Functional consequences of *TGFB1* polymorphisms and their role in haematopoietic stem cell transplantation

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Declaration

I, Esteban Roberto Arrieta Bolaños, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Haematopoietic stem cell transplantation (HSCT) is used to treat malignant and non-malignant diseases. Apart from clinical and human leukocyte antigen (HLA)-related factors, non-HLA Immunogenetics is increasingly recognized to play a role in the outcome of HSCT. A gene that may be relevant for the outcome of HSCT is *TGFB1*, which encodes transforming growth factor β-1 (TGF-β1), a cytokine that is central in the regulation of numerous immune processes. In order to understand the role of *TGFB1* polymorphism in HSCT, I studied the effect of this variation in the production of this cytokine by regulatory T cells (Treg) and also in the outcome of a cohort of HSCT patients and donors.

This study has found evidence of differential surface expression of TGF-β1 by activated Treg bearing *TGFB1* +29C as compared with those lacking this variant. Also, the analysis of a cohort of HSCT patients and donors revealed that patients carrying *TGFB1* allele p001 had reduced overall survival and increased non-relapse mortality. Interesting additional discoveries include the lack of induction of *TGFB1* messenger ribonucleic acid upon T cell receptor-mediated activation in Treg, the presence of preformed intracellular latent TGF-β1 both in Treg and effector cluster of differentiation (CD)4+CD25- cells, and the kinetics of transient surface latent TGF-β1 expression on Treg in the context of an *in vitro* suppression assay. The typing of *TGFB1* alleles in more than 1000 unrelated samples gives the first large-scale glimpse of the diversity of *TGFB1* polymorphism in the population. Likewise, this study discovered the presence of novel polymorphic positions and novel alleles of the human *TGFB1* gene, adding to the current knowledge on the diversity associated with it. Additional contributions are also the design of a novel *TGFB1* regulatory region and exon 1 sequencing-based typing strategy and the generation of an informatics tool for the easy assignment of allelic genotypes for this ~3000 base region based on the individual variant genotypes at 18 polymorphic positions.

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Abbreviations

5'UTR	5’-untranslated region
a	acute
Ab	antibody
ALK	activin receptor-like kinase
ALL	acute lymphoblastic leukaemia
allo	allogeneic
AML	acute myeloid leukaemia
APC	Antigen-presenting cell
APC	allophycocyanin
ARMS	amplification-refractory mutation system
ATG	anti-thymocyte globulin
B	bursa-derived
BM	bone marrow
c	chronic
cAMP	cyclic adenosine monophosphate
CB	cord blood
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CI	confidence interval
CML	chronic myelloid leukaemia
CMV	cytomegalovirus
Ct	threshold cycle
CTLA	cytotoxic T-lymphocyte antigen
DC	dendritic cells
DFS	disease-free survival
DLI	donor lymphocyte infusions
ECP	extracorporeal photophoresis
EDTA	sodium ethylene-diaminotetraacetate
EFS	event-free survival
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
G-CSF	granulocyte colony-stimulating factor
GARP	glycoprotein A repetitions predominant
GI	gastrointestinal
GITR	glucocorticoid-induced tumor necrosis factor receptor family-related gene
GVHD	graft-versus-host disease
GVL	graft-versus-leukaemia
GVM	graft-versus-malignancy
HAT/HDAC	histone acetyl transferase/histone deacetyl transferase complex
HLA	human leukocyte antigen
HSC	haematopoietic stem cells
HSCT	haematopoietic stem cell transplantation

HWE	Hardy-Weinberg equilibrium
i	induced
ICOS	inducible costimulatory
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobin
IHWS	International Histocompatibility Workshop
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
kb	kilobases
LAG-3	lymphocyte activation gene 3
LAP	latency-associated peptide
Leu	leucine
LFS	leukaemia-free survival
LLC	large latent complex
LTBP	latent TGF-β1 binding protein
MAF	minor allele frequency
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MMF	mycophenolate mofetil
mRNA	messenger ribonucleic acid
n	natural
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NK	natural killer
NRM	non-relapse mortality
OR	odds ratio
OS	overall survival
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	percidin chlorophyl
PFS	progression-free survival
Pro	proline
RORγt	retinoic acid receptor-related orphan receptor γt
RT	real-time
Runx1/AML-1	runt-related transcription factor 1
SBT	sequence-based typing
SLC	small latent complex
SNP	single nucleotide polymorphism
SSP	sequence-specific primer

T	thymus-derived
TBI	total-body irradiation
TCR	T cell receptor
TGF	transforming growh factor
Th	T helper
TNF	tumor necrosis factor
Treg	regulatory T cells
TRM	transplant-related mortality
TβRII	TGF-β receptor II
UD	unrelated donor
VOD	venoocclusive disease

Chapter 1. Introduction

1.1 The human immune system

The human immune system is a complex array of tissues, cells and molecules whose combined aim is to detect, respond to and eradicate invasion by potentially pathogenic organisms and malignant autologous cells. It is composed of two interwoven types of immune response: an innate response and an adaptive response.

The immune system is organised in primary and secondary organs (Boehm *et al.*, 2012). Primary organs are the sites where immune cells are generated and, in the case of lymphocytes, acquire the expression of receptors consistent with functional and phenotypic maturity. Primary organs include the bone marrow, where most mature immune cells are generated from a common haematopoietic stem cell in the adult individual, and the thymus, where a subset of lymphocytes mature and are selected based on their capacity to recognise 'foreign' molecules known as antigens.

Secondary lymphoid organs are the sites where lymphocyte responses to foreign antigens are initiated and developed. These organs include the spleen and the peripheral lymph nodes (Ruddle & Akirav, 2009). The spleen surveys the central circulation while the lymph nodes are served by a network of specialized vessels that drain fluid from tissues, which is later poured into the central circulation. Other secondary lymphoid organs with special regional characteristics are the skin and the mucosae such as that of the gastrointestinal (GI) tract. These sites also concentrate the different cells and soluble factors required for the construction of immune responses.

1.1.1 Cytokines

Cytokines are secreted proteins that bind to receptors on other cells and elicit a response. These proteins allow the communication between cells and are soluble mediators and regulators of the immune response. Broadly, they include the interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), stem cell and precursor growth factors, and transforming growth factor (TGF)-β. Cytokines can be pleiotropic or redundant, antagonic or synergistic. Cytokines usually act as short distance signaling molecules, in autocrine or paracrine fashions (Abbas, 2012). However some can act in an endocrine manner if sufficient amounts are produced. Cytokines are relevant actors in the immune system in the production, development, maturation, activation and differentiation of immune cells. They also participate actively both as mediators and regulators of immune responses. Cytokines can be classified according to various structural and functional characteristics.

Functionally, immune cytokines can be inflammatory or anti-inflammatory, innate or adaptive, humoral or cellular. Other cytokines act as growth factors and participate in haematopoiesis. Of note, the same cytokine can participate both in innate or adaptive responses and be produced by different kinds of cells. Moreover, much of their action is context dependent, limiting the applicability of these classification schemes.

1.1.2 Cells of the immune system

The cells of the immune system are classified into several categories related to their function, but also to their maturation status. Phagocytes, i.e. cells that can recognise, engulf and destroy whole foreign organisms, include macrophages and neutrophils (Dale *et al.*, 2008). Mononuclear phagocytes called macrophages are derived from circulating monocytes that enter tissues and are ready to respond to microorganisms that enter these tissues. Macrophages also produce cytokines and can act as antigen-presenting cells (APC). They also play a role in tissue repair and angiogenesis. Neutrophils, also known as polymorphonuclear leukocytes, are part of the granulocytic lineage (neutrophils, basophils and eosinophils). Neutrophils are very abundant and respond quickly to bacterial infection by phagocytosis, but also by release of microbicidal components of their intracellular granules.

Other granulocytes, such as basophils and eosinophils, contain granules that secrete inflammatory and antimicrobial substances, and play a role in the immune response to specific threats such as helminthes but also in allergic reactions.

Dendritic cells (DC) are specialised in the uptake of pathogenic antigens and their transportation from the tissues to the secondary lymphoid organs, where they present them to lymphocytes. They are the most important APC and play a central role in linking innate and adaptive immune mechanisms (Palucka & Banchereau, 1999).

Lymphocytes can be either naïve (not having previously encountered the antigens for which they are specific for) or mature. Naïve lymphocytes circulate through the peripheral lymphoid organs where they can encounter antigens and mature to form short-lived effector or persistent memory lymphocytes (Surh & Sprent, 2008). Mature lymphocytes circulate in the blood and can translocate to and be kept at sites of microbial entry. Lymphocytes express clonally distributed receptors for foreign antigens and this diversity is generated by genetic recombination mechanisms unique to these cells (Nemazee, 2006)

Lymphocytes can be classified into various subsets: thymus-derived (T)-lymphocytes: the mediators of cellular immunity, bursa-derived (B)-lymphocytes: producers of antibodies, and innate cytotoxic natural-killer (NK) cells and glycolipid-specific NK T cells. T-lymphocytes express the highly diverse T-cell receptor (TCR) and are classified into αβ

and γδ TCR-expressing cells. αβ T-lymphocytes can be further classified into cluster of differentiation (CD) 4 (helper) and CD8-expressing (cytotoxic) subsets.

At least 4 different kinds of CD4-expressing T helper (Th) lymphocytes are known, each of which plays a different role in the immune system: Th1, Th2, Th17 and regulatory T cells (Treg) (Zhu & Paul, 2008). Each of these subsets expresses a characteristic master transcription factor thought to confer them their specific characteristics and roles. Th1 cells express T-bet, produce IFN-γ and participate mainly in the coordination of cell-mediated responses against intracellular pathogens, but also in autoimmunity. Th2 cells express GATA-3, produce IL-4 and coordinate mainly humoral responses and immunity against parasites, but also play a role in allergy and asthma. Th17 cells express retinoic acid receptor-related orphan receptor γt (RORγt), produce IL-17 and IL-21 and are thought to play a role in the responses against fungi and extracellular bacteria, with a supposed participation in some autoimmune diseases as well. Finally, Treg express forkhead box P3 (FOXP3), produce TGF-β1 and play a central role in immune tolerance, lymphocyte homeostasis and regulation of immune responses (Zhu & Paul, 2008).

1.1.3 Innate and adaptive immune responses

The innate immune responses constitute the first line of defense against pathogens. It consists of cellular and biochemical factors that are in most cases ready before the encounter with the pathogen. This readiness ensures that the threat is faced immediately, whilst a more specific response is set up. Innate immunity is elicited by epithelial barriers, phagocytes (neutrophils and monocytes), DC, NK cells and humoral factors such as the complement system and cytokines. Innate responses are triggered not only by signature pathogen components, but also by molecules suggestive of cellular damage.

Adaptive immunity involves the participation of lymphocytes, to which antigens from foreign organisms are presented by APCs. The adaptive immune response involves specificity towards the particular agent facing the immune system. It also implies the construction of immunological memory, which allows for stronger and quicker responses upon re-exposure to the same pathogen, which are subsequently cleared with little damage to the host (Litman *et al.*, 2010). The central effectors of the adaptive immunity are lymphocytes and their products, such as antibodies. Moreover, adaptive immune responses can be classified in two types, namely, humoral responses and cell-mediated immunity. Humoral responses involve the activation of B-lymphocytes and their production of antibodies specific to epitopes present in molecular components of pathogens. Cell-mediated immunity is mediated by T lymphocytes and is mostly aimed at pathogens capable of escaping humoral mechanisms, such as intracellular pathogens.

A cardinal feature of adaptive immunity is the capacity to recognise a huge repertoire of pathogen components by the generation of a diverse array of molecules that bind these components. This diversity is attained by genetic recombination mechanisms that produce variation in molecules such as antibodies or TCR. Additionally, most nucleated cells, and especially APC, express molecules that are able to present a large repertoire of foreign peptides by means of the Human Leukocyte Antigens (HLA) to lymphocytes. The HLA molecules are very polymorphic with over 12,000 known alleles (Holdsworth *et al.*, 2009; Robinson *et al.*, 2013), allowing for the accommodation of a very large diversity of peptides from potentially pathogenic organisms. In this way, lymphocytes that carry TCR specific for that pathogen peptide can be activated and start a response against it.

1.1.4 The human leukocyte antigens

The HLA molecules are part of the Major Histocompatibility Complex (MHC), and are classified as classical and non-classical HLA. There are two types of classical HLA: class I HLA, including the products of genes *HLA-A*, *HLA-B* and *HLA-C*, are expressed by most nucleated cells and present peptides derived from the intrinsic peptide catabolic way to CD8-expressing T-lymphocytes. Class II HLA, including the products of genes *HLA-DRA*, *HLA-DRB1,3,4,5*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DPA1*, and *HLA-DPB1*, are expressed mainly by APC and present peptides derived from an extrinsic, phagocytic, catabolic way to CD4-expressing T-lymphocytes (Klein & Sato, 2000). The activation of CD8 and CD4-expressing lymphocytes promotes the destruction of the infected cell by cytotoxic lymphocytes or the coordination of an antibody-mediated response by B-lymphocytes. In addition to their role in the presentation of foreign and self peptides, HLA molecules and their high polymorphism constitute the main barrier to transplantation of cells, tissues and organs by eliciting a strong response in recipients that are not matched for the specific variants expressed by the transplanted cells. HLA incompatibility can cause rejection in organ transplantation, as well as the inverse effect (graft-versus-host) in stem cell therapy.

Both adaptive and innate immune responses work together in order to provide defense against potentially harmful organisms and the development of malignant cells. Innate mechanisms will trigger the first line response, but will also promote the activation of the adaptive mechanisms. For example, a phagocyte such as a macrophage can recognize and engulf a pathogen and destroy it, but it will also activate and present peptides from this pathogen to CD4 lymphocytes, which will, in turn, coordinate the activation of cytotoxic and B-lymphocytes that will mount an adapted response, specific for the pathogen involved.

1.2 Haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is a medical technique used to treat malignant and non-malignant haematological diseases, congenital immunodeficiency syndromes, solid tumors, and metabolic diseases (Ljungman *et al.*, 2010), and in many cases it is the only available therapy to treat these patients.

Allogeneic HSCT (allo-HSCT) aims to replace the patient's immune and haematological systems with those of a donor, which can be a relative, an unrelated donor (UD) or cord blood unit. Due to the greater degree of genetic disparity between patient and donor in the UD allo-HSCT setting, such transplants may have a higher incidence of immunological complications than those using sibling donors. Alternatively, this disparity may contribute to a decrease in disease relapse mediated through the graft-versus-malignancy (GVM) effect.

1.2.1 Uses of haematopoietic stem cell transplantation (HSCT)

According to a survey of transplant use worldwide (71 countries) in 2006 (Gratwohl *et al.*, 2010), out of 50,417 transplants performed 42.7% of the transplants were allogeneic and 57.3% autologous. Fifty-four point five percent were indicated for lymphoproliferative disorders, 33.8% for leukaemias, 5.8% for solid tumors, and 6.1% for non-malignant disorders and other conditions (e.g., immune deficiencies, haemoglobinopathies, autoimmune and disorders inherited diseases of metabolism). Among the allo-HSCT, 57.3% were performed with cells from UD and 70.7% were used to treat leukaemias (Gratwohl *et al.*, 2010).

1.2.2 Stem cell sources in haematopoietic stem cell transplantation (HSCT)

Allogeneic HSCT can be performed with HLA-identical sibling (or other family member), or unrelated donors (UD). In general, UD donors are selected firstly on the degree of high-resolution HLA matching, 10/10 or 9/10 being the best choice. In the cases where several donors are available, preference is usually given to young, male, AB0-matched, cytomegalovirus (CMV) infection status-matched donors (Gluckman, 2012).

Stem cells for transplantation were originally harvested from bone marrow by aspiration from the posterior iliac crests in most cases. However, nowadays about 71% of allo-HSCT are performed with mobilized peripheral blood stem cells (PBSC) (Gluckman, 2012). An HLA-matched sibling donor is still the preferred stem cell source. However, matched UD-HSCT show comparable survival rates as those performed with HLA-identical siblings,

despite some studies reporting higher GVHD rates for the former (Kiehl *et al.*, 2004; Hows *et al.*, 2006; Walter *et al.*, 2010; Zhang *et al.*, 2012).

Overall, when peripheral blood is used as a source of stem cells, the median CD34+ cell content as well as the median CD3+ content are several times higher than when bone marrow is used (2-3 x 10^6 vs 8 x 10^6 / kg CD34+ content; 25 x 10^6 / kg vs 2.5 x 10^6 / kg CD3+ content, respectively) (Gluckman, 2012).

Studies in HLA-identical sibling transplants have associated the use of PBSC with higher risk of cGVHD when compared to bone marrow (Flowers *et al.*, 2011). A large recent study has also found the use of PBSC in combination with myeloablative conditioning regimes as a significant risk factor for the development of aGVHD after sibling and UD-HSCT (Jagasia *et al.*, 2012). However, in a large multi-center study, no survival difference between PBSC and bone marrow was found in UD-HSCT (Anasetti *et al.*, 2012).

1.2.3 Conditioning regimes in haematopoietic stem cell transplantation (HSCT)

HSCT involves a conditioning step in which the patient is treated with chemo and/or radiotherapy in order to eliminate the original haematopoietic cell, including malignant ones, and prepare a space for the graft. The goals of conditioning are immunosuppression of the recipient and long-term disease control, especially in malignancies and disorders that involve a hyperplastic marrow. Because of their significant toxicity, conditioning regimes are often a cause of mortality in HSCT, and can significantly modify the way that immune reconstitution and other outcomes of the transplant develop. Moreover, a degree of immunosuppression is required in order to reduce rejection of the graft. Of note, the risk of rejection is increased in T cell-depleted HSCT and in patients that have received multiple blood products prior to the transplant, but it is in turn decreased with high stem cell and T cell doses (Gratwohl, 2012). Although in the past most patients received the same conditioning regime, nowadays it has become clear that different regimes should be applied to patients with different disease-related and transplant-related risks.

Classic intensified conditioning regimes usually involve the use of total-body irradiation (TBI) in combination with one or two drugs, most commonly cyclophosphamide, or a combination of cyclophosphamide and busulfan instead of TBI (Lowsky, 2010).

Because of the detrimental effects of these intensified regimes, reduced-intensity conditioning (RIC) regimes based on the use of fludarabine in combination with low-dose TBI or low doses of other cytotoxic drugs supported by donor-lymphocyte infusions, are becoming more common in clinical practice, especially for elderly patients and patients with co-morbidities (Gratwohl, 2012).

T cell depletion is used in some HSCT protocols aiming at reducing GVHD, but it is also associated with increases in graft rejection (when performed ex vivo) and disease recurrence. T cell depletion can be achieved by *in vitro* or *in vivo* techniques. *In vitro* depletion options usually involve either the selection of CD34+ cells or the depletion of CD3+ and CD19+ cells using magnetic cell separation columns. Anti-thymocyte immunoglobins, polyclonal antibodies against antigens on human T cells (and other immune cell subsets) produced in animals, are used both as *in vitro* and *in vivo* means of T cell depletion. Monoclonal antibodies against lymphocyte antigens like CD52 (alemtuzumab) are also used as a means of T cell depletion (Barge *et al.*, 2006; von dem Borne *et al.*, 2006).

Anti-thymocyte immunoglobins and monoclonal antibodies are mainly used for GVHD prevention (Sheng *et al.*, 2013). However, they can also be used as a conditioning agent to facilitate engraftment, especially in high failure risk transplants such as T cell-depleted grafts, CB-HSCT, RIC-HSCT or in cases where there is allo-immunisation of the patient. However, its use is hampered by side effects such as anaphylaxis, cytokine release syndrome, viral reactivations, lymphoprolipherative disorders, and increased risk of relapse (Chakraverty *et al.*, 2001; Seidel *et al.*, 2005; Gratwohl, 2012). On the contrary, the use of alemtuzumab as an *in vitro* depleting agent has been associated with durable engraftment and reduced GVHD without affecting immune recovery to the same extent as its *in vivo* use (Chakrabarti *et al.*, 2004).

1.2.4 The outcome of haematopoietic stem cell transplantation (HSCT) and its complications

The aim of HSCT is to achieve engraftment and immune reconstitution in patients. However, several potentially fatal complications can also affect patients treated with HSCT. Complications post-HSCT include graft failure, relapse, acute (a) and chronic (c) graft-versus-host disease (GVHD), infections, and organ damage. Long-term complications such as secondary malignancies such as solid tumors may also occur.

For statistical purposes, the outcome of HSCT can be categorized in several probability measures. The key events that take place after an HSCT are neutrophil and platelet engraftment, aGVHD , relapse or progression, death, and cGVHD.

Early complications after HSCT are usually caused by the conditioning regimens include mucositis, haemorragic cystitis, veno-occlusive disease of the liver (sinusoidal obstructive syndrome), capillary leakage syndrome, engraftment syndrome, diffuse alveolar haemorrhage, and HSCT-associated thrombotic microangiopathy, and idiopathic pneumonia syndrome (Carreras, 2012).

Intermediate complications include infections, GVHD and rejection, while long-term complications include cataracts, kerato-conjunctivitis, thyroid dysfunction, gonad dysfunction and infertility, bronchiolitis obliterans and other pulmonary conditions, heart failure, cerebrovascular and cardiovascular disease, chronic kidney disease, iron overload, avascular necrosis of the bone and osteoporosis, and late malignant complications and solid tumors (Tichelli, 2012).

Infections post-HSCT occur pre and post-engraftment. In the former case, they are promoted by neutropenia and natural barrier breakdown, while in the latter low lymphocyte counts, functional asplenia, and GVHD and its treatment predispose HSCT patients. Both Gram-negative and positive bacteria, fungi such as *Aspergillus* spp, *Candida* spp and *Pneumocystis jiroveci*, and viruses, especially herpesviridae, are main etiologic agents in HSCT patients (Rovira, 2012).

1.2.4.1 Graft-versus-host disease (GVHD)

GVHD is the consequence of three events: (1) the administration of a graft containing immunocompetent cells, (2) immunological disparity between the donor and the recipient, and (3) an immunosuppressed recipient (Billingham, 1966). Immunological or tissue disparity arises from differences in major (i.e. HLA) and minor histocompatibility antigens.

Acute (a)GVHD is directly or indirectly the main cause of short term mortality after allo-HSCT and occurs in approximately 40% of all recipients of allo-HSCT, albeit with variable frequencies depending mainly on the type of donor and the prophylaxis used. One paper has stated that aGVHD occurs in 35-45% of HLA-matched sibling HSCT and in 60-80% of one-antigen mismatched UD-HSCT despite the use of prophylaxis (Ferrara *et al.*, 2009).

aGVHD develops as a three-stage process. Firstly, conditioning regimes damage the host's tissues. This in turn activates the host's APC, which activate donor T cells. Host APC activation is boosted in mucosae (e.g. the GI tract) by means of the resident microorganisms and the release of their inflammatory stimuli. Donor T cells proliferate and produce an inflammatory cytokine storm. Finally, these cytokines cause tissue necrosis, while activated effector cells migrate to target tissues and also directly attack the host's tissues (Ferrara *et al.*, 2009).

The main risk factor for the development of aGVHD is HLA disparity between the donor and the recipient, increasing with increasing degrees of incompatibility. Other well-known risk factors are older patient age, prior allo-immunisation of the patient, and the type of GVHD prophylaxis employed (Apperley, 2012). Moreover, increasing intensity of the preparatory regime, and recipient seropositivity for CMV have also been proposed as risk

factors for aGVHD. Recent large multi-center studies have found that, with current practice, donor age, use of PBSC, and sex-incompatibility (i.e. a female donor for a male recipient) are rather associated with chronic (c)GVHD risk (Flowers *et al.*, 2011; Jagasia *et al.*, 2012).

aGVHD is clinically characterised by the sole or combined presence of an erythematous skin reaction, cholestatic liver disease and gastro-intestinal dysfunction (Apperley, 2012). The clinical grading of aGVHD is made based on a revised set of criteria combining organ-specific staging in an overall 4-category grading system (Przepiorka *et al.*, 1995). aGVHD grade I is usually limited to the skin and does not require systemic treatment. Clinically relevant (moderate to severe) aGVHD includes grades II-IV, while severe aGVHD is limited to grades III and IV and is associated with high risk of mortality.

cGVHD occurs in 30-70% of recipients of allo-HSCT. It involves features of both autoimmunity and immunodeficiency and is the main cause of late NRM and morbidity after allo-HSCT, mainly by infection. Its pathophysiology is less well understood, but seems to involve not only T cells but also donor B cells. Risk factors for cGVHD include previous aGVHD, HLA mismatch, older patient age, previous splenectomy, CMV seropositivity, female-to-male donation, and use of PBSC (Apperley, 2012).

Clinically, it involves manifestation in the skin, mucosae, eyes, GI tract, liver, lungs, muscles, fascia and joints. Diagnosis and staging criteria have been proposed (Filipovich *et al.*, 2005), but the classical extension-based characterisation (i.e. limited vs extensive) is still commonly used. cGVHD occurs more frequently and has an earlier onset in UD-HSCT when compared to HLA-identical sibling HSCT. In about 73% of the cases, cGVHD appears after a hiatus following aGVHD resolution or is transformed from it directly, but it also has a de novo onset in 27% of the cases (Apperley, 2012).

1.2.4.2 Immune reconstitution

The ultimate goal of HSCT is to achieve immune reconstitution in the patient. Several factors influence the immune reconstitution. Host factors include age, sex, type of conditioning regimen, and the initial pathology. In RIC, despite a milder myeloid suppression, lymphoid depletion tends to be as deep as with myeloablative regimens. Immune reconstitution is also affected by the degree of genetic incompatibility between the recipient and the graft. Stem cell source, T cell depletion and transplant manipulation can also modify immune reconstitution (Toubert, 2012). In fact, T cell depletion is associated with delayed immune reconstitution. Finally, post-HSCT events such as GVHD, relapse and infections play a role in immune reconstitution.

Myeloid engraftment, evaluated via neutrophil counts, usually occurs within 21 days post-HSCT. Platelet engraftment can occur much later. A haemoglobin level of at least 8 g/dL without transfusion is also considered a threshold for engraftment (Lowsky, 2010).

NK cells are the first lymphocyte population to be reconstituted, usually within 3 months after transplant. The fast recovery of NK cells after transplant is due to expansion of the $CD56^{hi}CD16-$ cytokine-producing subset. The process of NK cell education (also known as licensing), in which they acquire mature characteristics, is fully active after HSCT and is dependent on donor ligands for NK receptors, allowing for a long-term GVM effect (Toubert, 2012).

B cell reconstitution occurs after a decline in the first several months after HSCT, and antibody levels against antigen typically found after transplantation (e.g. anti-CMV) return to normal levels within the first year. However, antibodies against other antigens such as vaccines continue to decline, and re-vaccination is usually needed. In general, serum total IgG and IgM levels return to normality by 1 year after transplantation, but IgA production can take longer to normalize (Toubert, 2012). Moreover, defects in IgA production in patients with cGVHD are related with mucosal infections in the respiratory and digestive tracts, and the use of immunosuppressive drugs for cGVHD treatment can further delay immunoglobulin recovery. CD19+ B cells usually normalize within the first year after HSCT (Lowsky, 2010).

The vast majority of HSCT recipients see their lymphocytes almost eradicated, and must rely on mature cell expansion and lymphoid progenitors in the graft. T cell reconstitution takes place initially by expansion of memory subsets present in the graft or left over in the recipient despite the conditioning regime in cases where T cell depletion of the graft was used. Donor lymphocyte infusion (DLI) is also a source of T cell expansion. Donor-derived T cells drive expansion in myeloablative HSCT as well as in those receiving DLI; in RIC-HSCT it is usually also driven by recipient cells. T cell expansion is triggered both by antigen encounter and homeostatic proliferation in response to lymphopenia (Krenger *et al.*, 2011).

However, the expanded memory subsets will have a skewed repertoire of specificities with limited capacity of response against infections. Hence, for long-term reconstitution, it is necessary that a full repertoire of naïve T cells is educated in a functional thymus. Because the thymus itself can be a target of allo-reactivity, it is crucial that this is controlled for optimal recovery of the T cell ontogeny. GVHD targeting the thymus directly affects the reconstitution of the naïve compartment, and thymic involution affects immune reconstitution in older patients. De novo T cell production is more efficient in younger patients and can usually be readily detected from day 100 onwards. However, T cell

reconstitution is a long process, usually taking more than a year to reach a plateau. Pre-HSCT irradiation and chemotherapy also negatively affect the quantity and quality of thymopoiesis, mainly by disruption of thymic epithelial cells (Krenger *et al.*, 2011).

1.2.4.3 Graft failure

Graft failure is defined upon the engraftment measures mentioned before. Graft failure can be defined as primary or secondary. Late failure and can be related to graft rejection, persistent or progressive disease, low donor yield, medication side effects, infection or GVHD. Because RIC-HSCT is associated with persistence of recipient cells, graft failure assessment relies not only on neutrophil, platelet and haemoglobin measurements, but also on the assessment of donor chimerism.

1.2.4.4 Relapse

Relapse in HSCT is the main cause of treatment failure. Between 40–45% of recipients of HLA-matched siblings and approximately 35% of recipients of UD-HSCT will relapse with their original malignancy (Barrett & Battiwalla, 2010). Relapse treatment after allo-HSCT has worse results, and second myeloablative HSCT have high toxicity-related mortality. DLI is successful in treating relapse in some diseases such as CML. Relapse can also occur after a period of anti-malignancy effect if the immune system is weakened or becomes tolerant to the residual disease, or the disease undergoes immune escape through clonal selection of immune-resistant progenitors. Prognosis in relapsed patients depends on the time from HSCT to relapse, the disease type, the disease burden and the conditions of the first transplant (Barrett & Battiwalla, 2010).

1.2.5 Factors that affect the outcome of haematopoietic stem cell transplantation (HSCT)

Various factors, both clinical and genetic, influence the outcome of HSCT. Clinical factors in patients and donors known to influence the outcome of HSCT are the type and stage of disease, age, comorbidities, sex mismatching, conditioning regime, CMV status, and stem cell source (Anasetti, 2008). Survival risk scores based on clinical factors have been validated to predict the chances and risks for HSCT patients (Gratwohl, 2012). Moreover, genetic factors affecting HSCT can be classified according to whether they belong to the human leukocyte antigen (HLA) system or to other genetic systems.

1.2.5.1 Genetic factors that affect the outcome of haematopoietic stem cell transplantation (HSCT)

The main obstacle when performing allo-HSCT is donor-patient compatibility as defined by the HLA system (Lee *et al.*, 2007; Petersdorf EW, 2007; Shaw *et al.*, 2010). The HLA system is part of the Major Histocompatibility Complex, a region of the genome that includes over 200 genes mostly involved in immune functions. The HLA system includes the most polymorphic genes in the human genome (Robinson *et al.*, 2011) and, since it plays a central role in the immune system, specifically in adaptive immune responses, self-recognition, and rejection of foreign antigens, it has been thoroughly studied and its role in allo-HSCT is now well characterised (Lee *et al.*, 2007; Loiseau *et al.*, 2007; Petersdorf EW, 2007; Shaw *et al.*, 2010). Antigenic and allelic differences between HLA molecules can be readily recognised as foreign by both the patient's immune system as well as by the graft, thus being able to elicit strong immune responses against them. It has become clear that an additive survival disadvantage is produced by any degree of HLA incompatibility at *HLA-A*, *-B*, *-C*, and *-DRB1*, with allelic (i.e. not recognised by serological techniques) having the same effect as antigenic ones (except for *HLA-C*) (Shaw, 2012). The effect of *HLA-DQB1* incompatibility has not been found to be as strong as that caused be the four previously mentioned loci and its effect seems to be relevant only in the presence of other mismatches. In turn, *HLA-DPB1* matching has been associated with increased relapse, while its mismatch has been shown to increase GVHD, and survival at least in some studies (Bettens *et al.*, 2012). This, in addition to the difficulty of finding matched donors due to its reduced linkage disequilibrium with other HLA genes, means that *HLA-DPB1* matching is not currently routinely performed in clinical settings. Nevertheless, recent evidence shows that epitope matching analysis for *HLA-DPB1* defines permissive and non-permissive mismatches that have an impact on the outcome of UD-HSCT (Fleischhauer *et al.*, 2012).

Extensive compatibility at *HLA-A*, *-B*, *-C*, *-DRB1* and *–DQB1* between patient and donor is usually sought in order to minimise the occurrence of graft rejection and GVHD (Shaw, 2012). The gold standard is an allele-level compatibility for the 5 loci (i.e. 10/10 match), but incompatibilities may be tolerated if the reduction on the probability of survival is not enough to justify a different therapeutic option (Lee *et al.*, 2007; Bray *et al.*, 2008). Moreover, in the interest of prioritizing speed of transplant over compatibility, defining permissive and non-permissive mismatches has attracted considerable attention, especially in epitope matching (Shaw, 2012).

Despite a high degree of compatibility in terms of HLA, GVHD occurs in a significant proportion of the transplants. In addition, GVHD occurs even in well-matched, sibling transplants. Thus, it is likely that the outcome of allo-HSCT is still hampered by other

genetic factors that intervene in the immune reconstitution process. The field that studies the effect of these other genetic systems is called non-HLA immunogenetics.

1.2.5.1.1 Non-HLA immunogenetics

The discovery of an important degree of variation between human genomes, reaching on average nearly 1 SNP every 300 base pairs (bp) (Kruglyak & Nickerson, 2001), as well as of several other forms of variation, has prompted the interest in the consequences of these differences on the expression and function of proteins in cells in both normal and pathological conditions. Consequently, nearly every field in Medicine has now seen the advent of the study of the effect of polymorphism on diseases and the response to medical therapies and treatments. HSCT is thus an area where a significant amount of research on the effects of genetic variation on its outcome has been carried out.

Many different genes or genetic systems other than HLA that are likely to influence several processes involved in HSCT have been recognised. For instance, tissue compatibility defined by minor rather than major (i.e. HLA) histocompatibility antigens has been implicated in organ and stem cell rejection and GVHD (Hambach *et al.*, 2007; Spierings, 2008).

Genes whose products are related to the immune system are evidently likely to be involved in the various immune processes that occur prior, during, or after the transplant. Importantly, the genes for cytokines and their receptors bear a considerable amount of polymorphism, and have been associated with transplantation outcome (Dickinson & Holler, 2008). Innate immunity genes that are polymorphic, such as toll-like receptors (Mensah *et al.*, 2009) or the NOD/CARD15 system (Mayor *et al.*, 2007), have been widely studied. Steroid receptors such as the vitamin D receptor (Bogunia-Kubik *et al.*, 2008), which are involved in the development of the immune system, and killer-cell immunoglobulin-like receptors, which play a central role in natural killer cell immunosurveillance (Cooley *et al.*, 2010), have been associated with different outcomes following HSCT.

Polymorphism in enzymes that are involved in the absorption, distribution, metabolism, and excretion of drugs used in the various stages of HSCT have been recently recognised to affect patients' response to these drugs, and, thus, the outcome of the treatment. Specifically, polymorphisms in the enzyme methyl-tetrahydrofolate reductase, which metabolises the immunosuppressive GVHD-prophylactic drug methotrexate, have been associated with complications and GVHD after HSCT (Murphy *et al.*, 2006; Kim *et al.*, 2007).

One of the genes that may play an important role on the outcome of allo-HSCT is *TGFB1*, which encodes TGF-β1. Polymorphism in *TGFB1* among individuals may determine the way their "old" immune system responds to conditioning regimens and chemotherapy, but also how their "new" immune system recovers after an allo-HSCT and potentially causes GVHD. In fact, as previously mentioned, several functional polymorphisms in *TGFB1* have been identified, and these SNPs are known to cause alterations in cytokine secretion in several settings (Shah *et al.*, 2006).

1.3 Regulatory T cells (Treg)

Regulatory activity exerted by T-lymphocytes has been known for several years now and constitutes one of the most important findings in immunobiology in the last twenty years. Treg are necessary for the prevention of autoimmune disease, immunopathology, and allergy, as well as for the maintenance of allograft and fetal-maternal tolerance as they are able to suppress the activation, proliferation and function of CD4+ and CD8+ lymphocytes, NK, NK-T and B cells (Sakaguchi *et al.*, 2010).

1.3.1 Discovery and history of regulatory T cells (Treg)

In 1995, the first description of a regulatory T-lymphocyte lineage was made upon the identification of a subset capable of preventing the development of autoimmune diseases in mice (Sakaguchi *et al.*, 1995). These cells were reported to express high levels of the IL-2 receptor alpha chain (CD25) and their depletion from an allograft caused the receptor athymic mice to develop autoimmune diseases. Moreover, reconstitution of the CD25+ subset could prevent the autoimmune manifestations in a dose-dependent manner, albeit only if reintroduction of the regulatory subset was performed within a restricted time window after the transplant. Foxp3 was found later on to be the master transcription factor of these murine cells (Fontenot *et al.*, 2003; Hori *et al.*, 2003), and mutations in the gene encoding this transcription factor in mice and humans were found to be the cause of both the autoimmune phenotype of the Scurfy mice (Brunkow *et al.*, 2001) as well as the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in a human patient whose symptoms included diarrhea, insulin-dependent diabetes, thyroid disorders and eczema (Bennett *et al.*, 2001). Human Treg were later discovered in 2001, also as CD4+CD25+ T cells (Baecher-Allan *et al.*, 2001; Jonuleit *et al.*, 2001; Levings *et al.*, 2001; Ng *et al.*, 2001).

1.3.2 Types of regulatory T cells (Treg)

A number of cells with the capability of antagonising effector cell functions have been described. Naturally occurring, thymic-derived Treg (nTreg) express a diverse TCR repertoire that is specific for self-antigens (Hsieh *et al.*, 2004) and were the first regulatory subset that was described. These are CD4+ cells that express high levels of CD25, low levels of CD127, and the transcription factor FOXP3. In addition to nTreg, the peripheral induction of Treg (iTreg) from naïve CD4 cells activated in the presence of TGF-β1 and the absence of inflammatory cytokines with the concomitant induction of FOXP3 expression has also been described and is now a well-established mechanism of Treg

generation (Chen *et al.*, 2003). Recently, the TGF-β-independent generation of iTreg has also been described (Schallenberg *et al.*, 2010). However, iTreg usually have a more restricted specificity for cells, tumors, or foreign antigens. Hence, iTreg are thought to be mediators of the contraction phase of specific immune responses, and believed to play a complementary role to that of nTreg by the expansion of the regulatory TCR repertoire demonstrated by little TCR subset overlap in murine colitis models (Haribhai *et al.*, 2011).

Apart from naturally occurring Treg, mature T cells can acquire immunoregulatory functions in the periphery. This is the case of CD4+ TGF-β1-secreting Th3 cells generated in murine oral tolerance models, IL-10 secreting Tr1 cells, and certain CD4-CD8- and CD8+CD28- T cells (Liu *et al.*, 1998; Vieira *et al.*, 2004; Fischer *et al.*, 2005). Th3 cells produce TGF-β1 and not IL-4 nor IFN-γ, and are generated by a mechanism involving oral tolerance (Chen *et al.*, 1994), while the main feature of Tr1 cells is their production of IL-10 and may represent a certain state of each of the other lineages rather than a distinct subset (Zhu & Paul, 2008). Unless stated otherwise, the term Treg will henceforward refer to nTreg.

1.3.3 Biological aspects of regulatory T cells (Treg)

Treg are the main actors of peripheral tolerance. Human Treg comprise 5-10% of peripheral CD4+ lymphocytes (Sakaguchi, 2004) and their most distinctive marker is transcription factor FOXP3 (Fontenot *et al.*, 2003). These cells are hyporesponsive to TCR-derived proliferation and have reduced cytokine production. Continuous expression of FOXP3 is necessary for Treg suppressive function (Williams & Rudensky, 2007).

1.3.3.1 FOXP3 as a master regulator of regulatory T cells (Treg)

FOXP3 forms a homo-oligomer and interacts with several transcription factors including the nuclear factor of activated T cells (NFAT), runt-related transcription factor 1/acute myeloid leukaemia 1 (Runx1/AML-1), the histone acetyl transferase/histone deacetyl transferase (HAT/HDAC) complex, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Piccirillo, 2008). NFAT and NF-κB jointly initiate the expression of genes encoding IL-2, IL-4 and cytotoxic T-lymphocyte antigen (CTLA-4) during T cell activation. FOXP3 suppresses the expression of IL-2 by binding to NFAT (Wu *et al.*, 2006) and to Runx1/AML-1 (Ono *et al.*, 2007). Consequently, Treg depend upon exogenous IL-2 for their thymic generation, survival and function. Unlike murine Treg, human Treg express 2 different FOXP3 splice variants, with one of the forms lacking exon 2, but both being independently able to induce anergy in CD4 cells (Allan *et al.*, 2005).

Of note, despite its fundamental role in Treg, FOXP3 messenger ribonucleic acid (mRNA) and protein has been discovered in human CD4+CD25- and CD8+ cells upon activation

(Morgan *et al.*, 2005; Pillai *et al.*, 2007). However, the *in vitro* regulatory properties of these cells have been disputed (Allan *et al.*, 2007; Tran *et al.*, 2007), and it seems that these expression is a side effect of activation rather than the start of a regulatory phenotype in these cells (Wang *et al.*, 2007). Moreover, the existence of these human non-regulatory FOXP3+ CD4 cells in the periphery has been confirmed *in vivo*, and the main difference between them and Treg seems to be the incomplete demethylation status of two enhancer elements of the *FOXP3* gene in the former (Miyara *et al.*, 2009). *FOXP3* is completely demethylated in nTreg and fully methylated in naïve and Th conventional T cells (Baron *et al.*, 2007). High and sustained expression of FOXP3 seems to be the key for the development of the Treg program (Gavin *et al.*, 2006).

The *FOXP3* locus contains 5 conserved non-coding regulatory sequences, including 3 enhancers, one promoter and one pioneer element, and it has been shown that nTreg and iTreg depend on distinct regions for their generation. TCR stimulation, CD28 costimulation, IL-2 and TGF-β1 are all extracellular signals that induce modification or interaction of cellular components with these regulatory regions (Regateiro *et al.*, 2011).

Recently, FOXP3 expression has been detected in epithelial cells, mesenchymal stromal cells, NK-T cells and macrophages (Monteiro *et al.*, 2010; Manrique *et al.*, 2011; Sundin *et al.*, 2011; Lal *et al.*, 2013), possibly indicating that its regulatory program can be mounted in other cells at least partially.

Of note, FOXP3 expression has been shown to be reversible in Treg under certain conditions (Zhou *et al.*, 2009; Addey *et al.*, 2011). Indeed, the differentiation of the Treg cell lineage is not necessarily considered terminal, as developmental and functional plasticity with the perturbation of FOXP3 and its complex at a transcriptional, translational and post-translational level by inflammatory signals in the periphery has been postulated (Gao *et al.*, 2012).

1.3.3.2 Thymic development of regulatory T cells (Treg)

nTreg are generated in the thymus and require high-affinity interaction between their TCR and HLA molecules presenting self-peptides on thymic stromal cells. Rather than undergoing clonal deletion during negative selection as their non-regulatory counterparts, nTreg are thought to upregulate FOXP3 upon recognition of self-antigen (Jordan *et al.*, 2001; Ribot *et al.*, 2006). However, a role for nTreg in the tolerance to antigens produced by intestinal commensals has recently been proposed (Cebula *et al.*, 2013). TCR stimulation and co-stimulatory signals are required for thymic development of Treg cells (Yuan & Malek, 2012). IL-2 and IL-7 are required for the development of Treg in mice, while in humans thymic stromal lymphoprotein secreted by Hassall's corpuscles in the

thymic medulla activate CD11c+ DC that induce FOXP3 expression in immature CD4+CD8-CD25- thymocytes (Hanabuchi *et al.*, 2010). These activated DC are responsible for the positive selection of Treg with high affinity for self-antigens. Human thymic Treg development starts in utero as early as the thirteenth week of gestation and Treg colonisation of peripheral organs can be found by the fourteenth week (Darrasse-Jeze *et al.*, 2005).

1.3.3.3 Phenotypic markers and regulatory T cell (Treg) subsets

Treg constitutively express high levels of CD25. However, adult human CD4+CD25+ cells can include not only FOXP3+ cells but also FOXP3- memory cells. Moreover, since FOXP3 is an intranuclear marker, it cannot be used for isolation of these cells. Consequently, other markers have been sought for their characterization. Low or absent IL-7 receptor α chain (or CD127) expression has been recognised to be a feature of Treg and a 90% correlation between low expression of CD127 and FOXP3 in CD4+CD25+ lymphocytes has been established (Liu *et al.*, 2006; Seddiki *et al.*, 2006). However, conventional (i.e. non regulatory) T cells tend to down-regulate CD127 upon activation, thus making this marker less useful in settings that implicate T cell activation (Mazzucchelli & Durum, 2007; Aerts *et al.*, 2008). Expression of CD62L by Treg can help to differentiate them from activated conventional T cells, which express low levels of this marker (Hamann *et al.*, 2000). CD27 expression has also been shown to discriminate between regulatory and non-regulatory cells after expansion of human Treg (Duggleby *et al.*, 2007). Treg also express CTLA-4 (constitutively), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and folate receptor 4, and CD95, all upregulated upon activation. However, these markers can also be induced upon activation of naïve CD4 cells (Zhu & Paul, 2008).

1.3.3.3.1 Regulatory T cell (Treg) subsets

Treg have been divided into subsets according to their expression of CD45RA and CD45RO. It has become clear that human CD45RA+ Treg are a naïve subset that possesses strong suppressive capacity and are prevalent in umbilical cord blood (Seddiki *et al.*, 2006). These naïve Treg express lower levels of FOXP3 and most express CD31, a cell marker specific for recent thymic emigrants. They also proliferate after *in vitro* TCR stimulation and are highly resistant to apoptosis, whereas 'mature' CD45RO+ Treg are hyporesponsive and susceptible to apoptosis upon activation (Miyara *et al.*, 2009). CD45RA+ Treg transform into CD45RO+ and upregulate FOXP3 after stimulation. Subsequently, it appears that Treg can attain a terminal state analogous to that of memory conventional T cells in which they express HLA-DR. About one third of mature Treg express HLA-DR and these cells are suppressive and more ready to secrete cytokines than

their HLA-DR- counterparts (Baecher-Allan *et al.*, 2006). Naïve and mature Treg follow an inverse proportion tendency throughout life, the former decreasing while the latter increase (Miyara *et al.*, 2009).

Another marker reported to allow discrimination of functionally different Treg subsets is the inducible costimulator (ICOS) molecule. ICOS+ Treg have been reposted to use primarily IL-10 for DC suppression and TGF-β1 for T cell suppression, whereas ICOS- Treg produce only TGF-β1 (Ito *et al.*, 2008).

Finally, a member of the Ikaros transcription factor family, Helios was initially found to be differentially expressed in nTreg and not in iTreg (Thornton *et al.*, 2010). Helios was reported to be upregulated in microarray studies of Treg (Sugimoto *et al.*, 2006; Getnet *et al.*, 2009). However, its specificity for nTreg was later challenged by its induction in transgenic murine CD4 cells stimulated in the presence of IL-2 and TGF-β1 (Verhagen & Wraith, 2010), and also by its absence in a proportion of human nTreg (Himmel *et al.*, 2013). Despite this controversy, murine Helios+ Treg have been shown to express higher levels of CD103 and GITR proteins, higher TGF-β1 mRNA, and higher suppressive capability when compared to Helios- Treg (Zabransky *et al.*, 2012).

1.3.4 Regulatory T cell (Treg) control of immune responses

Treg are able to control the proliferation and effector functions of many immune cells. Evidence suggests that Treg can control conventional CD4+ and CD8+ T cells (Takahashi *et al.*, 2000), NK T cells (Azuma *et al.*, 2003), B cells (Lim *et al.*, 2005), DC (Fallarino *et al.*, 2003), monocytes/macrophages (Taams *et al.*, 2005), and NK cells (Ralainirina *et al.*, 2007). Consequently, Treg must possess a diverse arsenal of molecular mechanisms in order to be able to control such a diverse group of cells. Although there are still gaps in the knowledge of Treg mechanisms of action on some of these cells, much progress in the control of T cell responses has been made.

1.3.4.1 Mechanisms of action of regulatory T cells (Treg)

Several mechanisms have been proposed to contribute to Treg control of immune responses. At least 4 general models of Treg-mediated modulation of effector T cell activity have been identified: a) metabolic disruption, b) cytolysis, c) targeting of DC, and d) production of inhibitory cytokines (Vignali *et al.*, 2008). These mechanisms can be broadly divided into those that target T cells (inhibitory cytokines, metabolic disruption, cytolysis) and those that primarily target antigen-presenting cells (APC) (decreased costimulation or decreased antigen presentation). Most of these mechanisms are thought to require cell contact or proximity between Treg and their target cells, and none of them

have resulted in complete abrogation of regulatory activity when blocked or deleted despite significant reductions thereof.

1.3.4.1.1 Suppression by metabolic disruption

Metabolic disruption of effector T cells by Treg has been proposed as one of the mechanisms used by these cells to control immune responses. The first proposal of such a mechanism was derived from the fact that Treg express high levels of CD25, part of the IL-2 receptor. Since effector T cell expansion is dependent on IL-2 supply, local consumption of IL-2 by Treg has been hypothesised as an effect of their high receptor levels and its consequence on IL-2 availability would have a quenching effect on effector cell activation. Whether high CD25 levels are a suppressive characteristic of Treg or just a phenotypic trait derived from their impaired IL-2 production has been a matter of debate. Some studies have suggested that Treg can exert cytokine deprivation and subsequent apoptosis (Pandiyan *et al.*, 2007), while others have not confirmed this assumption (Oberle *et al.*, 2007).

Apart from IL-2 deprivation, the release of adenosine nucleosides has been proposed as another metabolic mechanism of inhibition by Treg. Adenosine is synthesized by CD39 and CD73, ectoenzymes expressed on Treg (Deaglio *et al.*, 2007). Adenosine suppresses effector T cell function including via binding to adenosine receptor 2A (Hoskin *et al.*, 2008). Additionally, Treg have been shown to be able to transfer cyclic adenosine monophosphate (cAMP), which is a potent inhibitory messenger molecule, to effector cells via membrane gap junctions (Bopp *et al.*, 2007). Moreover, inhibition of cAMP production by inhibition of adenylate cyclase activity or augmentation of cAMP degradation through ectopic expression of a cAMP-degrading phosphodiesterase reduced the suppressive activity and anergic state of human Treg both *in vitro* and in a humanized mouse model *in vivo* (Klein *et al.*, 2012). Prevention of cAMP degradation was shown to improve Treg suppressive function (Bopp *et al.*, 2009).

1.3.4.1.2 Suppression by cytolysis

Cytotoxic T-lymphocytes and NK cells use the protease granzyme in order destroy virus-infected cells by unleashing apoptosis within them. Granzyme is aided by the cytolytic protein perforin to reach the target cell's cytoplasm and thereupon activate caspases. Activated nTreg have been shown to produce granzyme and perforin (Grossman *et al.*, 2004), and these proteins have been shown to be important for Treg-mediated suppression of tumor clearance in mouse models (Cao *et al.*, 2007). While murine Treg express granzyme B, their human counterparts express the A isoenzyme. Also, studies on B-lymphocyte control by Treg showed that the latter are able to kill B cells in a granzyme-

perforin-dependent way (Zhao *et al.*, 2006). Granzyme has also been implicated in Treg control of pulmonary immune responses against viral pathogens such as respiratory syncytial virus, and the deficiency of granzyme appears to play an immunopathogenic role in children with bronchiolitis secondary to this viral infection (Loebbermann *et al.*, 2012).

Other studies have identified additional molecules used by Treg to induce apoptosis of their target cells. Induction of apoptosis in autologous CD8+ T cells by human Treg has been shown to be mediated by death receptor family member CD95/CD95L interaction (Strauss *et al.*, 2009). Another study aimed at analysing the role of tumor necrosis factor-related apoptosis inducing ligand/death receptor 5 as one of the mechanisms for the suppression and cytotoxicity induced by Treg confirmed that CD95/CD95L-independent apoptosis was one of the effects of Treg and that blocking of tumor necrosis factor-related apoptosis inducing ligand/death receptor 5 reduced their immunosuppressive power *in vitro* (Ren *et al.*, 2007). The researchers confirmed that this Treg cytolytic mechanism also occurred in an *in vivo* model of allogeneic skin grafts, resulting in reduced Treg-mediated survival of the grafts by addition of death receptor 5 blocking antibodies.

Finally, a protein called galectin-1, member of the family of beta-galactoside binding proteins and known to cause apoptosis in T cells (Perillo *et al.*, 1995), has been found to be upregulated in murine and human Treg (Garin *et al.*, 2007). Blockade of galectin-1 binding was found to reduce the inhibitory effects of murine and human Treg, and galectin-1-homozygous null mutant mice showed reduced suppressive action by Treg.

1.3.4.1.3 Suppression by dendritic cell (DC) function targeting

As previously mentioned, DC are the most efficient activators of naïve T-lymphocytes through their uptake and presentation of foreign antigens and provision of the adequate secondary signals for this end. Consequently, modulation of their function is likely to be a strategy to control adaptive immune responses, as was suggested early in Treg history (Cederbom *et al.*, 2000).

There is evidence that Treg can exert influence on DC, both on their maturation and function. Studies have reported that interaction between DC and CTLA-4-expressing Treg hampers the stable DC-naive T cell interactions (Tadokoro *et al.*, 2006). Just as the co-stimulatory second signal receptor CD28 does when T cell activation occurs, CTLA-4 binds to CD80 and CD86 (B7-1 and B7-2 antigens, respectively) on APC. However, unlike CD28, CTLA-4 transmits an inhibitory signal to T cells. Moreover, conditioning of DC with Treg has been shown to induce down-modulation of CD80 and CD86 molecules on DCs *in vitro*, resulting in a diminished capacity of naïve T cell stimulation (Oderup *et al.*, 2006). Experimental blocking of CTLA-4 with antibodies or the use of CTLA-4-deficient Treg

cause reduction of DC-mediated effector cell suppression, implying a role of this molecule in this type of Treg suppression mechanism.

Another strategy that Treg might use in order to modify DC function is the induction of the expression of indoleamine 2,3-dioxygenase by DC. This molecule favors the depletion of tryptophan in local tissue microenvironments and the production of proapoptotic metabolites of tryptophan, known as kynurenines, which can suppress effector cell activation (Fallarino & Grohmann, 2011). Kynurenines and tryptophan starvation can induce Treg from CD4+CD25- cells (Fallarino *et al.*, 2006), and Treg-induced expression of indoleamine 2,3-dioxygenase can make the DC develop a regulatory phenotype.

A further mechanism in which Treg could modulate DC function may involve lymphocyte activation gene 3 (LAG-3, CD233), which is expressed upon Treg activation. LAG-3 is a molecule capable of binding MHC class II in a fashion similar to that of CD4, and its expression by Treg has been shown to be required for full suppressive capacity (Huang *et al.*, 2004). Binding of LAG-3 to HLA class II expressed on immature DC suppresses their maturation and stimulatory capacity (Liang *et al.*, 2008). Similarly, differential expression of neuropilin-1 by nTreg (Weiss *et al.*, 2012; Yadav *et al.*, 2012) allows them to establish prolonged interactions with immature DC, which would strengthen their capacity to modulate DC before naïve T cell stimulation (Sarris *et al.*, 2008).

Apart from the effects of Treg on DC, there is some evidence that Treg might also affect the function of monocytes (Pommier *et al.*, 2013) and macrophages (Taams *et al.*, 2005; Tiemessen *et al.*, 2007), possibly by CTLA-4.

1.3.4.1.4 Suppression by inhibitory cytokines

Suppressive cytokine-mediated mechanisms of Treg action include the production of cytokines such as IL-10, IL-35 and TGF-β. These cytokines have also been implicated in the stimulation of Treg development and the induction of peripheral iTreg populations. The role of Treg derived IL-10 in suppression was shown in colitis (Annacker *et al.*, 2003) and allergy and asthma models (Hawrylowicz & O'Garra, 2005; Rubtsov *et al.*, 2008), although the source of IL-10 has been disputed as potentially being the suppressed effector cells rather than the Treg (Kearley *et al.*, 2005). The role of Treg-derived IL-10 has also been recognised in infectious disease models, in tumor microenvironments, and in feto-maternal tolerance, and its importance appearing to depend on the target organism or disease (Vignali *et al.*, 2008).

The IL-12 family member IL-35 is a novel inhibitory cytokine associated with Treg function. This heterodimeric cytokine is preferentially expressed by murine FOXP3+ Treg and upregulated upon activation of these cells, and not by resting or activated effector

cells (Collison *et al.*, 2007). Its role in Treg function is not completely clear and currently under scrutiny (Chaturvedi *et al.*, 2013).

Early studies exploring the role of TGF-β1 in Treg function by using neutralisation of the cytokine or TGF-β-deficient Treg suggested a limited role of TGF-β1 in Treg function (Piccirillo *et al.*, 2002). However, other studies, especially *in vitro* ones, suggested that TGF-β1 plays an active role in suppression of effector cells by Treg (Fahlen *et al.*, 2005; Li *et al.*, 2007). The relationship between Treg and TGF-β1 will be presented in a subsequent section.

1.3.5 Regulatory T cells (Treg) in disease

As shown in immune dysregulation, polyendocrinopathy, enteropathy, X-linked **syndrome** (IPEX) patients, FOXP3 and, consequently, Treg dysfunction can be a cause for disease as FOXP3+ Treg are dominant effectors of self-tolerance. Consequently, Treg have been involved in autoimmune/inflammatory diseases such as psoriasis (Mattozzi *et al.*, 2013), atherosclerosis (Pastrana *et al.*, 2012), juvenile systemic sclerosis (Reiff *et al.*, 2013), autoimmune hepatitis (Muratori & Longhi, 2013), type I diabetes (Kornete *et al.*, 2013), multiple sclerosis (Buc, 2013), immune thrombocytopenia (Nishimoto & Kuwana, 2013), inflammatory bowel disease (Mayne & Williams, 2013) and allergy (Thorburn *et al.*, 2013), as well as some infectious diseases (Rai *et al.*, 2012; de Lima Silva *et al.*, 2013; Larson *et al.*, 2013). CD25 deficiency has also been reported as causing Treg deficiency and chronic and severe inflammatory lung disease (Bezrodnik *et al.*, 2013).

Nonetheless, because of its role in suppression cytotoxic immune cells, Treg normal function has also been related to pathogenic processes such as tumor-induced immune suppression. FOXP3 expression and Treg have been related to progression in melanoma (Gerber *et al.*, 2013), breast cancer (Lal *et al.*, 2013), lymph node metastasis in gastric cancer (Zhou *et al.*, 2013), colon cancer (Xu *et al.*, 2013), ovarian cancer (Govindaraj *et al.*, 2013), human non small cell lung cancers (Ganesan *et al.*, 2013), and haematological malignancies (Kelley & Parker, 2010), amongst others.

In addition, problems with Treg function appear to be involved in pregnancy loss and miscarriage (Lee *et al.*, 2011; Inada *et al.*, 2013; Lu *et al.*, 2013).

1.3.6 Regulatory T cells (Treg) in transplantation

Transplantation of organs, tissues or cells inherently involves the participation of the immune system. Consequently, Treg are likely to play a role in the outcome of these medical procedures. In fact, donor Treg are believed to be able to generate recipient tolerogenic DC, which have an immature phenotype with low expression of HLA class II,

CD40, CD80/86 and IL-12, limiting their immunogenicity upon encountering donor antigen (Ichim *et al.*, 2003; Min *et al.*, 2003). These tolerogenic DC are in turn believed to induce Treg development, and this positive feedback loop is thought to play an important role in the maintenance of graft tolerance. Moreover, Treg have been identified as tolerance mediators in murine kidney (Hu *et al.*, 2013), cardiac (Fu *et al.*, 2013), and skin grafts (Graca *et al.*, 2002), with evidence of their involvement in containing graft rejection (Shalev *et al.*, 2012).

Transplantation of haematopoietic stem cells bears the peculiarity that immune cells are the main product of these procedures, and the restrictions and specific immune events that can develop after the transplant makes it inherently different from solid organ transplantation. Because of this difference and because it is the aim of this thesis, Treg role in this setting is treated in a separate section.

1.3.6.1 Regulatory T cells (Treg) in haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) has a number of complications that affect its success, some of which are immune derived. Graft-versus-host disease (GVHD) is one of the most important complications of HSCT. HSCT conditioning regimes create a massive release of proinflammatory cytokines that activate the patient's innate immune system, including APC. When the donor-derived T cells in the graft interact with activated APC, they recognise patient antigens as 'foreign' and become activated, unleashing a systemic immune response from the graft against the host, or GVHD (Billingham, 1966; Ferrara *et al.*, 2009).

1.3.6.1.1 Regulatory T cells (Treg) in graft-versus-host disease (GVHD) models

Studies in mice have identified a decrease in Treg during acute GVHD (aGVHD), allowing the proliferation of autoreactive inflammatory Th1 and Th17 donor T cells and the transition to chronic GVHD (cGVHD) (Chen *et al.*, 2007). Moreover, depletion of CD25+ T cells from the graft both before the transplant (ex vivo) and *in vivo* after the transplant were associated with worsening of GVHD, while adoptive transfer of ex vivo expanded Treg was beneficial in the control of the disease in this murine model (Taylor *et al.*, 2002). It was later confirmed that the adoptively transferred Treg must be of donor origin and able to produce IL-10 in order to prevent lethal aGVHD in murine models (Hoffmann *et al.*, 2002). Additionally, Treg suppression of GVHD was shown to be more effective if Treg were supplied early in the process (Edinger *et al.*, 2003). Later studies identified $CD62L^{hi}$ and not $CD62L^{lo}$ Treg as the main mediators of this *in vivo* protective effect against GVHD, implying that Treg trafficking to secondary lymphoid organs was necessary for alloreactive T cell control (Taylor *et al.*, 2004; Ermann *et al.*, 2005). Nonetheless, efficient

Treg expression of chemokine receptors such as CXCR3, CCR5, and CCR6, which enable T cells to travel to GVHD target organs such as the liver, the lungs or the intestine, has also proven necessary for Treg protection from GVHD (Wysocki *et al.*, 2005; Varona *et al.*, 2006; Hasegawa *et al.*, 2008).

Using a human *in vitro* skin GVHD model, the role of Treg in CD8 T cell-mediated GVHD prevention was confirmed, and it became clear that the presence of the Treg at the priming stage of the immune response was necessary for it to ensue (Wang *et al.*, 2009). Moreover, murine models of GVHD have also shown the necessity of alloantigen expression by the host APC, which is sufficient for induction of GVHD protection by donor Treg (Tawara *et al.*, 2010).

In a different HSCT model, transplantation of haematopoietic stem cells (HSC) performed with bone marrow-derived facilitating cells (defined as CD8+/TCR-) was shown to promote splenocyte TGF-β1 expression and induction of Treg-related genes *CTLA-4*, *GITR* and *FOXP3*, allowing for improved engraftment and prevention of GVHD by *in vivo* induction of Treg (Colson *et al.*, 2004).

The role of iTreg has also been studied in the context of GVHD by *in vitro* induction of these cells followed by adopted transfer into mice. Interestingly, there have been contradicting results with respect to the nTreg experiments mentioned previously. In fact, administration of *in vitro* generated iTreg along with bone marrow grafts has not resulted in significant protection from GVHD, and these cells have been shown to have limited survival and to revert their FOXP3 expression *in vivo* shortly after transplantation (Koenecke *et al.*, 2009). Notably, this FOXP3 expression reversion has also been determined for murine nTreg, a phenomenon that appears to be driven by the inflammatory cytokine milieu to which these cells are infused (Laurence *et al.*, 2012). Furthermore, FOXP3-expressing CD8+ T cells, initially described to suppress T cell responses in murine models of autoimmunity and allergic diseases (Hahn *et al.*, 2005; Laurence *et al.*, 2012), have recently been reported to arise by induction early in the development of GVHD. These cells have been shown to mitigate the severity of this disease along with CD4+ Treg in MHC-mismatched HSCT murine models, in which they comprise up to 70% of the iTreg population (Beres *et al.*, 2012; Robb *et al.*, 2012; Sawamukai *et al.*, 2012). These findings have been also confirmed with human CD8+ FOXP3+ T cells in a humanized mouse model (Zheng *et al.*, 2013).

1.3.6.1.2 Regulatory T cells (Treg) in graft-versus-host disease (GVHD) patients

The important role of Treg in the control of immune responses and the evidence of their capacity to ameliorate GVHD from murine models have also prompted research into their

quantitative and qualitative behavior in patients that undergo HSCT aiming at identifying early biological signals that could precede the onset of this complication. A number of studies have reported a decrease in the frequency of Treg in the peripheral blood of HSCT patients that develop high-grade aGVHD in comparison with those of HSCT patients with no or mild disease (Li *et al.*, 2010; Bremm *et al.*, 2011). In another study, patients that underwent autologous HSCT or allogeneic HSCT without GVHD had higher Treg frequencies (defined as CD4+CD25^{hi}FOXP3+ cells) in peripheral blood when compared to allogeneic HSCT patients with GVHD (Magenau *et al.*, 2010). Treg frequencies and absolute numbers in this study were reported to decrease linearly with increasing grades of GVHD at onset, and to correlate with the maximum grade of GVHD. Also, the frequency of Treg at the onset of GVHD was able to predict the response to GVHD treatment. Moreover, patients with Treg frequencies less than the median had higher non-relapse mortality (NRM) than patients with Treg greater than the median, resulting in an inferior survival 2 years after transplantation. Another study also reported a marked depletion of naïve Treg in patients developing aGVHD compared with tolerant patients. Despite their lower frequency, single-cell profiling showed that CD4+FOXP3+ T cells maintain the Treg gene expression signature and Treg suppressive activity was preserved (Dong *et al.*, 2013). As a proxy for Treg persistence and reconstitution, FOXP3 mRNA expression in PBMC after HSCT has been studied. FOXP3 mRNA expression was reported to be significantly decreased in PBMC from patients with either allogeneic GVHD or autologous GVHD compared with patients without GVHD. Expression of Foxp3 correlated negatively with the severity of GVHD but did so positively with recent thymic emigrants (Miura *et al.*, 2004).

Findings similar to those reported for aGVHD have also been published in the setting of cGVHD (Zorn *et al.*, 2005). Moreover, patients transplanted from HLA-identical sibling donors with higher nTreg (defined as FOXP3+Helios+ cells) counts and higher thymic output of Treg presented lower incidence of cGVHD when compared to patients whose donors had lower levels (McIver *et al.*, 2013).

One recent study assessed the role of Treg reduction before GVHD onset (Fujioka *et al.*, 2013). According to this report, the authors measured Treg (CD4+FOXP3+ cells) in peripheral blood at the second week after transplantation, and found that patients with aGVHD had significantly lower Treg/CD4+ T cell ratios than those without aGVHD. Moreover, the cumulative incidence of aGVHD in patients with ratios lower than 9% was significantly higher than that in patients with ratios higher than 9%. Since these differences were seen before the development of aGVHD, the authors propose that these ratios could actually predict the incidence of aGVHD.

In a study of the genetic profile of Treg in patients after HSCT (Ukena *et al.*, 2011), the authors monitored the transcriptome of these cells in consecutive monthly blood samples for a six-month period. Using microarray technology, the genetic expression profile of isolated Treg ($CD4^+CD25^+CD127^{lo}$) was shown to be stable and highly correlated between GVHD and no GVHD groups at several time points after transplantation. However, detailed analysis of the transcriptomes of these groups revealed that Treg isolated from patients with severe GVHD showed downregulation of molecules related to migration and homing to sites of inflammation and secondary lymphoid organs such as CCR5, CXCR3, CCR3, CXCR6 and CCR1. Moreover, a downregulation of the expression of molecules associated with Treg suppressive mechanisms, such as LAG3, CD44, galectin-1, and granzyme A, was also identified in cells from GVHD patients. On the contrary, the upregulation of molecules such as granzyme A, CXCR3 and CCR5 at the mRNA and protein levels was observed for immune tolerant patients early after HSCT (less than 100 days). Taken together, these data are proposed as evidence that impaired migration of Treg to GVHD development sites could prevent control of alloreactive T cells.

Another study of Treg in GVHD focused on telomere length and telomerase activity in HSCT patients with severe cGVHD. The authors found that telomere length was reduced and telomerase activity increased in Treg when compared to conventional T cells. Moreover, Treg number and telomerase activity were inversely correlated with the severity of cGVHD, suggesting that failure to activate Treg telomerase could restrict their proliferative capacity and, consequently, hamper control of allo-reactive T cell (Kawano *et al.*, 2011).

Another study investigating Treg behavior in cGVHD after myeloablative HSCT found that thymic generation of naive Tregs was markedly impaired, and reconstituting Tregs showed a predominantly activated/memory phenotype. The authors also report that Treg underwent higher levels of proliferation than conventional T cells in response to $CD4^+$ lymphopenia after HSCT, but Treg undergoing homeostatic proliferation also showed increased susceptibility to Fas-mediated apoptosis. Monitoring of $CD4^+$ T cell subsets at 3, 6, 9, 12, 18, and 24 months post-transplant revealed that Treg expanded rapidly and achieved normal levels by 9 months after HSCT, but Treg levels subsequently declined in patients with prolonged $CD4^+$ lymphopenia. The authors claim that this resulted in a relative deficiency of Treg, which was associated with a high incidence of extensive chronic GVHD in their study cohort (Matsuoka *et al.*, 2010).

Other studies have examined the role of Treg in tissues rather than in peripheral blood of HSCT patients with GVHD. In one study, the authors assessed the number of infiltrating Treg in the intestinal mucosa by using intestinal biopsies from HSCT patients and

compared them to healthy controls and patients with infectious inflammation. Patients with infectious intestinal inflammation showed a concomitant increase of CD8+ T cells and Treg (CD3+FOXP+ cells). However, patients with acute and cGVHD displayed a FOXP3+/CD8+ T-cell ratio identical to control subjects screened for other intestinal conditions. In contrast, specimens without histologic signs of GVHD demonstrated increased numbers of FOXP3+ per CD8+ T cells (Rieger *et al.*, 2006).

In contrast, a number of studies evaluating Treg in GVHD in humans have had opposite results as to those aforementioned. Some studies have actually found an increase in peripheral blood Treg in HSCT patients with cGVHD. In an early study (Clark *et al.*, 2004), the authors evaluated the number of Treg more than 100 days after allogeneic hematopoietic stem cell transplantation. Patients with cGVHD showed markedly elevated numbers of donor-derived CD4+CD25^{hi} T cells as compared to patients without GVHD. Interestingly though, in contrast to controls, CD4+CD25^{hi} T cells in patients with cGVHD were characterized by lower surface CD62L expression. As mentioned earlier, CD62L expression was reported as necessary for GVHD mitigation in mouse models. Also, their characterisation of Treg did not involve FOXP3 or CD127, and the possibility remains that non-regulatory T cells were included in their analysis. In another small study, the recovery of CD4+CD25^{hi}CD127^{lo} Treg in the peripheral blood of patients was assessed every 30 days for the first 6 months after HSCT (Ukena *et al.*, 2011). Despite progressively improving, the Treg count in GVHD patients always remained lower than in patients who never developed GVHD. In contrast, cGVHD patients who had not developed aGVHD displayed significantly increased Treg cell numbers at the onset of chronic inflammation. The authors interpret these results by stating that Treg counts after HSCT are associated with the development of acute but not chronic GVHD. In a study that used FOXP3 mRNA quantification in peripheral blood after HSCT as a proxy for Treg reconstitution, the authors found that the relative expression levels of FOXP3 mRNA did not significantly correlate with the occurrence of GVHD, and suggested that Treg frequency in peripheral blood relative to total leukocytes or T cells is not indicative of the development of GVHD (Arimoto *et al.*, 2007). Finally, with respect to Treg in tissue, one report examining FOXP3+ Treg in blood and in gastric antral biopsies in a cohort of 60 allogeneic HSCT patients did not find any difference in the expression of this marker with or without GVHD involving the upper gut. Furthermore, there was no correlation of Treg frequency with the histologic or clinical severity of GI GVHD in these patients (Lord *et al.*, 2011).

1.3.6.1.3 Regulatory T cells (Treg) as prognostic factor for graft-versus-host disease (GVHD)

Despite the evidence of the potential usefulness of monitoring Treg frequencies or absolute numbers early after HSCT as a means of identifying those in higher risk of developing GVHD, the evidence for the role of Treg in GVHD pathogenesis is hampered by the fact that Treg behaviour before, during and after GVHD onset could be the cause or part of the cause, but also a consequence of GVHD. GVHD involves the massive expansion of activated effector T cells, which could mean that the Treg population is overwhelmed by this outburst. Also, the inflammatory milieu characteristic of GVHD could impair the generation of iTreg, and could also potentially revert FOXP3 expression in nTreg, further affecting these subsets' numbers. Additionally, damage to tissues such as the thymus during GVHD (or previously during conditioning regime application) could hamper their capacity to generate Treg. Furthermore, the place of Treg assessment (i.e. peripheral blood, lymph nodes, thymus, GVHD target tissues, etc.) and the timing of these measurements could give different pictures and have different relevance in terms of the specific GVH process. All these caveats must be taken into account when critically analysing data from studies that report changes in Treg related to GVHD and/or HSCT outcome. More precise research into this matter is necessary.

Apart from the post-HSCT assessment of Treg frequency or counts, the Treg content of the graft before transplantation by measurement of Treg in the blood of stem cell donors has also been explored as a prognostic factor for GVHD. Some reports have found a correlation between higher frequencies of CD4+FOXP3+ Treg in peripheral blood of the donors (Rezvani *et al.*, 2006) or in cryopreserved aliquots of the donor grafts both in unrelated (Pabst *et al.*, 2007) and sibling transplants (Wolf *et al.*, 2007) and lower incidences of GVHD in their recipients, especially in myeloablative transplants. However, in one of the studies, the correlation did not hold in multivariate analysis (Pabst *et al.*, 2007). A study examining $CD62L^{hi}$ Treg ($CD4+CD25^{hi}CD45RA+$) in HSCT grafts found that patients infused with lower concentrations of these naïve Treg exhibited an increased incidence of aGVHD, and that delayed reconstitution of these subset in patients was associated with the development of aGVHD (Lu *et al.*, 2011).

1.3.6.1.4 Regulatory T cells (Treg) in the prevention and treatment of graft-versus-host disease (GVHD)

The evidence of Treg involvement in GVHD control has evidently prompted research into the manipulation, production or promotion of Treg function in order to help improve the outcome of HSCT (Edinger & Hoffmann, 2011).

In an animal model study, the authors used recipient spleen cells to stimulate a mixed leukocyte reaction to generate antigen-induced Treg in the presence of TGF-β1 and retinoic acid, and found that the $CD11c^{hi}$ DC fraction induced high numbers of alloreactive Foxp3+ cells. These induced CD4+CD25+Foxp3+ cells were purified and shown to be suppressive *in vitro* in an antigen-specific manner. After transfer into allogeneic mice, the induced Treg persisted for 6 months and prevented GVHD caused by co-transferred effector T cells. Similar findings were made when the Treg cells were transferred after onset of GVHD (Sela *et al.*, 2011).

For human trials, strategies such as *in vivo* induction of Treg or Treg ex vivo expansion followed by re-infusion have been explored as potential therapeutic alternatives. However, concerns about the correct way to phenotypically define Treg, Treg source, heterogeneity (e.g. nTreg vs iTreg), stability both *in vitro* and *in vivo*, the right timing for Treg infusion, as well as the efficiency of industrial and clinically-approved production of Treg derivatives continue to hold back broader application of this therapy. Nevertheless, progress in the first small clinical trials has been promising in some areas, proving the safety and preliminary efficacy of Treg transfusion (Brunstein *et al.*, 2011; Di Ianni *et al.*, 2011; Di Ianni *et al.*, 2011). Also, the sub-cutaneous application of daily low-dose IL-2 has been explored as a way to favor Treg development *in vivo*, altering the Treg:Tcon ratio in glucocorticoid-refractory cGVHD patients with some success, albeit in a small pilot study (Koreth *et al.*, 2011). Also, in a mouse HSCT model, the transfer of *in vitro* expanded human cord blood Treg was shown to significantly prevent aGVHD and to correlate with increases in TGF-β1 production and FOXP3 expression in CD4+ cells, polarizing the Treg/Th17 balance toward Treg (Yang *et al.*, 2012).

1.4 Transforming growth factor-β1 (TGF-β1)

TGF-β is a cytokine that has three homologous isoforms, which are encoded by three different genes in mammals: TGF-β1, TGF-β2, and TGF-β3. TGF-β1, together with TGF-β2 and TGF-β3, is a member of a highly pleiotropic family of proteins, including bone morphogenic proteins, activins, and growth differentiation factors that are involved in the regulation of numerous immunomodulatory processes, in addition to having pleiotropic effects on cell proliferation, differentiation, migration and survival (Li *et al.*, 2006). TGF-β1 is produced by, and acts upon, a wide variety of cells. The processes in which TGF-β1 plays a central role include embryogenesis, hematopoiesis, angiogenesis, fibrosis, wound healing, carcinogenesis, immune responses, and transplantation (Li *et al.*, 2006).

The TGF-β system is evolutionarily very old, thought to have appeared approximately one billion years ago, previous to the divergence between arthropods and vertebrates and to the development of lymphocyte-based adaptive immunity (Newfeld *et al.*, 1999). TGF-β1 is the predominant isoform expressed in the immune system (Li *et al.*, 2006), and its role in the control of immunity was confirmed since the 1990s when experiments in mice with a homozygous mutation preventing TGF-β1 production showed no major developmental abnormalities, but died early after birth because of a wasting syndrome accompanied by a multifocal inflammatory cell response with tissue necrosis, leading to organ failure (Shull *et al.*, 1992).

1.4.1 Biology, structure and function of transforming growth factor-β1 (TGF-β1)

1.4.1.1 Production, structure and activation of TGF-β1

As all three TGF-βs, TGF-β1 is synthesized as a 390-amino acid pre-pro-TGF-β1 (Annes *et al.*, 2003). The pre region contains a signal peptide (amino acids 1-29). The 75-kilo Dalton (kDa) pro-TGF-β1 is cleaved in two portions by furin-like proteases in the trans Golgi apparatus. The N-terminal portion is called latency-associated peptide (LAP, amino acids 30-278), and a homodimer of this molecule binds non-covalently to a homodimer of the mature TGF-β1 (amino acids 279-390) of 24 kDa. This forms an inactive complex called the small latent complex (SLC) that can be secreted as such or form the large latent complex (LLC) when bound to a latent TGF-β1 binding protein (LTBP). Cysteines in LAP form disulphide bonds with specific cysteines in the LTBP. The LLC covalently targets TGF-β1 to the extracellular matrix. **Figure 1.1** shows a schematic representation of TGF-β1's precursor as well as its SLC and LLC.

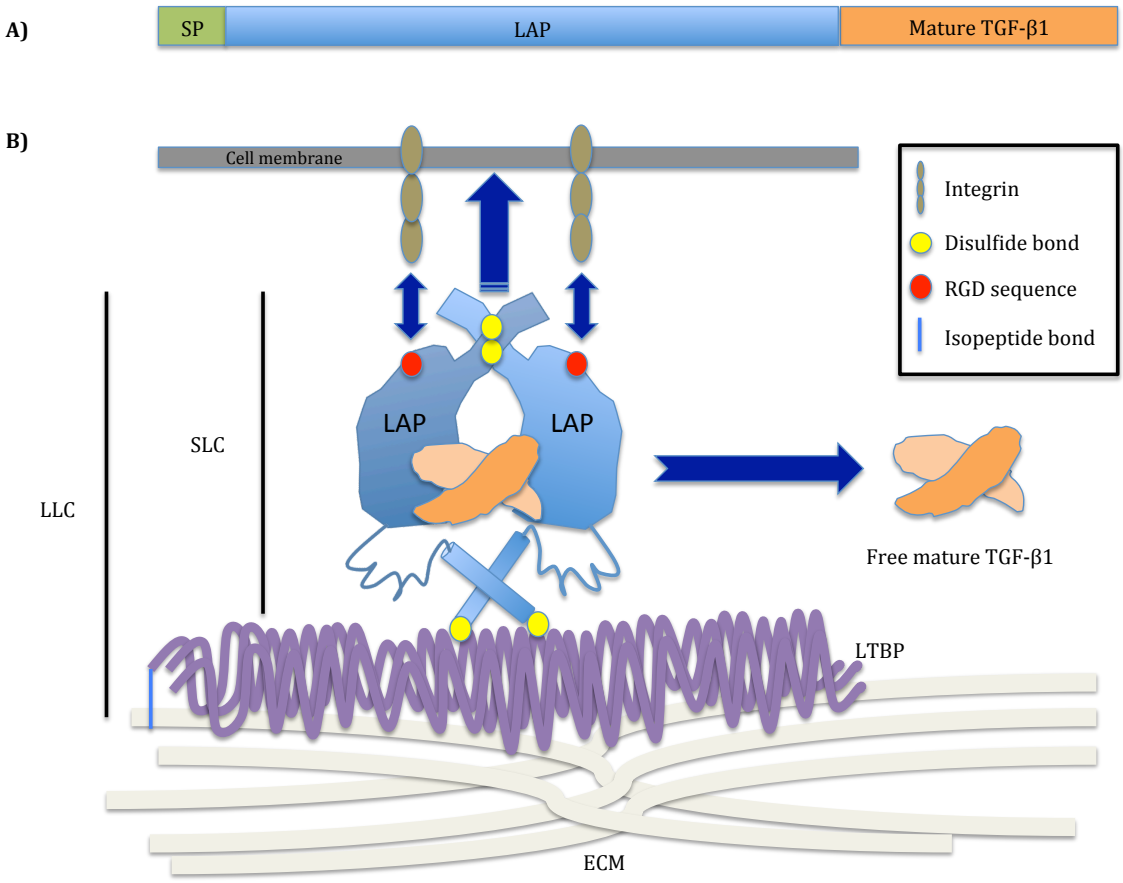


Figure 1.1 Molecular organization of transforming growth factor-β1 (TGF-β1). (A) The TGF-β1 precursor and its components. (B) Molecular organization of the processed TGF-β1 precursor and its activation by integrins. Cleavage of the TGF-β1 precursor produces LAP and mature TGF-β1. Mature TGF-β1 dimers are enclosed in a protective straightjacket structure by LAP dimers (the SLC), covalently linked by disulphide bonds. The SLC can be in turn attached to a LTBP, also by disulfide bonds (the LLC). The LLC is bound to the ECM by isopeptide bonds. Integrins on the surface of cells bind RGD motifs on LAP and exert a force capable of extending LAP and opening the SLC's straightjacket, thus releasing the mature and biologically active TGF-β1 dimer. ECM, extracellular matrix; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β1–binding protein; SLC, small latent complex; SP, signal peptide.

Hydrophobic residues on one side of the LAP molecule, which form an amphipatic α-helix, interact with the mature TGF-β1 to form the SLC, while TGF-β1-induced conformational changes in LAP expose ionic residues on the other side of the helix, which, in turn, interact with the LTBP (Walton *et al.*, 2010). Covalent dimerization of LAP is necessary for the stability of the LLC. Recent progress in defining the crystal structure of the TGF-β1 latent complex has revealed that its conformation is that of a ring-shaped straightjacket, in which LAP shields the mature cytokine and prevents it from interacting with receptors. Release of TGF-β1 from this structure requires tensile force and the unfastening of the straightjacket (Shi *et al.*, 2011), concomitant with LLC binding to the extra-cellular matrix (Buscemi *et al.*, 2011) (See **Figure 1.1**).

TGF-β1 is inactive whilst bound to LAP or in the LLC. These complexes form intracellularly, and TGF-β1 that fails to form a complex with these structures is inefficiently secreted (Kim *et al.*, 1992). Activation of latent TGF-β1 is dependent on its liberation from these molecules by the action of other molecules, extreme pH or temperature. Physiologically, the activation may be carried out by interaction with thrombospondin, proteolytic cleavage by transglutaminase, plasmin, or traction mainly by $\alpha_v\beta_6$ or $\alpha_v\beta_8$ integrins, as well as by reactive oxygen species (Annes *et al.*, 2003; Shi *et al.*, 2011).

LAP contains integrin binding sites bearing the sequence RGD (arginyl-glycyl-aspartic acid). Six integrins can bind the SLC: $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_v\beta_1$, $\alpha_8\beta_1$, the first four being actually able to release TGF-β1 (Worthington *et al.*, 2011). $\alpha_v\beta_6$-mediated activation involves a conformational change in LAP and not the release of mature TGF-β1 from the complex, and assumes a cell-contact dependent presentation of active TGF-β1 (Munger *et al.*, 1999). A different strategy is employed with $\alpha_v\beta_8$ integrin, in which participation of metalloproteinases that degrade LAP is necessary, and that allows for a more distant reach of the released active TGF-β1 (Mu *et al.*, 2002). $\alpha_v\beta_6$ integrin is expressed in a subset of epithelial cells, whereas $\alpha_v\beta_8$ is expressed in many immune cells, including T cells and myeloid DC. Of note, an indispensable role for integrins in the *in vivo* activation of TGF-β1 has been recognised in mice with a homozygous mutation of *TGFB1* that disrupts the integrin-binding motif in LAP and causes a lethal inflammatory phenotype indistinguishable of TGF-β1-deficient mice (Yang *et al.*, 2007).

1.4.1.2 Signaling and function of transforming growth factor-β1 (TGF-β1)

The members of the TGF-β1 superfamily of cytokines mediate their effect via receptors that are type I or type II serine/threonine kinases. Five type I (activin receptor-like kinase, ALK1-5) and seven type II TGF-β superfamily receptors have been described. TGF-β1, 2,

and 3 mediate their signaling mainly through ALK5 and TGF-β receptor II (TβRII) (Chang *et al.*, 2002). For the signaling process to take place, active dimeric TGF-β1 molecules bind TβRII first, and then, recruit ALK5 molecules to form a tetrameric receptor complex. The binding of the TGF-β1 molecules causes TβRII to activate recruited ALK5 by phosphorylation. Subsequently, the type I receptor phosphorylates intracellular transcription factors Smad2 and 3, which then associate with Smad4 and this complex translocates to the nucleus in order to bind to a Smad-binding element in DNA and to other transcription factors, activating or repressing target genes. On the contrary, Smads 6 and 7 are inhibitory for TGF-β1 signaling. Smad7, which is not phosphorylated by the receptors, is induced by and suppresses TGF-β1 signaling by competition with Smad2 and 3 for the binding of ALK5, and by recruitment of Smurf- containing E3 ubiquitinase complexes that cause ALK5 degradation, thus participating in a negative feedback loop to control TGF-β1 responses (Nakao *et al.*, 1997; Kavsak *et al.*, 2000; von Gersdorff *et al.*, 2000). Smad7 is also induced by inflammatory cytokines INF-γ, TNF-α, and IL-6 (Ulloa *et al.*, 1999; Bitzer *et al.*, 2000; Dominitzki *et al.*, 2007). In mice, Smad6 interferes with the phosphorylation of Smad2 and the subsequent heteromerization with Smad4, but does not inhibit the activity of Smad3 (Imamura *et al.*, 1997). Of note, Smad-independent signaling pathways using mitogen-activated protein kinase, PI3K kinase, PP2A phosphatase, Rho family proteins, and the epithelial polarity protein Par6 have also been described for TGF-β1 (Yu *et al.*, 2002; Derynck & Zhang, 2003), but involvement of Smad6 in the regulation of some of these non-canonical pathways suggests cross control with Smad-dependent signaling (Jung *et al.*, 2013).

TGF-β1 signaling inhibits the proliferation of epithelial, endothelial and hematopoietic cells, and regulates the differentiation of neural, immune, mesenchymal and epithelial cells. TGF-β1 signaling can also initiate apoptosis in specific contexts, and participates in chromosomal stability maintenance (Massague, 2000). However, depending on the local environment and concentration, as well as the target cell, TGF-β1 can also stimulate proliferation and be anti-apoptotic, as well as increase or decrease the function of terminally differentiated cells (Ruscetti *et al.*, 2005). Accordingly, TGF-β1 signaling has been implicated in the pathogenic processes of various cancers and haematopoietic malignancies by targeting of the malignant cell, the tumor stromal cells or by modulation of angiogenesis (Ikushima & Miyazono, 2010). TGF-β1 is the most potent inhibitor of cell cycle progression in committed hematopoietic progenitors, potentially through an autocrine mechanism (Ruscetti *et al.*, 2005), and the disruption of TGF-β1 signaling is known to play a role in malignant transformation. The current paradigm suggests that

TGF-β1 acts as a tumor suppressor in early stages of tumorigenesis, but promotes tumor growth during cancer progression (Bierie & Moses, 2006).

The presence of receptors for TGF-β1 on early haematopoietic progenitors throughout all their maturation stages suggests that this cytokine plays an important role in their biology. Indeed, bone marrow cells from neonatal mice with deleted TGF-β1 showed impaired hematopoietic stem cell short- and long-term reconstitutive activity. This was associated with a parallel decrease in *in vivo* homing capacity of these cells, as well as reduced survival of immature progenitors *in vitro* (Capron *et al.*, 2010). Moreover, myeloid and lymphoid stem cells seem to respond differently to TGF-β1: TGF-β1 stimulates myeloid stem cell proliferation but inhibits their lymphoid counterparts (Challen *et al.*, 2010).

Another relevant target of TGF-β1 are thymic epithelial cells. As mentioned previously, epithelial cells in the thymus participate in the education of lymphoid precursors and the production of naïve T cells. TGF-β1 signaling has been shown to promote thymic senescence in mice, and to hinder early thymic reconstitution after myeloablative conditioning and subsequent HSCT. Moreover, inhibition of TGF-β1 signaling appears to decelerate age-related thymic involution and could favor the reconstitution of thymic output after the transplant (Hauri-Hohl *et al.*, 2008).

1.4.1.3 Regulation of transforming growth factor-β1 (TGF-β1) production and activity

In line with its relevance in many biological processes, the regulation of the expression of TGF-β1 has been shown to be tightly controlled. In T cells, TGF-β1 production upon activation has been known since early studies (Kehrl *et al.*, 1986). CD69, a marker of activation, has been shown to induce TGF-β1 production upon cross-linking, both in CD4+ and CD8+ cells (Esplugues *et al.*, 2003). CTLA-4 engagement has been a disputed induction stimulus for TGF-β1 production (Chen *et al.*, 1998; Sullivan *et al.*, 2001). Also, Th1 conditions inhibit TGF-β1 production, while Th2 conditions appear to enhance it (Seder *et al.*, 1998). In fact, T-bet-deficient CD4+ cells produce more TGF-β1 than normal ones in colitis models (Neurath *et al.*, 2002).

Little is known about the transcriptional control of TGF-β1 expression. The activator protein 1 complex (jun and fos proteins) has been implicated in TGF-β1's autoinduction of transcription (Kim *et al.*, 1990). However, negative regulation of *TGFB1* expression by the activator protein 1 complex has also been proposed (Shah *et al.*, 2006). Recently, the early growth response 3 transcription factor has also been recognised to intervene in the expression of TGF-β1 in both murine and human CD4+ T cells *in vivo* (Sumitomo *et al.*, 2013).

Several studies have suggested the presence of post-transcriptional regulation for TGF-β1 and an unusually long and GC-rich 5'-untranslated region (5'UTR) in its mRNA has been implicated in these processes (Kim *et al.*, 1992). TGF-β1 mRNA is poorly translated, and its 5'UTR has been shown to form stable secondary structures that inhibit translation by altered binding to the YB-1 protein (Jenkins *et al.*, 2010). Moreover, the 3'UTR in TGF-β1 mRNA has also been implicated in post-transcriptional regulation, with two forms (short and long) that are differentially expressed and serve as targets for micro RNA 744, which strongly represses TGF-β1 translation (Martin *et al.*, 2011).

The production of TGF-β1 in the form of the SLC and the LLC, in which it is inactive and requires specific stimuli or molecules for its activation, are another level of control that is added to this cytokine's system. In fact, human tissues contain relevant amounts of latent TGF-β1, but only a minor fraction is normally activated in biological processes.

1.4.1.4 Genetics of transforming growth factor-β1 (TGF-β1)

TGF-β1 is encoded by the *TGFB1* gene located in chromosome 19q13.1-q13.3 (Shah *et al.*, 2006). *TGFB1* has 7 exons, and regulatory activity for this gene has been mapped to approximately 3.0 kilobases (kb) from positions -2665 to +423 (+1 being the translation start site). This region includes two promoter sites, flanking the major transcription site, two negative regulatory elements and two enhancers lying upstream of the first promoter. The *TGFB1* promoters contain activator protein 1 binding sites, allowing for the binding of transcription factors such as c-jun and c-fos. In addition, stimulatory protein 1, early growth response gene 1 and upstream stimulating factor 1 are thought to also participate in *TGFB1* gene regulation (Shah *et al.*, 2006).

1.4.1.4.1 TGFB1 polymorphism and its functionality

According to GeneCards Human Gene Database (http://www.genecards.org/ v. 3.10.108, 24 October 2013) (Safran *et al.*, 2010), 624 polymorphisms have been described in *TGFB1* and its surrounding and regulatory regions, most of which (65%) are located in introns. At least 57 of these polymorphisms cause amino acid changes. Most of the polymorphisms are single nucleotide polymorphisms (SNPs), and at least 20 of them lie within the 2.7 kb regulatory region and exon 1 and have been described to be arranged in at least 17 alleles (Shah *et al.*, 2006; Shah *et al.*, 2009). **Figure 1.2** shows a schematic representation of these polymorphisms in *TGFB1*'s regulatory region. The variant id numbers of these positions and their location in chromosome 19 are given in **Appendix A**.

Many of the polymorphisms found within *TGFB1*'s regulatory region appear to influence the expression of TGF-β1 and to explain inter-individual differences in the levels of this cytokine. The -1347C>T (also known as -509C>T) of the transcriptional start site is

considered as +1; rs1800469) located in the proximal negative regulatory region, has been shown to mediate differences in TGF-β1 plasma and transcription levels (Grainger *et al.*, 1999; Silverman *et al.*, 2004). Individuals bearing the T allele present higher plasma levels than those who bear the -1347C. In fact, normal individuals with a homozygous TT genotype for this SNP had almost twice the amount of circulating TGF-β1 than CC individuals (Grainger *et al.*, 1999). Moreover, the -1347T allele has been associated with a 30% increase in transcription as analysed by reporter constructs and transient transfection in experiments using human lung carcinoma cells and bronchial epithelial cells (Silverman *et al.*, 2004). Additionally, this polymorphism, as well as -2726G>A and -2389_-2391dupAGG, is predicted to alter DNA-protein complex formation in electrophoretic mobility shift assays (Healy *et al.*, 2009). In *in silico* modeling analyses, alleles -2726A and -1347T were predicted to cause the loss of specific binding sites for repressor complex proteins AP1 and AP4, and thus promoting *TGFB1* expression. However, in a study carried out in a cohort of Chinese individuals, the -1347C allele showed higher reporter gene activities in plasmid experiments, as well as higher plasma TGF-β1 concentration in hepatitits B virus-infected liver cirrhosis patients that had this allele (Wang *et al.*, 2008).

Another two SNPs in the regulatory region of *TGFB1*, -1287G>A and -387C>T, which are common in African Americans, have been shown to modify the binding affinity of transcription factor complexes and, consequently, the expression levels of this gene in reporter vector experiments (Shah *et al.*, 2006). The -1287A allele showed a 78% increase in gene expression with respect to those constructs containing the G allele, which was explained by the higher affinities of two DNA binding factors to the former. The -387 C>T allele showed differential binding of nuclear proteins, including Sp1 and Sp3, which are known regulators of *TGFB1* expression (Kim *et al.*, 1989).

Two polymorphisms within *TGFB1*'s signal peptide region, +29T>C (rs1982073) and +74G>C (rs1800471), cause changes to the amino acid sequence of the pre-pro-TGF-β1, and have also been associated with differential production of this cytokine. The +29T>C causes a change from a leucine (Leu) to a proline (Pro) in *TGFB1*'s codon 10, and experiments with HeLa cells have shown that cells bearing the C allele see a 2.8-fold increase in TGF-β1 production (Dunning *et al.*, 2003). Another study found that among Japanese male myocardial infarction patients and controls, the serum concentration of TGF-β1 was significantly increased in +29 CC individuals when compared to +29 TT or TC subjects (Yokota *et al.*, 2000). Conversely, in a study performed in postmenopausal German women, Hinke *et al*. (2001) found that serum levels of TGF-β1 were higher in women with the +29 TT genotype than in those with a CC (Hinke *et al.*, 2001).

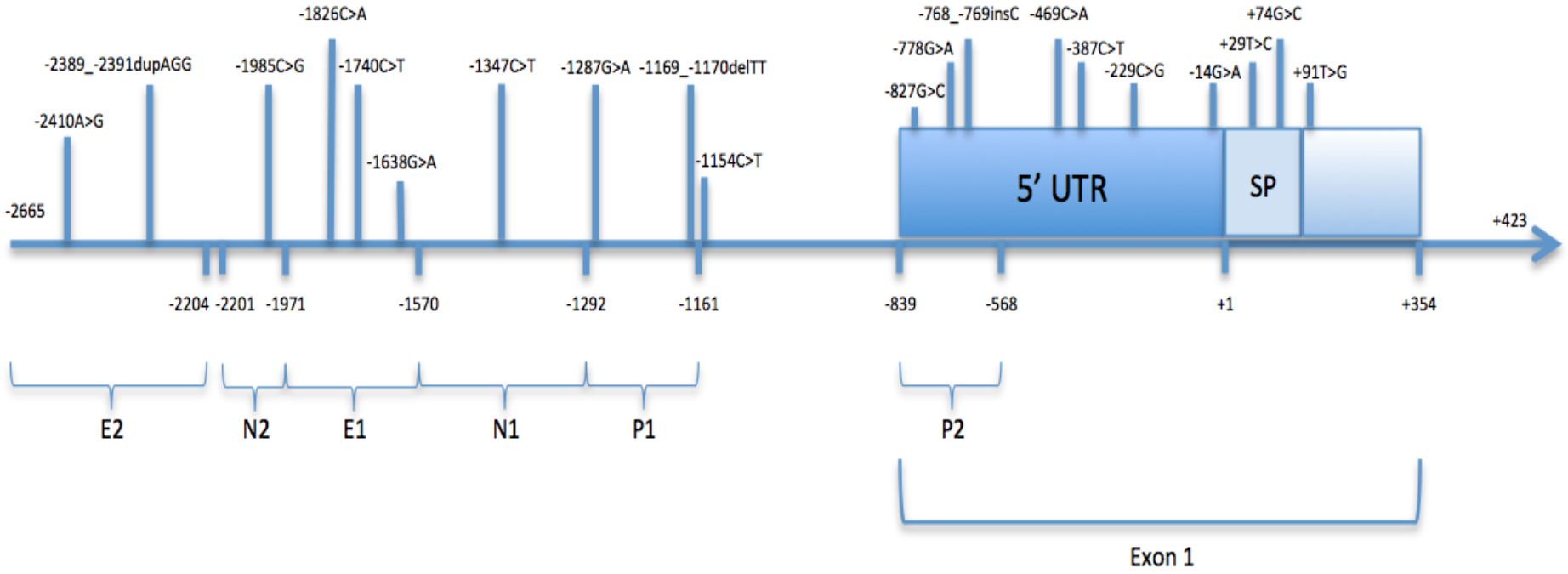


Figure 1.2 The *TGFB1* regulatory region and exon 1 (-2,665 to +423). Position +1 is the translation start site. Exon 1 (box) encodes the 5' untranslated region (5' UTR), signal peptide (SP) and part of the pro-protein. Regulatory elements include the promoters (P1 and P2), two negative regulatory regions (N1, N2) and the enhancer regions (E1, E2). The relative positions of SNPs are indicated. Figure is not to scale. Modified from Shah R. *et al.* (2006).

Similarly, lymphocytes isolated from individuals homozygous for the +74G allele, which encodes an arginine at position 25 of the TGF-β1 precursor, and stimulated *in vitro* produced significantly higher amounts of TGF-β1 after stimulation when compared to lymphocytes from heterozygous individuals who bear a Pro (Awad *et al.*, 1998). The functional effect of other polymorphisms has not been sufficiently addressed yet.

Given the diverse involvement of TGF-β1 in physiological processes, changes in its levels have been associated with disease generation and progression. Consequently, polymorphisms in *TGFB1* have in turn been associated with the development or modification of several diseases, mostly related to fibrotic (Arkwright *et al.*, 2000; Gewaltig *et al.*, 2002; Eurich *et al.*, 2011), immune (Arkwright *et al.*, 2001; Pulleyn *et al.*, 2001; Crilly *et al.*, 2002; Sugiura *et al.*, 2002; Silverman *et al.*, 2004), endocrine (Yamada 2000; Jia *et al.* 2011), neurologic (Luedecking *et al.*, 2000), vascular (Yokota *et al.*, 2000; Holweg *et al.*, 2001; Holweg *et al.*, 2001; Niu, 2011) or neoplastic processes (Saha *et al.*, 2004; Berndt *et al.*, 2007; Wei *et al.*, 2007). *TGFB1* polymorphism has also been associated with pregnancy loss (Magdoud *et al.*, 2013).

More specifically, the -1347C>T has been associated with increased susceptibility and severity of asthma (Silverman *et al.*, 2004), risk of developing breast cancer (Saha *et al.*, 2004) and Alzheimer's disease (Luedecking *et al.*, 2000). Also, polymorphism at codon 25 has been associated with atopic dermatitis (Arkwright *et al.*, 2001) and progression of graft fibrosis after liver transplantation (Eurich *et al.*, 2011).

The presence of a Pro at codon 10 of TGF-β1 has been associated with protection from osteoporosis (Yamada, 2000), reduced incidence of rheumatoid arthritis (Sugiura *et al.*, 2002), increased risk of systemic sclerosis (Crilly *et al.*, 2002), asthma (Pulleyn *et al.*, 2001), pulmonary dysfunction with cystic fibrosis (Arkwright *et al.*, 2000), progression of liver fibrosis in hepatitis C (Gewaltig *et al.*, 2002), myocardial infarction and heart failure (Yokota *et al.*, 2000; Holweg *et al.*, 2001), hypertension (Niu, 2011), graft vascular disease after heart transplant (Holweg *et al.*, 2001), advanced colorectal carcinoma (Berndt *et al.*, 2007), esophageal squamous cell carcinoma (Wei *et al.*, 2007) and diabetic nephropathy (Jia *et al.*, 2011).

1.4.2 Transforming growth factor-β1 (TGF-β1) in immune responses

All leukocytes produce at least one form of TGF-β (Li *et al.*, 2006), and TGF-β1 is the predominant isoform expressed in the immune system. This cytokine intervenes in multiple processes during immune responses, and nearly all leukocytes respond to it.

TGF-β1 inhibits B cell proliferation, induces apoptosis of immature and resting B cells, and blocks of B cell activation and antibody class switching (except to immunoglobin (Ig) A, which is promoted in human cells) (Lebman & Edmiston, 1999). TGF-β1 prevents both B lymphocyte progenitors and mature B cells from proliferating by cell cycle arrest. By preventing antibody class switching, TGF-β1 plays an important role in preventing B cell responses to low-affinity antigens and the promotion of B cell tolerance to self-antigens *in vivo* (Li *et al.*, 2006). On the contrary, TGF-β1 strongly promotes IgA when B cells are optimally stimulated by antigen and cytokines, playing a central role in the prevention of mucosal infection and immunity to orally or intranasally administered antigens (Borsutzky *et al.*, 2004). Moreover, polyclonal stimulation of B cells induces TGF-β1 production, and autocrine TGF-β1 signaling seems to be important in promoting IgA class switching (Zan *et al.*, 1998). *In vitro* experiments have also allotted some relevance to the amount of TGF-β1 present: low doses seem to promote rather than to inhibit antibody secretion (Snapper *et al.*, 1993).

TGF-β1 exerts inhibition of NK cell functions via attenuation of cytolytic activity and antagonisation of IL-12-induced IFN-γ production (Bellone *et al.*, 1995; Hunter *et al.*, 1995). TGF-β1 also inhibits NK expression of the INF-α receptor and α chain of the IL-2 receptor (Rook *et al.*, 1986; Ortaldo *et al.*, 1991), thus impairing cytokine-driven activation of these cells. TGF-β1 regulates NK cell cytolytic activities via inhibition of the expression of receptors NKp30 and NKG2D (Castriconi *et al.*, 2003). NK cells also constitutively produce TGF-β1 (Gray *et al.*, 1998), thus opening the possibility of autocrine or paracrine control of NK immune function.

TGF-β1 supports the development of Langerhans cells, specialised DC in skin and other epithelia, from monocytes, and regulates the maturation of differentiated DC and DC-mediated responses (Strobl & Knapp, 1999). TGF-β1 affects the maturation status of DC, promoting the generation of an immature phenotype in these cells. When TGF-β1 is added to *in vitro* cultures in which DC are stimulated with bacterial components, antigen presentation is attenuated. Moreover, TGF-β1 prevents DC maturation and IL-12 production induced by IL-1 and TNF-α (Geissmann *et al.*, 1999). Once more, TGF-β1 is produced by DC in general and at higher levels by bone marrow-derived immature DC (Morelli *et al.*, 2001), and it has been proposed that DC-derived TGF-β1 could regulate T cells in a paracrine way, or that the immature state of DC is maintained in an autocrine way by this cytokine (Li *et al.*, 2006).

TGF-β1 has a dual effect on monocytes/macrophages depending on their maturation state. TGF-β1 stimulates and recruits resting monocytes, but inhibits phagocytosis, activation, and antigen presentation in activated macrophages (Ashcroft, 1999). TGF-β1 functions as

a chemotactic attractant to monocytes and induces expression of adhesion molecules and matrix metalloproteinases, promoting their attachment to the extracellular matrix and their migration to and through tissues (Wahl *et al.*, 1993). Of note, TGF-β1 induces IL-1, IL-6 and leukotriene C4 synthase in monocytes, thus promoting inflammation through these cells (Turner *et al.*, 1990; Wahl *et al.*, 1993; Riddick *et al.*, 1999). On the contrary, TGF-β1 reduces phagocytosis by macrophages and their activation through the down-regulation of IgG and scavenger receptors, as well as by the inhibition of the expression of inflammatory cytokines and chemokines, the down-regulation of the production of reactive oxygen and nitrogen species, and the attenuation of toll-like receptor-4 signaling (Bottalico *et al.*, 1991; Bogdan *et al.*, 1992; Vodovotz *et al.*, 1993; Tridandapani *et al.*, 2003; Naiki *et al.*, 2005). TGF-β1 also affects macrophage antigen presentation by inhibiting IFN-γ-induced expression of MHC class II molecules and of costimulatory CD40 and IL-12 by these cells (Nandan & Reiner, 1997; Takeuchi *et al.*, 1998). Finally, phagocytosis of apoptotic bodies (Huynh *et al.*, 2002) and infection by certain microorganisms (e.g. *Leishmania* (Barral *et al.*, 1993)) can trigger TGF-β1 production by macrophages, attenuating inflammatory responses in normal programmed death cell removal or as a means of control of host immunity by pathogens.

Other effects of TGF-β1 on cells of the immune system include being a potent chemo attractant for mast cells (Olsson *et al.*, 2000) and for neutrophils (Reibman *et al.*, 1991) and eosinophils (Luttmann *et al.*, 1998), while limiting their transmigration through endothelium by down-regulation of adhesion molecules (Reibman *et al.*, 1991; Smith *et al.*, 1996).

1.4.2.1 Transforming growth factor-β1 (TGF-β1) and T cells

Despite its various and diverse effects on other immune cells, TGF-β1's greatest impact in the immune system is on T cells by affecting their proliferation, differentiation and survival. In fact, the production of TGF-β1 by human T lymphocytes and its role in the regulation of T cell growth have been known for almost 30 years (Kehrl *et al.*, 1986). Despite thymic education, autoreactive T cell clones are present in the periphery of healthy individuals (Danke *et al.*, 2004), and the control of these cells is dependent on a regulatory network that is coordinated by TGF-β1. Moreover, in TGF-β1-deficient mice, an inflammatory disorder characterised by hyperactivation of T cells and progressive infiltration of multiple organs likely driven by self-antigens develops, suggesting that TGF-β1 is fundamental for peripheral tolerance (Kulkarni *et al.*, 1993).

In the thymus, TGF-β1 seems to promote the differentiation of T cells into NK T cells, nTreg and CD8+ cells, whereas in the periphery, TGF-β1 promotes the survival of low-

affinity CD4+ and CD8+ cells and inhibits high-affinity T cell proliferation and differentiation into Th1, Th2 and cytotoxic T lymphocyte effectors. Moreover, TGF-β1 impairs nTreg proliferation, but promotes their persistence in peripheral organs, while in certain cytokine milieus it promotes iTreg (in the presence of IL-2 and retinoic acid and induction of STAT5) or resting Th17 cells (in the presence of IL-6 and induction of STAT3) (Li & Flavell, 2008). TGF-β1 is necessary for the induction of Th17 cells from naïve T cells, and both iTreg and Th17 differentiation pathways seem to be mutually exclusive and to play complementing roles in mucosal immune homeostasis.

Resting human T cells have been described to be constitutively inhibited by TGF-β1 under steady-state conditions, most probably by circulating TGF-β1 in human serum (Classen *et al.*, 2007). Indeed, TGF-β1 signaling through Smad3 has been shown to impair the effect of CD28 stimulation on T cell proliferation by modulating T cell activation and anabolic metabolism similar to the effect of Rapamycin (Delisle *et al.*, 2013). TGF-β1 blocks T cell proliferation by inhibition of IL-2 production, and by down regulation of cyclins, upregulation of cyclin-dependent kinase inhibitors and down-regulation of c-myc (Brabletz *et al.*, 1993; Hannon & Beach, 1994; Li *et al.*, 2006). TGF-β1's effect on T cell proliferation seems to be relevant only on naïve T cells and minimal on activated T cells (Cottrez & Groux, 2001).

TGF-β1 inhibits the differentiation of Th1, Th2 and cytotoxic T cells through suppression of expression or function of their signature transcription factors (Ranges *et al.*, 1987; Gorelik *et al.*, 2000; Gorelik *et al.*, 2002). TGF-β1 impairs tec kinase Itk activation and calcium influx caused by TCR/CD28 stimulation, which in turn compromises the nuclear translocation of NFAT and its induction of GATA-3 and Tbet (Chen *et al.*, 2003). While TGF-β1 can inhibit IFN-γ expression in differentiated Th1 cells, fully mature Th2 cells are refractory to TGF-β1's inhibition of cytokine production (Ludviksson *et al.*, 2000). In cytotoxic CD8+ T lymphocytes, TGF-β1 has been shown to inhibit their production of perforin (Smyth *et al.*, 1991), IFN-γ (Bonig *et al.*, 1999) and Fas ligand (Genestier *et al.*, 1999). TGF-β1 induces the expression of transcription factor FOXP3 and generation of regulatory T cells (Treg) (Xu *et al.*, 2010).

Despite its inhibition of T cell proliferation, once they are activated, TGF-β1 inhibits apoptosis and promotes the survival of T cells during their expansion. If strong TCR stimulation and CD28 co-stimulation are present, TGF-β1 promotes T cell expansion (Gunnlaugsdottir *et al.*, 2005). By synergizing with IL-2, TGF-β1 blocks activation-induced cell death in Th1 and Th2 CD4+ cells (Zhang *et al.*, 1995).

Finally, a study reported the promotion of the reprogramming of a Th2 phenotype and the generation of IL-9-producing inflammatory Th9 cells by TGF-β1 n the presence of IL-4.

(Veldhoen *et al.*, 2008). Recent reports have shown that TGF-β1 signaling can induce FOXP3 expression and regulatory phenotypes in murine γδ-T lymphocytes (Casetti *et al.*, 2009), CD8+ T cells (Mayer *et al.*, 2011), and human and murine NK T cells (Monteiro *et al.*, 2010). However the biological relevance of these findings awaits more clarification.

It is important to point out that the effects of TGF-β1 on different cells of the immune system are highly dependent on the context, being affected both by the differentiation states of the target cells and the presence of co-stimulatory molecules and inflammatory cytokines in their milieu.

1.4.3 The relationship between transforming growth factor-β1 (TGF-β1) and regulatory T cells (Treg)

TGF-β1 plays a central role in the development, maintenance, and induction of Treg. Deletion of TGF-β1 from Treg reduces their suppressive capacity and their survival *in vivo* (Marie *et al.*, 2005). TGF-β1 can convert CD4+CD25- cells into Treg *in vitro* (Chen *et al.*, 2003). However, data from some groups show that TGF-β1-dependent FOXP3 expression by TCR stimulation in naïve CD4+FOXP3- cells does not to confer a functional regulatory phenotype on these cells, which instead maintain their proliferative capacity, fail to suppress effector cells and produce high levels of inflammatory cytokines (Tran *et al.*, 2007). However, murine antigen-specific TGF-β1-producing Th3 cells have been shown to induce the differentiation of antigen-specific FOXP3+ Treg in peripheral tissues, and these *in vivo* induced Treg do show potent suppressive capabilities *in vitro* and *in vivo* (Carrier *et al.*, 2007).

TGF-β1 appears to play a fundamental role in maintaining the expression of FOXP3 by nTreg (Pyzik & Piccirillo, 2007). TGF-β1 appears to mediate FOXP3 expression by the recruitment of Smad3 to a *FOXP3* enhancer element (Tone *et al.*, 2008). Smad 3 synergises with IL-2-induced STAT5 for the induction of FOXP3 expression and iTreg induction (Burchill *et al.*, 2007). TGF-β1-induced Smad3 and NFAT activation of this *FOXP3* enhancer causes the demethylation of DNA and acetylation of histones in both nTreg and iTreg. TGF-β1 signaling has also been implicated in interaction with transcription factors FOXO1/FOXO3a, which positively regulate *FOXP3* expression by binding its promoter (Kerdiles *et al.*, 2010).

As previously mentioned, TGF-β1 has been associated with the immunomodulatory function of Treg. In fact, TGF-β1 produced by Treg has been identified as a fundamental mechanism for their function (Chen & Wahl, 2003; Nakamura *et al.*, 2004; Andersson *et al.*, 2008). Neutralising anti-TGF-β1 antibodies and recombinant LAP have been shown to reverse suppression of effector CD4+ cells by murine and human Treg (Nakamura *et al.*,

2004). Moreover, while Treg from TGF-β1-deficient mice were able to suppress CD4+CD25- cell proliferation *in vitro*, they failed to protect recipient mice in a colitis transfer model *in vivo*, and only CD4+LAP+ and not CD4+LAP- cells from normal mice were able to confer protection to recipient mice (Nakamura *et al.*, 2004). Indeed, effector cells rendered irresponsive to TGF-β1 by a double negative TGF-β1 receptor escaped control by Treg in another murine colitis model (Fahlen *et al.*, 2005). Additionally, membrane-bound TGF-β1 on Treg was reported to induce de novo CD4+FOXP3+ cells from naïve T cells *in vitro*, conferring the latter with suppressive capacity (Andersson *et al.*, 2008). This induction occurred in a contact-dependent, integrin and APC-independent way in this study.

In murine tumor models, Treg-derived TGF-β1 has been shown to directly inhibit NK cell effector functions and down-regulate NKG2D receptors on the NK cell surface. Adoptive transfer of wild-type Treg but not TGF-β1-deficient Treg into nude mice suppressed NK cell-mediated cytotoxicity and accelerated the growth of tumors that were normally controlled by NK cells (Ghiringhelli *et al.*, 2005). Also, in a murine model of allo-HSCT in which parental marrow grafts were rejected by NK cells of irradiated offspring recipients, *in vivo* TGF-β1 derived from Treg was shown to mediate inhibition of NK cell-mediated rejection (Barao *et al.*, 2006).

Additionally, TGF-β1 has been implicated in the Treg control of the immune response against *Mycobacterium tuberculosis* (Roberts et al., 2007), prevention of colitis in an inflammatory bowel disease model (Li *et al.*, 2007), control of allergic responses (Joetham *et al.*, 2007), control of anti-islet responses in type 1 diabetes (Green *et al.*, 2003), and limitation of anti-tumor immunity in head and neck carcinoma (Strauss *et al.*, 2007) and follicular lymphoma (Hilchey *et al.*, 2007). Using a commercially available fusion protein composed of the Fc region of IgG1 fused to the extracellular domain of CTLA-4, CTLA-4 has been seen to inhibit activated T cells via a CD28-independent, Treg and TGF-β1-dependent pathway coupled with nitric oxide produced by macrophages both *in vivo* and in an allergic airway inflammation model (Deppong *et al.*, 2013). However, as previously mentioned, double negative TGF-β1 Treg are able to suppress T cell proliferation *in vitro*, questioning the requirement of TGF-β1 in this system (Piccirillo *et al.*, 2002).

1.4.3.1 Membrane expression of transforming growth factor-β1 (TGF-β1) by regulatory T cells (Treg)

The discovery of membrane-tethered TGF-β1 on Treg and not on conventional T cells further supported its involvement in contact dependent suppression of CD4+CD25- cells (Nakamura *et al.*, 2001). Treg also inhibited IgG production by B cells by means of TGF-β1.

Interestingly, tumor exosome membranes bearing TGF-β1 have been shown to enhance the suppressive function of Treg and induce regulatory functions in effector cells (Clayton *et al.*, 2007).

Recently, a transmembrane protein member of the leucine-rich repeat family of proteins termed glycoprotein A repetitions predominant (GARP or LCRRC32) has been shown to be upregulated in activated human Treg (Wang *et al.*, 2009) and proposed to be the anchor for LAP on human Treg and platelets (Stockis *et al.*, 2009; Tran *et al.*, 2009).

GARP was first identified as a novel molecule expressed specifically by Treg upon TCR stimulation, and which, if expressed ectopically in human naïve T cells, inhibited their proliferation and cytokine secretion upon TCR stimulation (Wang *et al.*, 2008). Naïve T cells with ectopic GARP expression also expressed FOXP3 and conferred them with some suppressive capacity. Moreover, exposure of naïve T cells to soluble GARP has also been found to induce FOXP3, decrease proliferation and repress IL-2 and IFN-γ production, resulting in differentiation of naïve T cells into iTreg (Hahn *et al.*, 2013). These findings were confirmed *in vivo* in a xenogeneic GVHD model. Later on, further experiments revealed that GARP was not induced in T cells activated in the presence of TGF-β1, which did not have suppressive function despite high levels of FOXP3 expression, and that ectopic FOXP3 expression in naïve T cells was insufficient to induce expression of this molecule (Wang *et al.*, 2009). Moreover, CD25+ T cells that were selected upon high GARP expression showed stronger suppressive capacity in the aforementioned study. Although GARP and LAP colocalisation has also been found on murine cells, unlike human CD4 cells, surface LAP expression on murine cells does appear to be controlled by FOXP3 expression. However, exposure to TGF-β1 can induce LAP expression on FOXP3- murine cells as well (Oida & Weiner, 2010). Further evidence on the role of GARP in FOXP3 induction and Treg function came from gene-profiling experiments (Probst-Kepper *et al.*, 2009).

The previous observations about GARP expression in Treg prompted the hypothesis that it could be related to TGF-β1 membrane expression in these cells. Indeed, two reports showed almost simultaneously that GARP was critical for the tethering of TGF-β1 (LAP) to the surface of activated Treg. One of the studies showed that knockdown of GARP mRNA prevented surface TGF-β1 expression on activated Treg (Tran *et al.*, 2009). GARP colocalisation with TGF-β1 on the membrane of Treg was confirmed by microscopic and immunoprecipitation experiments. The second study showed that LAP and GARP were expressed in the same cells, and reciprocal immunoprecipitation confirmed the cytometry results (Stockis *et al.*, 2009).

Recently, it has been shown that GARP associates non-covalently to the SLC, outcompeting the LTBP and regulating the bioavailability and activation of TGF-β1. Indeed, GARP

impedes the secretion of the SLC, and integrins $\alpha_v\beta_6$, $\alpha_v\beta_8$ are able to activate TGF-β1 bound to GARP in the cell membrane (Wang *et al.*, 2012). A study in which GARP was overexpressed in murine T cells showed that this resulted in diminished Treg development in the thymus and diminished differentiation of CD4+ cells into memory subsets, as well as reduced proliferative capacity because of enhanced TGF-β1 signaling (Zhou *et al.*, 2013). This study also provided evidence for a down-regulation of GARP as a consequence of TGF-β1 signaling, thus suggesting a control loop. Moreover, another recent study has discovered that GARP is also the anchor for TGF-β1 in murine Treg, and that GARP expression is independent to that of TGF-β1. The authors also showed that GARP can load pro-TGF-β1 without furin processing, and that GARP-deficient murine cells can suppress conventional T cell proliferation *in vitro* (Edwards *et al.*, 2013). Of note, one report claims to have identified a population of human CD4+ cells that expresses LAP on its membrane and has hypoproliferative and regulatory characteristics but does not express FOXP3+, this meaning, according to the authors, that this subset is different from regular Treg (Gandhi *et al.*, 2010). Finally, LAP/TGF-β1 has been shown to form a complex with a molecular chaperone called glucose-regulated protein 78, but this association was independent of GARP, thus providing evidence of another possible anchor for membrane TGF-β1 in human cells (Oida & Weiner, 2010).

1.4.3.2 The role of membrane-bound transforming growth factor-β1 (TGF-β1) in regulatory T cell (Treg) function

The mechanism by which membrane-bound TGF-β1 mediates suppression by Treg requires TCR engagement from an APC and close contact with the target cells. A three-cell model formed by the Treg, DC and naïve T cells, has been proposed as the one allowing for TGF-β1-dependent control of immune responses. Following this model, Treg would express LAP on their surface upon TCR activation by presentation of antigens by the DC. In turn, integrin $\alpha_v\beta_8$ on DC would be implicated in the activation of latent TGF-β1 on Treg, allowing it to reach its target cells in the periphery of the DC (Li & Flavell, 2008). In this way, the DC could activate naïve T cells in inflammatory conditions where Treg function is inhibited, and activation of Treg-derived TGF-β1 will only occur at the site of potential naïve T cell activation. The proponents of this model point out, however, that given the evidence that T cell-specific TGF-β1 deficiency and DC $\alpha_v\beta_8$ deficiency mouse models show a less severe phenotype when compared to mice that lack TGF-β1 completely or that express a mutated LAP that does not bind integrins, there must be other biologically relevant sources for TGF-β1 and other activating integrins that play a role in T cell response regulation (Li & Flavell, 2008).

Freshly isolated Treg express surface TGF-β1 and TβRII, both of which are upregulated upon TCR stimulation. By contrast, naïve CD4+ responder cells initially do not express TGF-β1 or its receptor, but become positive for TβRII after TCR stimulation. The co-expression of TGF-β1 and TβRII on Treg might explain their anergy (Chen & Wahl, 2003). In addition, both CD4+ and CD8+ LAP+ Treg have been identified as a subset with enhanced suppression in murine models (Chen *et al.*, 2008; Chen *et al.*, 2009), and LAP expression has been successfully used to purify FOXP3+ Treg from expansion cultures (Tran *et al.*, 2009). Even though the role of TGF-β1 in some *in vitro* suppression settings has been disputed and this cytokine appears not to be required for it, there is stronger evidence for its involvement in *in vivo* settings (Li *et al.*, 2007).

An interesting report on HSCT for the treatment of systemic lupus erythematosus found that HSCT in these patients generates a post-transplant population of $LAP^{hi}CD103^{hi}$ CD8+ Treg cells, which repairs the Treg deficiency in human lupus and maintains patients in true immunological remission. Moreover, these cells showed contact independent and predominantly TGF-dependent autoantigen-specific and nonspecific suppressive activity (Zhang *et al.*, 2009). Furthermore, CD8+LAP+ T cells have also been reported to suppress murine experimental autoimmune encephalomyelitits in a partially TGF-β1-dependent way, despite only a small fraction of these cells expressing FOXP3 (Chen *et al.*, 2009).

Finally, TGF-beta and LAP have been shown to be present on the surface of ex vivo immature human DC and to be lost upon DC Maturation. The presence of LAP on immature DC appears to be required for differentiation and survival of FOXP3+ Treg (Gandhi *et al.*, 2007).

1.4.4 Transforming growth factor-β1 (TGF-β1) and its role in disease

TGF-β1 and other components of this cytokine superfamily are involved in the pathogenesis of cardiovascular disease, connective tissue diseases, skeletal and muscular disorders, reproductive disorders, hereditary cancer syndromes, sporadic cancer, and developmental disorders (Blobe *et al.*, 2000; Gordon & Blobe, 2008)

In addition, a number of diseases are at least partly modified by TGF-β1. In particular, autoimmune diseases such as systemic lupus erythematosus (Xing *et al.*, 2011), rheumatoid arthritis (Mieliauskaite *et al.*, 2009), insulin-dependent diabetes mellitus (Olivieri *et al.*, 2010), and multiple sclerosis (Meoli *et al.*, 2011) are modified by this cytokine. Moreover, atherosclerosis (Oklu *et al.*, 2010), asthma (Sandford, 2010), inflammatory bowel disease (Monteleone *et al.*, 2008), and infections (Taylor *et al.*, 2000; Korten *et al.*, 2010; Thornburg *et al.*, 2010; Wang *et al.*, 2010) have been reported to be affected by TGF-β1.

Finally, an example of a disease directly caused by TGF-β1 is Camurati-Engelmann disease. This condition is inherited as an autosomal dominant disease, resulting from mutations in the LAP sequence (Kinoshita *et al.*, 2004), and is characterised by hyperostosis and sclerosis of the base of the skull and long bones. The mutations associated with Camurati-Engelmann disease apparently alter the susceptibility of LAP to proteolysis by furin and other proteases, and some of them have been shown to produce constitutively active TGF-β1 (Saito *et al.*, 2001; Janssens *et al.*, 2003).

1.4.5 Transforming growth factor-β1 (TGF-β1) and its role in transplantation

As with its role in the immune system, TGF-β1 is also likely to play a central role in transplantation. TGF-β1 has been associated with renal damage and has been implicated in the process of acute rejection of kidney grafts due to its chemo attractant properties and its capacity to induce CD103 expression, an integrin that binds to E-cadherin present in epithelia, on activated cytotoxic T lymphocytes, thus facilitating infiltration of the graft by T cells. TGF-β1 has also been involved in chronic solid organ graft rejection, mainly by its association with fibrosis and arteriosclerosis of the lung, heart, liver and kidney. Interestingly, the use of cyclosporine and not tacrolimus as immunosuppressive drug in solid organ patients has been associated with increases in TGF-β1 production *in vitro* and in animal models, as well as in clinical settings (Hutchinson, 1999).

The role of TGF-β1 in the generation of iTreg and of Th17 cells suggests that this cytokine might play a role in transplant rejection or tolerance. IL-17 and Th17 cells have been recognised to mediate solid organ graft rejection (Yuan *et al.*, 2008; Chen *et al.*, 2009).

1.4.5.1 Transforming growth factor-β1 (TGF-β1) and its role in haematopoietic stem cell transplantation (HSCT)

TGF-β1 has been studied in the context of HSCT and its outcomes and complications such as pulmonary fibrosis, graft rejection and infections, because of its role in immune responses and its widespread involvement in diverse cellular processes (Coomes & Moore, 2010). The fact that GVHD is still the most important complication for patients who undergo HSCT has attracted interest in the role this cytokine plays in the pathogenesis, protection or resolution of this complication. There is increasing evidence suggesting that TGF-β1 could play a role both in the allogeneic response leading to aGVHD as well as in the pathological manifestations of cGVHD, especially in fibrosis (Carli *et al.*, 2012).

1.4.5.1.1 Transforming growth factor-β1 (TGF-β1) in murine models of graft-versus-host disease (GVHD)

In GVHD murine models, the neutralisation of TGF-β1 with antibodies has been shown to have different effects on GVHD progression depending on the underlying pathophysiology. In an aGVHD mouse model, the neutralisation of TGF-β1 early after transplantation led to an increase in T cell expansion and organ damage. On the contrary, late blockade of TGF-β1 in a cGVHD model ameliorated clinical scores, partly by reduced fibrosis (Banovic *et al.*, 2005). In turn, early TGF-β1 neutralisation was found to ameliorate skin and lung fibrosis in a mouse cGVHD model (McCormick *et al.*, 1999). Because aGVHD pathophysiology is mainly driven by lymphoid cells and fibrosis is generated by myeloid cells, TGF-β1's target cell in GVHD settings could modify the outcome of its activity. Moreover, in a mouse model of aGVHD through minor histocompatibility antigens disparity, the authors found that mice engrafted by Smad3-deficient donors experienced fatal disease in their colon mediated by Th1 and neutrophilic infiltrate, while their littermates transplanted with wild type donors did not develop discernible GVHD (Giroux *et al.*, 2011), thus suggesting a role for TGF-β1 control of donor cells. Interestingly, another study exposed recipient mice DC to TGF-β1 ex vivo and reinfused them prior to HSCT, and found that this prevented aGVHD in the recipient mice (Mou *et al.*, 2004).

1.4.5.1.2 Transforming growth factor-β1 (TGF-β1) levels and transplant outcome

Many studies have analysed HSCT patient TGF-β1 serum levels and cellular expression and attempted to link these measurements with transplant outcome. Studies have shown that circulating TGF-β1 levels in recipients of HSCT suffer a sharp decrease of up to one fourth of the normal levels in plasma during the conditioning regimen, recovering these levels after 20 to 50 days after the transplant (Liem *et al.*, 1999; Visentainer *et al.*, 2003; Malone *et al.*, 2007). The recovery of TGF-β1 levels has been correlated with the recovery of leukocyte and platelet populations (Liem *et al.*, 1999).

Malone *et al*. (2007) found that plasma levels of TGF-β1 were significantly reduced (-43.4% change per week) during the 15-day interval before the clinical onset of aGVHD grades II-IV in comparison to levels in patients who did not develop the complication. In another study, higher levels of TGF-β1 were found during conditioning and in the first week after transplantation in patients with mild or absent aGVHD compared with those with clinically relevant aGVHD after examining serum TGF-β1 before and after HSCT with unrelated donors (Remberger *et al.*, 2003). This study also revealed that low levels of this cytokine during conditioning correlated with GVHD, eradication of recipient hematopoietic cells and full donor chimerism. Additionally, patients with aGVHD or

cGVHD showed lower plasma levels of TGF-β1 in combination with higher levels of Th17-associated cytokines when compared to patients without GVHD and healthy donors in a cohort of 39 HSCT patients from China (Wang *et al.*, 2011).

Similar results were obtained in a study by Li *et al*. (2010) that examined serum cytokine levels and Treg frequency in peripheral blood mononuclear cells (PBMC) in 56 patients and correlated these measurements with the development of GVHD. With a single measurement done at early haematological reconstitution (2-4 weeks) after HSCT, results revealed that both the percentage of circulating Treg from CD4+ cells and the serum levels of TGF-β1 were significantly lower in patients who suffered aGVHD and cGVHD when compared to those of patients who did not (Li *et al.*, 2010). Similar results were reported by Niu and collaborators in an independent, although much smaller (13 patients), study performed in subjects of Chinese origin (Niu *et al.*, 2010).

Serum levels of TGF-β1 were measured weekly in another study carried out in 13 patients during pre and post conditioning, engraftment and post engraftment until 15 weeks post-HSCT or death or relapse (Visentainer *et al.*, 2003). The study showed a sharp decrease in the levels of this cytokine from the pre-conditioning up to the second week after transplantation both in patients that developed aGVHD and those who did not. Subsequently, TGF-β1 levels started to increase in both groups, and coincided with the engraftment period (2nd to 4th weeks). Up to the 8th week after transplantation, the levels of TGF-β1 were consistently higher among the aGVHD group. After that point and up to week 15, the relationship was inverted and patients who did not develop aGVHD now had significantly higher levels of circulating cytokine. Interestingly, the median time for aGVHD in this study was 51 days (7-8 weeks) after HSCT, which correlated with the time for the inversion of the relationship in TGF-β1 levels between the two groups.

TGF-β1 levels have also been shown to be augmented during cGVHD (Liem *et al.*, 1999; Kyrcz-Krzemien *et al.*, 2011), and organ-specific increases in this cytokine occur in skin being affected by GVHD (Wu *et al.*, 2010) and radiation-mediated injured lung tissue following HSCT (Anscher *et al.*, 1998). In addition, TGF-β1 overexpression after HSCT has been associated with impaired antiviral immunity in the lung (Coomes *et al.*, 2010; Coomes *et al.*, 2011), and TGF-β1 released from platelets contributes to hypercoagulability in veno-occlusive disease caused by HSCT (Pihusch *et al.*, 2005).

Conversely, Lunn *et al*. (2005) examined serum TGF-β1 levels between groups of patients who underwent autologous, allo- or no HSCT and found no significant differences between these groups (Lunn *et al.*, 2005).

Using genetic expression profiling, Baron *et al*. (2007) found that the pre-transplant genetic expression profile of isolated CD4+ and CD8+ cells from sibling donors was

predictive of the incidence of GVHD in recipients of myeloablative T cell-replete HSCT. Seven genes belonging to TGF-β1's intracellular signaling pathway and induced proteins were markedly up-regulated in donors whose recipients did not develop GVHD compared to those of patients who did. In particular, *SMAD3* was shown to be the most up-regulated. This prompted the investigators to question whether increased TGF-β1 signaling in these cells pre-HSCT correlates with decreased incidence of GVHD (Baron *et al.*, 2007). A later study from the same group found no association between polymorphisms covering the *SMAD3* gene and the 6-fold variation in Smad3 expression found in normal subjects, suggesting that variation in expression of this molecule could be controlled by upstream elements in a *trans* manner (Busque *et al.*, 2009). Interestingly, the transcripts for TGF-β1 and its receptors were included in the microarrays used by the researchers in the former study but were not found to be differentially expressed in T cells from cGVHD-positive (+) or cGVHD-negative (-) donors.

Wu *et al*. (2010) performed a study in which skin biopsies from allo-HSCT recipients suffering grades 0-III, lichenoid, and sclerodermoid GVHD were assessed for *TGFB1* RNA expression transcript levels. Interestingly, the expression of *TGFB1* was correlated with shorter survival in these patients. These results contrast with those of Imamura *et al*. (1999), which showed decreased TGF-β1 mRNA expression in PBMC during aGVHD (Imamura *et al.*, 1996).

Petersen *et al*. (2006) developed a study in which they isolated PBMC from patients and donors before and after HSCT and cultured them in mixed lymphocyte reactions, but failed to identify clear differences in TGF-β1 mRNA expression between patients who developed aGVHD and those who did not (Petersen *et al.*, 2006). However, in another study, the expression of TGF-β1 mRNA was increased in both PBMC from allo-HSCT patients with GVHD and after *in vitro* stimulation. This increase in TGF-β1 mRNA was concomitant with a decrease in FOXP3 mRNA expression in PBMC (Miura *et al.*, 2004).

Looking simultaneously at TGF-β1 mRNA expression levels and circulating cytokine, Krycz-Krzemien *et al*. (2011) were able to confirm a sharp drop in *TGFB1* mRNA levels in PBMC as well as serum TGF-β1 in patients from day -10 to day 0 followed by a slow recovery of levels up to day +100 when levels reached pre-conditioning levels. However, although the mRNA levels of *TGFB1* were lower at days 0 and +30 in patients who developed aGVHD, this difference was not significant. Serum TGF-β1 levels followed the general trend but were not significantly different between GVHD+ or – patients either. Moreover, as mentioned before, although a significant increase in the levels of *TGFB1* mRNA expression was detected when comparing the levels on days 0 and +100 within the cGVHD+ group, both the levels of mRNA and the levels of circulating TGF-β1 were not

significantly different between patients with or without cGVHD (Kyrcz-Krzemien *et al.*, 2011).

Another study based on PBMC cytokine mRNA comparisons in aGVHD found that TGF-β1 expression in severe aGVHD patients (grades 3-4) was significantly reduced compared to the patients without aGVHD. TGF-β1, but not IL-6 expression was positively associated with FoxP3 expression in PBMC from these patients (Liu *et al.*, 2013).

Finally, apart from TGF-β1's effect on immune tolerance or GVHD after HSCT, another less-explored area of influence on the outcome of transplantation is the graft-versus-leukaemia (GVL) effect. TGF-β1 is known to be present in the microenvironment of tumors and haematological malignancies serving as an agent for immune evasion by the malignant cells. as mentioned previously, TGF-β1 plays a complicated role in the homeostasis of normal and malignant hematopoietic cells. Consequently, TGF-β1 levels in the recipient of HSCT could have a role in how efficient the GVL effect is. Indeed, a small pediatric study found that relapsing patients had higher TGF-β1 serum levels as well as lower percentages of IFN-γ-producing T cells before and early after transplantation (Carli *et al.*, 2012).

Overall, these studies have shown contradicting results regarding the role of TGF-β1 serum or mRNA levels after HSCT, most likely due to differences in the timing and the interpretation of the measurements, as well as the tissues or compartments evaluated. Apart from the consistent drop in the levels of the cytokine after conditioning, it is not yet clear whether higher or lower levels of TGF-β1 correlate with better or worse outcome or the incidence of complications after HSCT.

1.4.5.2 TGFB1 polymorphism in haematopoietic stem cell transplantation (HSCT)

Due to the central role that TGF-β1 plays in immune responses and, consequently in HSCT, and the functional consequences and clinical associations identified for polymorphisms within its gene, the association of *TGFB1* polymorphisms with HSCT outcome has also received attention by a number of groups. The majority of the studies have examined the relationship between *TGFB1* polymorphisms and the risk of developing GVHD, and were performed in cohorts of patients who received myeloablative conditioning regimens. **Table 1.1** compiles the studies that have been carried out to date, their characteristics and design, and their major findings.

The first report on *TGFB1* polymorphisms and HSCT outcome, published thirteen years ago, included 50 donor–recipient pairs, half of whom suffered no or mild GVHD (Leffell *et al.*, 2001). The authors evaluated the effect of *TGFB1* +74G>C and classified patients and donors as being high (GG), intermediate (GC) or low (CC) TGF-β1 producers following previously determined categories. No distinction between aGVHD or cGVHD was stated in

this study. The authors found a significant increase in the frequency of "high producer" patients in the severe GVHD group compared to patients with no or mild GVHD ($p<0.0005$).

Another publication obtained opposite results despite performing a similar analysis as that done in the former study (Tambur *et al.*, 2001). In this study a smaller cohort of pediatric recipients of HSCT from siblings, chosen such that half developed GVHD was the analysed population. No data on donor genotype was presented. Polymorphisms at *TGFB1*'s codon 10 and codon 25 were genotyped and the participants were again classified into 3 predetermined categories of cytokine production. Their results showed that only a minority of GVHD patients were "high producers", whereas 11/12 pediatric patients with no GVHD were classified as high producers ($p=0.03$).

It is important to remark that these first two studies (as well as that performed by Laguila Visentainer *et al*. (2005), see below) used a preconceived classification of patients into three categories of TGF-β1 production following a commercial cytokine genotyping kit as the basis for their analyses, but the association of these genotypes and production has not always been supported by functional studies.

In a study by Hattori *et al*., the authors analysed the association between *TGFB1* -1347C>T and +29T>C in addition to a polymorphism in the gene encoding TβRII (*TBRII* +1167 C>T) in Japanese patients and their donors (Hattori *et al.*, 2002). The authors found that donor +29T>C polymorphism was associated with the risk of developing aGVHD. Patients receiving stem cells from donors bearing a C allele developed aGVHD more frequently than those with cells from +29 TT donors (odds ratio (OR)=3.00; $p=0.04$). Interestingly, patients with the *TBRII* +1167T allele were more likely to suffer aGVHD than those without it. These results could point to a functional combination of polymorphisms in TGF-β1 producer and target cells.

A later study published by Laguila Visentainer *et al*. (2005) examined the outcomes of HSCT in 118 patients from Brazil and their sibling donors according to *TGFB1* +29T>C and +74G>C polymorphisms. The authors based their analysis on the same classification for high, intermediate and low TGF-β1 producers as Leffell *et al*. (2001) and Tambur *et al*. (2001), but found no association between *TGFB1* polymorphisms in patients or donors and aGVHD or cGVHD (Laguila Visentainer *et al.*, 2005). The Brazilian population examined by this study is likely to be heavily admixed (mixed European, African and American ancestries in individuals) and this could be an important factor to consider when interpreting these results since SNP allele frequency and/or linkage disequilibrium could be different in the ancestral populations that gave rise to this one. Of note, this study

did include multivariate analyses for the outcome data in contrast to earlier studies, strengthening their methodology.

The next three reports on *TGFB1* polymorphisms and HSCT outcome that were published were a series of studies in Iranian patients (Noori-Daloii *et al.*, 2007; Karimi *et al.*, 2010; Rashidi-Nezhad *et al.*, 2010). Both *TGFB1* +29T>C (Noori-Daloii *et al.*, 2007) and +74G>C (Rashidi-Nezhad *et al.*, 2010) were analysed in HSCT performed with HLA-identical sibling donors and both were also associated with aGVHD in these cohorts. Results from these analyses showed that recipients bearing the +29T allele were less likely to develop aGVHD than recipients with a +29 CC genotype (OR=0.334; p=0.026). In the latter study, there was a significant association when the severity of aGVHD was analysed. Recipients with a +74 GG genotype were more likely to develop severe (grades III-IV) aGVHD than those bearing a +74 C allele (OR=12.133; p=0.015). The third study from Iran (Karimi *et al.*, 2010) showed no association between *TGFB1* polymorphism and the risk of developing moderate to severe GVHD. Unfortunately, no data on the effect of donor genotype appeared in this paper.

Subsequently, in pilot study Shah *et al*. (2009) examined 38 patient-donor pairs and determined the independent and combined allelic distributions of SNPs in the *TGFB1* promoter in these pairs by direct sequencing. The region sequenced by the authors spanned from position -2265 to +423 (+1 being the translational start site), and included several SNPs and other types of genetic variation. This study failed to identify any association between *TGFB1* polymorphisms and the alleles formed by them, and aGVHD (Shah *et al.*, 2009).

Our group performed a much larger study on *TGFB1* polymorphisms and their effect in allo-HSCT (Berro *et al.*, 2010). This study included 427 patient-unrelated donor pairs and included analyses of several outcomes and comprehensive data on HLA matching and clinical factors analysed with univariate and multivariate methods. Results from this study showed that patients homozygous for the +29C allele had significantly increased NRM (46.8% *versus* 29.4%; p=0.014) and reduced OS (29.3% *versus* 42.2%; p=0.013) when compared to recipients bearing at least 1 +29T allele, and the presence of multiple +29C alleles within the pair was significantly associated with higher NRM (44% *versus* 29%; p=0.021) and decreased OS (33.8% *versus* 41.9%; p=0.033). An analysis of causes of mortality showed that the presence of a +29 CC genotype in the patient conferred significantly higher probability of dying due to infection (p=0.024). When the analyses were restricted to the 10/10 HLA-matched transplants (n=280), the results showed that donors bearing a +29C allele had a trend towards increased incidence of aGVHD grades II-IV, and +29C-bearing pairs had increased incidence of overall aGVHD. Of note, an analysis

of the levels of plasma TGF-β1 in patients before transplantation yielded no association with *TGFB1* +29T>C or with clinical outcome.

In another study, the role of polymorphisms in *TGFB1* and the outcome of HSCT was analysed in a cohort of 240 patient-donor pairs including both unrelated and sibling donors from China (Xiao *et al.*, 2011). Three TGF-β1-related polymorphisms were included.

The univariate analysis in UD transplants showed that a -1347 TT genotype in the donor (p=0.003) or the presence of a T allele in the recipient (p=0.01) was associated with protection from aGVHD grades II-IV. When restricting the analysis to the 10/10 HLA-matched pairs (n=96), the same findings were observed. In the confirmation analysis with the sibling donor cohort, the results showed a protective effect against aGVHD for the -1347 TT genotype, but this did not reach statistical significance. When a multivariate analysis including all the pairs in the study was performed, there was confirmation of the protective effect of donor -1347 TT genotype against aGVHD grades II-IV (OR for C allele carriage=2.879; p=0.009).

In a single center study investigating haematopoietic recovery and incidence of infections in 102 patients with thalassemia major that were transplanted with HLA-matched related donors with myeloablative conditioning, the authors evaluated the rate of neutrophil engraftment and platelet recovery, the presence of bacterial, viral and fungal infections, and OS in relation to cytokine polymorphisms including *TGFB1* +29T>C and +74G>C both in patients and donors (Sellathamby *et al.*, 2012). However, the authors did not report any association for these polymorphisms.

While most of the aforementioned studies have performed their analyses mostly in matched patient-donor pairs, one exploratory study reported by Harkensee and collaborators (Harkensee *et al.*, 2012) genotyped 41 SNPs in 2 independent cohorts of Japanese unrelated patient-donor pairs. The authors included pairs with different degrees of HLA matching. Of all the pairs, only 38.7% were 8/8 HLA matched (only 8.5% were 12/12 matched). Of note, the authors report that the selection of *TGFB1* polymorphisms was done using the HapMap database (www.hapmap.org), arguing that polymorphims previously analysed in non-Japanese populations were non-polymorphic in Japanese. They included the -1347C>T, as well as three downstream intronic variants for *TGFB1*: rs2241715, rs2241716 and rs4803455.

In univariate analysis, donor -1347CC genotype showed a significant association with increased risk of aGVHD grades 2-4 (p=0.035, OR=1.69) while donor -1347CT was protective for aGVHD (p=0.036, OR=0.66) in the discovery cohort (n=460 pairs). However, both associations did not withstand Bonferroni correction for multiple testing.

Consequently, they were not pursued in the confirmatory cohort. The same situation befell other donor genotype associations initially found for rs2241715. Regarding recipient genotypes, three SNPs showed initially significant associations: 'mismatch' for the -1347C>T was associated with increased risk of aGVHD (p=0.02, OR=1.63), 'mismatch' for rs2241715 was associated with increased risk of both aGVHD (p=0.015, OR=1.61) and cGVHD (p=0.035, OR=1.58), and AA genotype for rs2241716 was associated with increased risk of extensive cGVHD (p=0.0041, OR=2.58). However, only the latter retained statistical significance after Bonferroni correction. Nevertheless, in the confirmatory cohort it did not show a statistically significant association. When the analyses were performed in the HLA-matched subgroup, no *TGFB1* polymorphism showed any significant association in donors or in recipients. Likewise, multivariate analyses on the full combined cohort did not produce any significant association for *TGFB1* polymorphisms.

Finally, in an attempt to generate multiple SNP-based risk models for the outcomes of HSCT (GVHD, RFS, NRM, OS), Kim *et al*. have recently performed a SNP analysis in 394 patient-donor pairs including 22 polymorphisms of the TGF-β signaling family, -1347C>T being the only one in *TGFB1*. Their methodology included a discovery analysis in 307 pairs of related HSCT transplants in which the 259 SNPs were included. Predictive models were tested in a cohort of 87 pairs from unrelated HSCT. Finally, the authors made a comparison of the genetic and clinical predictive model against the clinical-only model in the whole cohort (394 pairs).

In univariate analysis, recipient *TGFB1* -1347C>T genotype was associated with aGVHD (p=0.0036, HR=1.2562). These results were confirmed in the multivariate analyses (p=0.0146). There was no association with any other HSCT outcome. The analyses revealed that the presence of a T allele at *TGFB1* -1347 in the recipients increased the risk of aGVHD in a dominant fashion (CC vs CT, HR=1.283; CC vs TT, HR=1.645). This SNP was successfully included in the final models for aGVHD risk assessment (Kim *et al.*, 2012).

The same group published a subsequent report in which they applied the same strategy in the same cohorts for the generation of risk stratification models for organ-specific GVHD incorporating genetic polymorphisms including *TGFB1* -1347C>T (Kim *et al.*, 2014). In this occasion, Kim and collaborators found that this SNP was associated with increased risk of skin aGVHD (donor TT+CT vs CC; p=0.007), whereas recipient and donor genotypes were associated with lung cGVHD (TT+CT vs CC; p=0.007). In multivariate models, donor genotype was associated with skin aGVHD (TT+CT vs CC; HR=1.50, p=0.023) and cGVHD of the lung (TT+CT vs CC; HR=0.52, p=0.002). As done in the previous study, *TGFB1* -

1347C>T was included in the risk models, which effectively stratified the patients according to their risk of overall and organ-specific acute and cGVHD.

Despite this body of work, the studies attempting to identify the role of this cytokine and its polymorphisms in HSCT have given inconsistent and sometimes contradicting results. Lack of homogeneity among studies and oversimplified experimental systems and interpretations of the data are among the reasons that could explain these discrepancies. Inter-study differences in aspects such as type of donor, stem cell source, conditioning regime, GVHD prophylaxis, and ethnicity of patients and donors, are likely to influence the results of these association studies. Studies aimed at measuring levels of circulating cytokines or gene expression by RNA and correlating these to HSCT outcome may be affected by the compartment they evaluate (i.e. blood vs. tissues), the timing of the samples, the composition of the cells from which RNA is extracted, tissue regulation of TGF-β1 activation, and stimulation of TGF-β1 synthesis by stem cell mobilization agents. Furthermore, most of these studies do not consider the potential effect of polymorphism as the source of variation in the reported cytokine levels. Also, the analysis of these data can be confounded by the fact that the observed cytokine levels could be a consequence rather than a cause of any given outcome. Moreover, because most studies on the effect of *TGFB1* polymorphisms in HSCT have been small, with diverse cohorts, of univariate nature, and focused on GVHD and its association with one or two polymorphisms, it is necessary that larger and more homogeneous studies with adequate power to analyse the potential combined effect of various *TGFB1* polymorphisms on the various outcomes of transplantation and in the presence of other well-known clinical and genetic modifiers of these endpoints are carried out. Finally, direct correlations between genetic association studies and functional evidence of the effect of *TGFB1* polymorphisms are also lacking.

Nonetheless, evidence generated so far in the largest studies seems to support the hypothesis of a clinically relevant effect of *TGFB1* polymorphisms on HSCT and this highlights the need for more research in this field. Consequently, this research project aims to address the functional effects of *TGFB1* polymorphisms on a specific cell subset likely to play a central role in immune processes in HSCT such as Treg, as well as the combined effect of various polymorphisms in *TGFB1* on HSCT outcome. The data derived from this project will potentially generate criteria for the selection of donors and the prevention of complications after HSCT.

Table 1.1 Summary of the studies analyzing the role of polymorphisms in *TGFB1* and *TBRII* and the outcome of HSCT.

Study	N[1]	Population	Ethnicity	SC source	Conditioning	GVHD prophylaxis[2]	Polymorphisms tested	Outcome tested	Associations found
(Leffell *et al.*, 2001)	50	44 HLA-identical siblings 6 UD	88% Caucasian	BM	Not stated	Not stated	+74G>C	GVHD	Recipient and P-D pair +74 GG and increased severe GVHD
(Tambur *et al.*, 2001)	24	HLA identical sibling (pediatric)	Not stated	BM	Not stated	Not stated	+29T>C +74G>C	GVHD	Recipient "High expression genotype" and reduced GVHD
(Hattori *et al.*, 2002)	67	HLA identical sibling (pediatric and adult)	Japanese	BM	TBI+Mel+Bu or thio-TEPA, or TBI+Cy, or TBI+ATG (32) non-TBI (35)	CsA+MTX (11) Other (55)	-1347C>T +29T>C +1167C>T (*TBRII*)	aGVHD cGVHD	Donor +29 TC or CC and increased aGVHD Recipient *TBRII* +1167 CT or TT and increased aGVHD No association
(Laguila Visentainer *et al.*, 2005)	118	HLA identical sibling (pediatric and adult)	Mixed (Brazilian)	82 BM 36 PBSC	Bu+Cy (97) Bu+Cy+VP (2) Cy (7) Cy+TBI (12)	MTX+CsA (109) Other (9)	+29T>C +74G>C	aGVHD cGVHD OS	No association (combined genotypes) No association (combined genotypes) No association (combined genotypes)
(Noori-Daloii *et al.*, 2007)	84	HLA-identical siblings (pediatric and adult)	Iranian	3 BM 81 PBSC	Bu+Cy	MTX+CsA	+29T>C	aGVHD	Recipient +29 TT or TC and reduced aGVHD
(Shah *et al.*, 2009)	38	UD	Caucasian	BM	"Myeloablative"	MTX+CsA (37) None (1)	-2389dupAGG -1347C>T +29T>C	aGVHD	No association (SNP or allele)
(Berro *et al.*, 2010)	427	UD	Not stated	321 BM 106 PBSC	"Myeloablative" TBI (346)	MTX+CsA (256) Other (132) None (25) Not known (14)	+29T>C +74G>C	OS, NRM, DFS, R, Ea,cGVHD	Recipient +29 CC and increased NRM and reduced OS Multiple +29C alleles in P-D pair and increased aGVHD, NRM and reduced OS

(Rashidi-Nezhad *et al.*, 2010)	86	HLA-identical siblings	Iranian	5 BM 81 PBSC	Bu+Cy	MTX+CsA	+74G>C	aGVHD	Recipient +74 GG and increased severity but no association with development of aGVHD
(Karimi *et al.*, 2010)	35	Not stated	Iranian	2 BM 33 PBSC	Bu+Cy (33) Cy+ATG (2)	MTX+CsA	+29T>C	aGVHD	No association
(Xiao *et al.*, 2011)	240	138 UD 102 HLA-identical siblings	Chinese	68 BM 172 PBSC	Bu+Cy (209) Flu-based (31)	MTX+CsA+MMF	-1347C>T +29T>C +1167C>T (*TBRII*)	aGVHD OS, NRM, R, cGVHD	Donor -1347 TT and recipient T allele and reduced aGVHD (UD group and combined cohort) No association
(Sellathamby *et al.*, 2012)	102	97 HLA-identical siblings 5 Other family member	Not stated	Not stated	Bu+Cy+ATG (72) Bu+Cy (30)	MTX+CsA	+29T>C +74G>C	E, OS infections	No association
(Harkensee *et al.*, 2012)	460[a] 462[b]	UD	Japanese	BM	Cy+TBI (656) Bu+Cy (100) TBI (156) Other (10)	MTX+CsA+Cor (433)[3] MTX+T+Cor (482)[4] Other (7)	-1347C>T[a] rs2241715[a] rs2241716[a,b] rs4803455[a]	a,cGVHD, R, OS	Recipient rs2241716 AA and increased extensive cGVHD (only in (a))
(Kim *et al.*, 2012)	394	307 related[a] 87 UD[b]	Not stated	276 PBSC[3] 118 BM[4]	"Myeloablative" (282) RIC (112)	MTX+CsA (263) CsA+MMF (97) CsA (22) Others (12)	-1347C>T	a,cGVHD, OS, RFS, NRM	Recipient -1347 CT and TT and increased aGVHD when compared to CC patients
(Kim *et al.*, 2014)	394	288 matched related 19 mismatched 84 matched UD 3 alternative	Not stated	276 PBSC[3] 118 BM[4]	"Myeloablative" (282) RIC (112)	MTX+CsA (263) CsA+MMF (97) CsA (22) Others (12)	-1347 C>T	a,cGVHD, (overall and organ-specific)	Recipient -1347 CT and TT and increased aGVHD Donor -1347 CT and TT and increased skin aGVHD and decreased lung cGVHD

[1] Patient-Donor pairs
[2] Other includes MTX, CsA, tacrolimus, CsA+Cor.
[3] More common in screening cohort ([a])
[4] More common in confirmatory cohort ([b])
ATG, anti-thymocyte globulin; BM, bone marrow; Bu, busulfan; Cor, corticosteroids; CsA, cyclosporine A; Cy, cyclophosphamide; DFS, disease-free survival; E, engraftment; Flu, fludarabine; (a, c)GVHD, (acute, chronic) graft versus host disease; HLA, human leukocyte antigen; Mel, melphalan; MMF, mycophenolate mofetil; MTX, methotrexate; OS, overall survival; PBSC, peripheral blood stem cell; P-D, patient-donor; R, relapse; RFS, relapse-free survival; RIC, reduced-intensity conditioning; SC, stem cell; T, tacrolimus; TBI, total body irradiation; thio-TEPA, N,N'N'-triethylenethiophosphoramide; UD, unrelated donor; VP, etoposide.

1.5 Hypothesis

Polymorphisms in *TGFB1* influence the outcome of allo-HSCT by affecting immunomodulatory functions within regulatory T cells in a post transplant setting.

1.6 Aims

- To identify differences in gene expression, protein production, protein secretion and/or protein functionality caused by *TGFB1* polymorphism.
- To explore the link between *TGFB1* polymorphism and regulatory T cell functionality.
- To correlate these biological parameters with the development of post-HSCT complications.
- To analyse the combined effect of various *TGFB1* polymorphisms on the outcome of UD-HSCT.
- To translate this knowledge into practical approaches by which transplant clinicians can foresee, assess and treat HSCT complications in patients.

Chapter 2. Materials and methods

2.1 Samples and subjects

2.1.1 Volunteer healthy donors

Volunteer healthy donors were used to obtain blood cells and nucleic acids for experiments. These were recruited among laboratory staff, and gave informed written consent (see **Appendix B**). Blood, saliva or buccal swabs were obtained and samples were given a unique identifier. No additional data were collected for these donors.

2.1.2 Patients and stem cell donors

HSCT patient and donor samples used in this thesis are part of Anthony Nolan Research Institute's sample biobank. This bank receives preconditioning blood samples from all recipients of an UD-HSCT performed at a transplant center in the United Kingdom who received a stem cell graft from a donor registered in Anthony Nolan's Stem Cell Donor Registry. Likewise, a sample from the respective donor is also obtained when their pre-transplant medical check takes place. When a preconditioning blood sample could not be obtained for the patient, a post-transplant buccal swab is requested instead. For donors, if it was not possible to obtain a sample during medical checks, then samples were obtained at the time of haematopoietic stem cell harvest.

The Patient-Donor Project staff at Anthony Nolan Research Institute coordinates the sample drawing with the transplant centers upon being notified of the transplant by the harvest coordinators. For this, a kit for sample collection and a consent form are shipped to the transplant center.

When the samples are shipped back to Anthony Nolan Research Institute, the data for the patient or donor are entered in a secure database, and samples are given and labeled with a unique internal consecutive identifier, henceforth used as the only means of sample identification. Afterwards, samples are processed accordingly in order to obtain DNA, PBMC, plasma, and a red cell and granulocyte pellets. The EDTA-anticoagulated blood is used for DNA extraction

2.1.3 Cell lines

A panel of B-lymphoblastoid cell lines was used as controls for experiments involving typing of molecular markers (Marsh, 1997). These cells are described by the International

Histocompatibility Workshop (IHWS) and are maintained at Anthony Nolan Research Institute by growing them in culture and storage of frozen stocks in liquid nitrogen. All cell lines had known HLA types. These cells were used for DNA extraction and this genetic material was used at 100 ng/µL for experiments. **Table 2.1** lists the cell lines that were used in this thesis and their basic information. More information on these cell lines can be found at the IPD-IMGT/HLA Database (http://www.ebi.ac.uk/cgi-bin/ipd/imgt/hla/cell_query.cgi, (Robinson *et al.*, 2013)).

Table 2.1 IHWS B-lymphoblastoid cell lines used in this thesis.

IHWS number	Common name	HLA class I type
9065	HHKB	HLA-A*03:01
		HLA-B*07:02
9077	T7527	HLA-A*02:01:01, 02:07
		HLA-B*46:01:01
9089	BOB	HLA-A*24:03:01
		HLA-B*51:01
9050	MOU	HLA-A*29:02
		HLA-B*44:03:01
9058	OMW	HLA-A*02:01
		HLA-B*45:01
9016	RML	HLA-A*02:04
		HLA-B*51:01:01
9035	JBUSH	HLA-A*32:01
		HLA-B*38:01:01
9021	RSH	HLA-A*68:02, 30:01:01
		HLA-B*42:01
9225	DOP-ND	HLA-A*02:01, 33:03
		HLA-B*42:01
9253	CJO-A	HLA-A*11:01:01
		HLA-B*35
9090	AWELLS	HLA-A*02:01
		HLA-B*44:02

2.1.4 Sample collection

Peripheral blood from healthy donors was obtained at Anthony Nolan Research Institute by venipuncture (60-110 mL) and stored 1.25:1 in transport media I (RPMI (Lonza Group Ltd., Basel) plus 1% sodium citrate (Sigma-Aldrich, Dorset, UK) and 0.05 µM

mercaptoethanol (Sigma-Aldrich, Dorset, UK)) in 50 mL Falcon tubes (BD Biosciences, Oxford, UK) until processed. 6-10 mL of the blood were anticoagulated in sodium ethylene-diaminetetraacetate (EDTA)-containing tubes (BD Biosciences, Plymouth, UK). Plasma and leukocyte buffy coat were separated from the EDTA-treated blood by centrifugation at 1800 rpm for 5 minutes, and stored at -80˚C.

For HSCT patient and donor peripheral blood samples, 50 mL are requested: 40 mL to be put in a tube with 40 mL of TM separated in two 20 mL aliquots, and 10 mL to be collected in a tube with 1.6 mg/mL EDTA (Sarstedt, Leicester, UK).

In both cases, PB collected in TM was used for separation of mononuclear cells, whereas the blood collected in tubes containing EDTA was used to obtain plasma and a buffy coat for DNA extraction.

In some cases, saliva samples from healthy volunteer donors were also collected for DNA extraction. For this purpose, the Oragene OG-500 sample collection kits (DNA Genotek Inc., Ontario, Canada) were used for the collection of 2 mL of saliva as indicated by the manufacturer. These kits contain preservatives and protease for DNA extraction from epithelial cells contained in human saliva and allow for long-term room temperature storage of collected samples.

For the collection of buccal swab samples, a nylon bristle cytology brush was rotated in the subject's mouth gutters located between the cheeks and the gums for 30 seconds. Two brushes per subject were collected, one used for the upper gutter, and the other for the lower one. Once the sample has been collected, the brushes can be kept at room temperature until DNA is extracted. However, sample processing was done promptly to avoid excessive desiccation and fragmentation of the DNA.

2.1.5 Clinical data collection

Clinical data on the outcome and status of the patients that underwent HSCT were collected by Anthony Nolan Research Institute in collaboration with the British Society for Blood and Marrow Transplantation. Originally, HSCT patients that consented to the study were followed and data were collected at six weeks, three months, six months, one year, eighteen months, two years and annually thereafter until loss of follow up because of death or failure to attend clinic. Later, all data collection was in line with the BSBMT process (100 days and annual follow up). All clinical data are stored under secure conditions on a protected Filemaker Pro database (Filemaker Inc., Santa Clara, California, USA), using the same unique identifier as the blood sample.

2.1.6 Ethical approval

All samples were collected according to the Anthony Nolan Research Institute's review board-approved guidelines and written informed consent was obtained from all the donors. Moreover, the Patient-Donor project has been granted ethical approval by the United Kingdom's National Research Ethics Service (www.myresearchproject.org.uk, application number MREC 01/8/31).

2.2 Cellular methods

2.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were separated from the transport media-treated blood by ficoll gradient (Lympholyte-H cell separation media, Cedarlane, Burlington, USA).

Diluted blood was layered onto ficoll 1.5:1 in 50mL Falcon tubes in sterile conditions, and was subsequently spun for 22 minutes at 2200 rpm with no brake applied. After this centrifugation, the mononuclear cell layer formed between the plasma and the ficoll was collected with plastic Pasteur pipettes (Fisher Scientific UK Ltd., Leicester, UK) and transferred to another sterile 50 mL Falcon tube, and granulocytes and cell debris were collected for DNA extraction from the bottom fraction of the gradient. The mononuclear layer was washed by addition of RPMI to complete 50 mL and spun for 10 minutes at 1800 rpm. After this second centrifugation, the supernatant was removed, and the cell pellet was resuspended in new RPMI. Once more, the cell suspension was spun for 5 minutes at 1600 rpm. After this final centrifugation, the supernatant was removed and the cell pellet was resuspended in 20-25 mL of RPMI.

Finally, the resuspended washed cells were counted using a Neubauer haemocytometer. For this, 10 µL of the resuspended PBMC were mixed with the same volume of a 0.4% solution of trypan blue (Sigma-Aldrich, Dorset, UK), and also with the same volume of Turk's solution (a 6% solution of acetic acid and Gentian violet) in a separate well. The viability of the cells was evaluated qualitatively with the trypan blue solution, and the nucleated cells were counted with Turk's solution.

2.2.2 Isolation of regulatory T cells (Treg)

PBMC isolated from transport media-anticoagulated blood were washed twice in RPMI and their number was determined by microscopic counting. In some experiments, 3-5x10^6 PBMC were separated for staining and/or irradiation purposes and the rest was

resuspended in chilled isolation buffer containing phosphate-buffered saline (PBS, Lonza Group Ltd., Basel), 1% bovine albumin (Sigma-Aldrich Ltd., Gillingham, Dorset, UK) and 2 mM EDTA (Gibco, Invitrogen, Life Technologies Ltd., UK). This isolation buffer was sterilized by filtration with 0.2 μL-filters (Minisart®, Sartorius Stedim biotech, Goettingen, Germany).

CD4+CD25- and CD4+CD25+ cells were then isolated by magnetic bead labeling with a human CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following a modified version of the manufacturer's instructions. Briefly, non-CD4+ cells were depleted from the PBMC by labeling with a cocktail of biotinylated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and CD235a, and anti-biotin microbeads in subsequent incubations at 4-8°C. The labeled cells were then retained in a bead column subject to a magnetic field, while non-labeled cells (the enriched CD4+ fraction) were collected from the flow-through by washing the column with chilled separation buffer. In a second step, CD4+CD25+ cells were separated from the CD4+CD25- cells in the CD4-enriched fraction from step 1 by direct labeling with anti-CD25 microbeads through incubation at 4-8°C. The cells were then subject to passing through tandem separation columns in a magnetic field, in which the CD4+CD25+ cells were trapped and later released after washing of the column when no magnetic field was present.

The modifications to the manufacturer's procedure aimed at increasing the yield but also protecting the purity of the isolation. These modifications included the use of a different scheme of depletion and selection magnetic columns (LS for depletion and LS-MS for selection, instead of LD and MS-MS respectively), and, in some experiments, a 20% reduction in the amount of anti-CD25 microbeads used in the selection step.

In order to assess the isolation of the cell subsets, PBMC, CD4-depleted, CD4+CD25- and CD4+CD25+ fractions were stained with flow cytometric antibodies against CD4 (PerCP, clone SK3, BD Biosciences, Oxford, UK; APC, clone RPA-T4, eBioscience, San Diego, USA), CD127 (FITC, clone eBioRDR5, eBioscience, San Diego, USA; PerCP, clone eBioRDR5, eBioscience, San Diego, USA), and CD25 (APC, clone 2A3, BD Biosciences, Oxford, UK; PerCP-Cy5.5, clone BC96, BioLegend, San Diego, USA).

2.2.3 Staining of cells with fluorescently-labeled antibodies

Staining of cells with fluorescently-labeled antibodies was performed for membrane (extracellular), cytoplasmic and nuclear antigens in different experiments. **Table 2.2** presents the information on the antibodies' clones, titrations and suppliers used for the experiments performed in this thesis. For the staining of antigens, 50,000-150,000

(depending on the experiment) freshly isolated cells or cells in culture were transferred to 96-well conical bottom polystyrene plates (Nunc, Thermo Scientific, Langenselbold, Germany) and subsequently transferred to 5 mL polypropylene tubes after the staining process in order to analyse the samples on the flow-assisted cell sorter. All centrifugations and incubations for cell staining were performed at 4˚C in the dark.

Table 2.2 Antibodies used for experiments in this thesis.

Antibody	Clone	Manufacturer	Titer
Mouse IgG1-PE IC	11711	R&DSystems	1/10
Rat IgG2a-FITC IC	eBR2A	eBioscience	1/20
αCD103-PE	Ber-ACT8	BD Pharmingen	1/50
αCD127-FITC	eBioRDR5	eBioscience	1/20
αCD127-PerCP-Cy5.5	eBioRDR5	eBioscience	1/20
αCD25-APC	2A3	BD Bioscience	1/25
αCD3-APC	UCHT1	BD Pharmingen	1/20
αCD3-FITC	UCHT1	BD Pharmingen	1/20
αCD3-PE	APA1/1	BD Pharmingen	1/20
αCD3-PerCP	SK7	BD Bioscience	1/50
αCD4-APC	RPA-T4	eBioscience	1/20
αCD4-FITC	EDU-2	ImmunoTools	1/100
αCD4-PerCP	SK3	BD Bioscience	1/20
αFOXP3	PCH101	eBioscience	1/20
αLAP-PE	27232	R&DSystems	1/10
αTGF-β1	9016	R&DSystems	1/10
αCD25-PerCP-Cy5.5	PC61.5	eBioscience	1/50

APC, allophycocyanine; Cy, cyanine; FITC, fluorescein isothiocyanate; IC, isotype control; PE, phycoerythrin; PerCP, peridinin chlorophyll.

2.2.3.1 Staining of extracellular antigens

For the staining of antigens present on the surface of cells, freshly isolated cells or cells in culture were transferred to 96-well conical bottom microwell plates and centrifuged for 3 minutes at 1800 rpm. All centrifugations for cell staining were performed at 4˚C. Thereafter, the supernatant was removed and the cell pellet was resuspended in 100 µL chilled staining buffer (PBS plus 2.5% fetal bovine serum, heat inactivated, Lonza Group Ltd., Basel) and spun again at 1800 rpm for 3 minutes. The supernatant was again removed completely and the cells were subsequently resuspended and incubated with 50 µL of the relevant titrated antibody mixture for 10 minutes at 4-8˚C. The cells were then spun as previously and washed twice by addition of 100 µL chilled staining buffer. After a

final centrifugation and removal of the supernatant, the stained cells were resuspended in 200 µL of staining buffer and transferred to 5 mL polypropylene tubes for acquisition on the flow cytometer.

2.2.3.2 Staining of intracellular antigens

In some experiments, intracellular (i.e. cytoplasmic or organelle-bound) antigens were targeted for immunofluorescent labeling. The intracellular staining was performed using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (Beckton, Dickinson and Company, BD Biosciences, San Jose, California) according to the manufacturer's instructions except for the final volume used for the washes, which was reduced from the recommended 250 µL to 200 µL but with prior complete removal of the previous supernatant, in order to avoid spillage when handling the micro-well plate.

Briefly, after removal of culture media, RPMI, isolation buffer or staining buffer, the cells were thoroughly resuspended in 100 µL chilled Fixation/Permeabilization solution and incubated for 20 minutes at 4˚C in the dark. Next, the cells were spun and washed twice in 200 µL BD Perm/Wash buffer. BD Perm/Wash buffer is supplied as a 10x concentrate, and was freshly prepared for each procedure by diluting it to 1x with distilled H_2O. After the second wash, the supernatant was removed completely, and the cell pellet was thoroughly resuspended in 100 µL of a 10% mouse serum (Sigma-Aldrich Ltd., Gillingham, Dorset, UK) solution in Perm/Wash buffer and incubated at 4-8˚C for 15 minutes, in order to block Fc receptors. The blocked cells were then spun and the diluted serum was removed. Next, the cell pellet was resuspended in 50 µL of BD Perm/Wash buffer containing the predetermined optimal concentration of the relevant fluorochrome-conjugated antibody or the relevant control. The suspension was incubated for 30 minutes at 4˚C in the dark. After the incubation with the antibodies, the cells were spun and the supernatant was removed. The cells were next washed twice by resuspending in 200 µL and centrifuging at 1800 rpm for 3 minutes. After the second wash, the supernatant was removed completely and the cell pellet was resuspended in 200 µL of standard staining buffer and transferred to 5 mL polypropylene tubes for acquisition on the flow cytometer.

2.2.3.3 Staining of intranuclear antigens

In some experiments, CD4+CD25- and CD4+CD25+ lymphocyte subsets were stained with antibodies against nuclear FOXP3 (FITC, clone PCH101, eBioscience, San Diego, USA) or the corresponding isotype control (FITC, clone eBR2a, eBioscience, San Diego, USA). The intranuclear staining was carried out after the extracellular staining (as previously detailed) using an Anti-Human FOXP3 staining set (eBioscience, San Diego, USA) following manufacturer's instructions.

Briefly, the cells were resuspended in 100 µL chilled freshly made Fix-Perm 1x solution and incubated at 4-8˚C for 30 minutes in the dark. The Fix-Perm solution is provided as a 4x concentrate, and thus the working solution was prepared on the day of staining by dilution with distilled H_2O. The cell suspension was then spun for 3 minutes at 1800 rpm and the supernatant was removed. The cells were washed twice by resuspending the cell pellet in 100 µL chilled freshly made Perm Buffer and spinning at 1800 rpm for 3 minutes. Perm buffer is supplied as a 10x concentrate, and was consequently diluted to a working solution with sterile distilled H_2O. After the second wash, the cells were resuspended in a solution containing 2% rat serum (Sigma-Aldrich Ltd., Gillingham, Dorset, UK), and incubated at 4-8˚C for 15 minutes in order to block Fc receptors in the cells. Rat serum was used because the antibodies against FOXP3 were generated in rats. After blocking, the cell suspension was spun and the supernatant was removed completely. Subsequently, the cell pellet was thoroughly resuspended in 50 µL of a pre-titrated dilution of the relevant antibodies in Perm buffer. The cells were incubated for 30 minutes at 4-8˚C in the dark. Once the incubation with the relevant antibodies was complete, the cells were spun and the supernatant was removed. The cells were subsequently resuspended and washed first with 100 µL of Perm buffer, and then with 100 µL of normal staining buffer. After a final centrifugation and removal of supernatant the stained cell pellet was resuspended in 200 µL of staining buffer and transferred to 5 mL polypropylene tubes for acquisition on the flow cytometer.

2.2.3.4 Antibody titration and controls

The antibody concentrations for the staining of cell surface, intracellular and intranuclear antigens were determined empirically by titration. For this purpose, PBMC were stained with various concentrations of the stock antibody solutions diluted with the relevant staining buffer. Normally, dilutions included 1/5, 1/10, 1/20 and 1/50 ratios. The cells were stained as previously described and the stained cells were analysed on the flow cytometer. The data from the titration experiments was analysed in order to determine the lowest concentration of antibody that allowed for a clear distinction between positive and negative cell populations.

Three types of staining controls were used depending on the type and nature of the experiments that were being carried out: isotype-matched control antibodies against an irrelevant antigen, fluorescence-minus-one antibody cocktails, and non-stained cells were used accordingly. Isotype controls were used mainly for controlling for the possibility of insufficient washing and trapping of the relevant antibody or nonspecific binding of the antibody to Fc receptors, especially in experiments involving intracellular or intranuclear

stainings. Fluorescence-minus-one cocktails were prepared by including all other antibodies except the relevant one and staining cells in the same manner as done with the regular cocktail. This procedure allowed for analysis of the interaction between the different fluorochromes and channels employed during acquisition of the data on the flow cytometer. Finally, non-stained cells (i.e. cells that underwent all the staining procedure except they were not incubated with any fluorescently-labeled antibody) were used for the establishment of cell autofluorescence and for setting up the cut-off for fluorescence positivity for stained cells.

2.2.4 Flow-cytometric analysis of fluorescently-labeled cells

All flow-cytometric data were generated by acquisition of single-cell suspensions on a florescence-assisted cell sorter FACScalibur (BD Biosciences, Oxford, UK). A lymphocyte gate and a live cell gate were established using the side-scatter and forward-scatter channels and based on cell size and internal complexity (granularity). A lymphocyte (restricted) gate was used in experiments that required exclusion of other cell types, whereas a live cell gate included all cells that did not appear to be dead or apoptotic, and was preferred in experiments that required the examination of non-lymphocyte populations or, more commonly, to allow for the inclusion of lymphocytes that had undergone or were undergoing blastic transformation upon activation.

Non-stained cells were used to set up the flow-cytometer's laser voltages in order to ensure that different cell subsets were clearly distinguishable on the side and forward-scatter, and to be able to differentiate between positive and negative fluorescence. Positive cut-offs for each fluorescence channel in different cell subsets were set on non-stained cells by allowing for a 0.5% positivity (i.e. 99.5% of the cells had a fluorescence that was lower than that cutoff).

For compensation between fluorescence channels, single-stained cells were used. These cells were stained with markers for which clearly distinguishable and frequent positive and negative populations could be obtained (most commonly CD3 or CD4) in that channel. Consequently, the mean fluorescence intensity of those two populations was calibrated to be approximately the same on another channel with which the stained channel interacts. For example, when compensating FL2 against FL1 leakage, lymphocytes stained for CD3 with an FL1-fluorescing antibody (usually fluorescein isothiocyanate (FITC)-labeled anti-CD3), CD3-negative lymphocytes were set to lie where the non-stained cells did with that laser voltage setting, while the CD3-positive population was adjusted so that their geometric mean-fluorescence intensity on FL2 was approximately the same as that of the CD3-negative population, and meant that, of all cells (CD3-positive and CD3-negative

together), no more than 0.5% trespassed the FL2 positivity cut-off set with non-stained cells. The same procedure was repeated for all 4 channels (FL1, FL2, FL3 and FL4), compensating for FL1 into FL2 and vice versa, FL2 into FL3 and vice versa, and FL3 into FL4 and vice versa leakages.

Once the laser voltages and compensation percentages had been set, the relevant samples were acquired with these settings and the data were collected and transferred from the flow cytometer to a computer for further analysis. Fifty-thousand events or as many as possible depending on the nature of the experiment were collected for each sample.

The flow cytometry results were analysed with Flowjo software (v. 6.4.7, Tree Star Inc., Ashland, USA).

2.2.5 Cell culture

All cell culture experiments were set up following sterile procedures. The cells (typically $100x10^3$) were cultured in 96-well round bottom plates (Microtest U-Bottom, Beckton Dickinson Labware, Le Pont de Claix, France) in 200 µL of culture media that consisted of RPMI supplemented with L-glutamine (2 mM, Sigma-Aldrich Ltd., Gillingham, Dorset, UK), HEPES (20 mM, Sigma-Aldrich Ltd., Gillingham, Dorset, UK), penicillin and streptomycin (100 U/mL each, Lonza Group Ltd., Basel), and 10% AB serum (heat inactivated, Lonza Group Ltd., Basel, or Sigma-Aldrich Ltd., Gillingham, Dorset, UK). In some experiments, the culture media was supplemented with IL-2, (final concentration at 100 U/mL, R&D Systems, Abingdon, UK). The cells were cultured at 37˚C in a 5% CO_2 atmosphere.

2.2.5.1 Activation assays

LAP and TGF-β1 expression and *TGFB1* mRNA production by Treg were assessed in cultured resting and activated isolated CD4+CD25+ cells. In parallel, isolated effector CD4 cells (CD4+CD25-) were also assessed in these experiments. Activation of the cells was carried out by means of TCR stimulation with antibodies against CD3 (NA/LE mouse, clone HIT3a, BD Biosciences, Oxford, UK) and CD28 (NA/LE mouse, clone CD28.2, BD Biosciences, Oxford, UK). Both antibodies were used at a final concentration of 10 µg/mL. Non-stimulated and plate-bound-antibody stimulated cells were used as controls. Coating of the wells for plate-bound stimulation was performed by addition of 50 µL of anti-CD3 and anti-CD28 at 10 µg/mL (each) in PBS and incubation at 4-8˚C overnight prior to culture. After incubation, and before set up of the culture, the antibody suspension was eliminated and the wells were washed thrice with 200 µL of sterile PBS.

In some experiments, the cells were harvested for assessment at different time points during culture (0-96h) and culture supernatants (as well as plain culture media) were

collected and stored at -80C˚ for future studies by centrifugation of the cell suspension in v-bottom plates or in microcentrifuge tubes.

2.2.5.2 Autologous suppression assays

In order to assess the immunoregulatory capacity of Treg with respect to *TGFB1* +29 T>C genotype, an autologous suppression assay was optimised.

After the isolation of CD4+CD25- and CD4+CD25+ subsets, the cells were resuspended in culture media that consisted of RPMI supplemented with L-glutamine (2 mM), HEPES (20 mM), penicillin and streptomycin (100 U/mL each), and 10% AB serum. The cells were cultured in a final volume of 200 µL in 96-well round bottom plates at 37˚C in a 5% CO_2 atmosphere.

During the optimisation process, several conditions differing in terms of cell numbers, cell subset ratios, costimulatory cells, positive proliferation controls, concentration of the stimulating antibodies, length of incubation, and method of assessment of proliferation, were tested. Problems with the proliferation of effector CD4+CD25- cells and lack of suppression by Treg in the system during the initial experiments prompted a thorough optimisation process. The details of this optimisation process are presented in Chapter 5.

After several experiments, the following conditions were selected: $100x10^3$ irradiated (30 Gy for 7 minutes) autologous PBMC were included as a source of costimulatory signals from antigen presenting cells. $100x10^3$ effector CD4 cells were used as responders, and up to 3 ratios of Treg were used in order to assess suppression (2:1, 4:1, 8:1). The stimulus was anti-CD3 (1 µg/mL) and, in some experiments, anti-CD28 (2 µg/mL) antibodies. Non-stimulated and phytohemagglutinin-stimulated (10 µg/mL, Sigma-Aldrich Ltd., Gillingham, Dorset, UK) effectors were included as controls. Also, stimulated irradiated PBMC on their own and with 25 or $50x10^3$ Treg were included as controls.

Two strategies for the assessment of cell proliferation were employed in different experiments, namely measurement of [^{3}H] thymidine incorporation, or of dilution of intracellular carboxyfluorescein succinimidyl ester (CFSE) in responding cells.

For the experiments involving [^{3}H] thymidine incorporation, the cells were incubated in culture for 3 days with different conditions, and then pulsed with [^{3}H] thymidine in RPMI (0.07 MBq, Perkin Elmer, UK) for the next 16-18 hours of incubation. The cultures were then frozen at -20˚C until harvested. Harvesting was performed using a TOMTEC Harvester 96 Mach III (TOMTEC, Hamden, USA), and the extent of proliferation assessed using liquid scintillation and β counting on a 1450 Microbeta Plus Liquid Scintillation Counter (Perkin Elmer, UK).

For those experiments based on CFSE dilution, the responding cells (typically isolated CD4+CD25- T cells) were stained with CFSE prior to the start of the culture, immediately after isolation, following the protocol described by Quah and collaborators (Quah *et al.*, 2007). Briefly, 5-10x10^6 responding cells were resuspended in 1 mL of PBS supplemented with 5% fetal bovine serum in a 15 mL Falcon tube. Next, a vial with lyophilized CFSE (Cell Trace™ CFSE Cell Proliferation Kit, Invitrogen, Eugene, Oregon, USA) was resuspended with 18 µL of dimethyl sulfoxide (supplied by the kit) for a 5 mM CFSE concentration. Then 1 µL of this CFSE stock was added to 1 mL of 1x PBS to get a 5 µM CSFE solution. This CFSE working solution was carefully transferred to the Falcon tube containing the cell suspension avoiding contact of the two liquids. This was achieved by placing the Falcon tube in a nearly-horizontal position and transferring the CFSE solution to the side of the tube while the cell suspension remained untouched at the bottom of the tube. The two solutions were then mixed instantly by immediate inversion of the tube, ensuring a uniform distribution of the CFSE among the cells. Consequently, the CFSE staining concentration was 2.5 µM. The mixed CFSE and cells were incubated at room temperature for 5 minutes in the dark. After this incubation, the tube was filled with PBS supplemented with 10% FBS to quench the CFSE, and the cells were spun for 5 minutes at 300 g. The supernatant was then removed and the cells were washed by resuspension in 15 mL of PBS+10% FBS three times, repeating the same procedure. After the third wash the cells were resuspended in the necessary amount of culture media and were ready for culture in the suppression assay. The dilution of CFSE by cell division was assessed by flow cytometry (CFSE fluoresces in the FL1 channel) after a 72-96 hour culture, and proliferation/suppression was determined by the analysis of the flow-cytometric data for each condition.

2.3 Molecular methods

2.3.1 Genomic DNA extraction

Genomic DNA for the experiments was obtained from three major sources: blood leukocytes and epithelial cells in buccal swabs or in saliva. The following protocols were used for extraction of this genetic material from its different sources.

2.3.1.1 Genomic DNA extraction from blood leukocytes

DNA was extracted from either a leukocyte buffy coat separated from EDTA-anticoagulated peripheral blood or from granulocytes and dead cells separated after the ficoll gradient of TM-anticoagulated peripheral blood. DNA was extracted by an in-house

salting-out protocol based on Miller's method (Miller *et al.*, 1988). All reagents for DNA extraction were obtained from Sigma-Aldrich Ltd., Gillingham, Dorset, UK.

Briefly, the concentrated blood containing the buffy coat or the granulocytes was transferred to a 15 mL Falcon tube (BD Biosciences, Oxford, UK), which was filled with chilled sterile lysis buffer (10 mM Tris-HCL, 5 mM $MgCl_2$, 10 mM NaCl) and thoroughly mixed by inversion. The tubes with concentrated blood and lysis buffer were incubated for 10 minutes at 4-8˚C in order to allow for the lysis of erythrocytes. After incubation, the tubes were centrifuged for 10 minutes at 3,200 rpm. Next the supernatant containing haemoglobin was discarded, and the cell pellet loosened by flicking. The cell pellets were then washed with lysis buffer by filling the Falcon tube and centrifuging with the same conditions as previously done. This was repeated (typically 3-4 times) until haemoglobin was eliminated (i.e. the supernatants and pellets had no reddish hue). Next, the supernatant was eliminated and the cell pellets loosened. Then, 80 µL of proteinase K buffer (10 mM Tris pH=7.5, 10 mM EDTA pH=8.0 and 50 mM NaCl), 30 µL of 10 mg/mL Proteinase K, 20 µL of 10% sodium dodecyl sulfate and 240 µL of sterile H_2O were added to the cells and mixed. The cells were digested in this mixture overnight at 37˚C. After the digestion the lysates were transferred to a clean 1.5 mL microcentrifuge tube. Subsequently, proteins were precipitated by addition of 100 µL of 5M NaCl and a short mixing by vortexing. The cell lysates were then spun for 5 minutes at 13,000 g and the supernatants containing the DNA were transferred to a clean 15 mL Falcon tube with 1 mL of cold absolute ethanol (VWR, Leicester, UK). The lysate and ethanol were gently mixed by inversion in order to precipitate the DNA. If the DNA was visible, it was collected with a glass rod and left to air-dry for 30 minutes. The ethanol-free DNA was then transferred to a clean microcentrifuge tube and sterile water was added to it. When no clearly visible precipitated DNA could be obtained, the samples were incubated overnight at -20˚C, and then transferred to a clean microcentrifuge tube and centrifuged at 13,000 g for 20 minutes. After centrifugation, the supernatant was discarded and the DNA pellet was left to air-dry for 30 minutes before adding the required amount of sterile H_2O and incubated at room temperature. DNA resuspension in water was ensured and its concentration and purity were assessed by ultraviolet spectrophotometry.

2.3.1.2 Genomic DNA extraction from buccal swabs

DNA was extracted from epithelial buccal cells collected with a brush using the Gentra Purgene Buccal Cell Kit (Qiagen, Manchester, UK), following the manufacturer's instructions, except for the fact that a different volume of proteinase K and a longer drying time were used. All reagents were supplied with the kit unless stated otherwise.

Briefly, the heads of the brushes were cut and placed in a microcentrifuge tube. Next, 300 µL of Cell Lysis Solution and 3.0 µL of 10 mg/mL Proteinase K (Sigma-Aldrich Ltd., Gillingham, Dorset, UK) were added to the tube, mixing and ensuring the liquid covered the whole brush head. Then, the tube was incubated in a water bath at 55˚C overnight. After incubation, the brush head was removed after removing as much liquid as possible from it and leaving the liquid in the tube. Subsequently, 100 µL of Protein Precipitation Solution were added to the lysates, and the mixture was vortexed for 20 seconds. The tube was then incubated for 5 minutes on ice, followed by a centrifugation at 15,000 g for 3 minutes. The supernatant was then removed and transferred to a clean microcentrifuge tube containing 300 µL of isopropanol and 0.5 µL of glycogen solution. The contents of the tube were mixed by gentle inversion, and centrifuged for 5 minutes at 15,000 g. The supernatant was now discarded and the tube was drained by inversion on an absorbent tissue. One wash with 300 µL of 70% ethanol followed, followed by a short centrifugation of 1 minute at 15,000 g. The supernatant was once more discarded and the tube drained as explained previously. The DNA pellet was left to dry for 15 minutes and 50 µL of sterile distilled H_2O were added for DNA rehydration.

2.3.1.3 Genomic DNA extraction from saliva

DNA was extracted from epithelial buccal cells collected via saliva using the Oragene OG-500 sample collection kits (DNA Genotek Inc., Ontario, Canada). The extraction process followed the manufacturer's instructions. All reagents were supplied with the kit unless stated otherwise.

Briefly, the kit tubes containing the saliva samples were incubated at 50˚C overnight in a water bath or in an air incubator. After this incubation, an aliquot of 500 µL of the collected saliva mixed with the collection kit liquid was transferred to a 1.5 mL microcentrifuge tube. Next, 20 µL of Oragene Purifier was added to the tube and mixed by vortexing for a few seconds. The tube was then incubated on ice for 10 minutes. After this, the tubes were spun at 15,000 g for 10 minutes, and the supernatant was collected and transferred to a clean microcentrifuge tube containing the same volume of room temperature absolute ethanol. The tube was mixed by inversion 10 times, and left to fully precipitate by incubating at room temperature for 10 minutes. The tube was next centrifuged at 15,000 g for 2 minutes, after which the supernatant was discarded and the tube was left to dry for 10 minutes. Finally, 50-150 µL of sterile distilled H_2O were added to rehydrate the DNA depending on the size of the pellet.

2.3.2 Total RNA extraction

Total RNA was extracted from PBMC or isolated cell subsets directly after separation or after culture. The cell suspension of interest was transferred to a microcentrifuge tube. Any separation buffer or culture media was completely removed by aspiration after centrifuging the cells at 300 g for 5 minutes. The cell pellet was the processed with the RNeasy® Mini Kit (Qiagen, Manchester, UK) following the manufacturer's instructions for total RNA extraction for animal cells. All RNA-related work was performed in RNAse-clean areas and on benches and equipment treated with RNAseZap (Ambion Inc., USA).

Briefly, the cell pellet was disrupted by flicking followed by addition of 350 µL of buffer RLT and repeated aspiration with a micropipette tip. After 25 aspirations, the lysed cells were agitated by vortexing for 60 seconds. If the extraction was not going to be performed immediately after lysis, the lysed cells were frozen and kept at -80˚C until used. For the capture of the RNA, 350 µL of 70% ethanol were added to the lysed cells and mixed by pipetting. This mixture was then transferred to an assembled RNeasy column in a collection tube. The loaded columns were spun at 10,000 g for 15 seconds. The flow-through was subsequently discarded, and the column reassembled. Next, 700 µL of buffer RW1 were added to the column, and it was spun again for 15 seconds at 10, 000 g. After centrifugation, the flow-through was discarded and 500 µL of buffer RPE were added to the reassembled column. The column was then centrifuged for 15 seconds at 10,000 g and the flow-through was discarded once more. This wash with buffer RPE was repeated but followed by a longer centrifugation for 2 minutes at 10,000 g. The column was then transferred to a new collection tube and spun without any addition for a further minute. After this step, the column was transferred to a clean microcentrifuge tube and 30 µL of RNase-free H_2O were added to it. The column underwent a final centrifugation for 1 minute at 10,000 rpm, and the eluted RNA solution was collected and frozen at -80˚C until use.

2.3.3 Assessment of the quality and quantity of nucleic acids

The concentration and purity of extracted nucleic acids was determined by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Labtech, Lewes, UK). Using 1.5 µL of the nucleic acid sample, the absorbance at 260 and 280 nm wavelengths was determined. The concentration of nucleic acids was calculated assuming equivalence of a 260 nm absorbance of 1.0 and 50 ng/µL of DNA or 30 ng/µL for RNA. The ratio of absorbances at 260 and 280 nm was used to assess the purity of the extraction, considering a ratio of 1.6-1.8 for DNA and of 1.8-2.0 for RNA as adequate. The sample's absorbance at 340 nm was used to evaluate its turbidity, and a correction to the 260 and

280 absorbances was applied if this was high. The samples were diluted to working solutions of 100 ng/µL if applicable, and stored at -20˚C until used.

In order to assess the integrity of nucleic acids, selected extracted samples were also run on an agarose gel alongside molecular weight markers (see below) to visualise the size and fragmentation of the respective nucleic acid molecules.

2.3.4 cDNA production

Copy (c)DNA production from total RNA was performed following an optimised protocol kindly supplied by Dr. Aurore Saudemont (Anthony Nolan Research Institute). In a first step, a mix including the relevant extracted total RNA (the volume for 200 ng or as much as possible with a maximum of 10.5 µL), distilled sterile H_2O (to complete 10.5 µL of RNA-H_2O mixture), 0.5 uL random primers (250 ng, Promega, Madison, Wisconsin, USA) and 1 µL of a premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP (Bioline Ltd., London, UK) each at 12.5 mM in sterile water (henceforward called dNTP mix). This mix (mix 1) was incubated at 65˚C for 5 min on an Eppendorf Mastercycler gradient thermalcycler (Eppendorf, Stevenage, UK), and was subsequently placed on ice for 5 minutes. In the meantime, a second mix (mix 2) was made by adding together 4 µL of SS II RT H 5x buffer (Invitrogen, Life Technologies, Paisley, UK), 2 µL DTT (0.1 M, Invitrogen, Life Technologies, Paisley, UK), and 1 µL RNAse inhibitor (40 UI/mL Promega, Madison, Wisconsin, USA) per RNA sample.

After mix 1 incubation on ice was over, 7 µL of mix 2 were added to mix 1 and the mixture was incubated on the thermalcycler at 25˚C for 10 minutes, followed by another incubation at 42˚C for 2 minutes. At this stage, 1 µL of the enzyme SuperScript™ II reverse transcriptase (Invitrogen, Life Technologies, Paisley, UK) was added to complete a 20 µL reaction. This mixture was further incubated on the thermalcycler at 42˚C for 50 minutes. After this final step, 45 µL of sterile distilled H_2O were added, and diluted cDNA was stored at -20˚C until use.

cDNA production was tested by conventional PCR with primers for β-Actin mRNA as reported by White *et al.* (White *et al.*, 2010). PCR was set up with Bioline reagents in a 30 µL reaction including 3 µL of 10x buffer, 2 µL of 50 mM $MgCl_2$, 0.6 µL of dNTPs, 1 µL of each primer, 0.2 µL of Taq polymerase, 20.2 µL of distilled H_2O and 2 µL of cDNA. The cycling protocol had an initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of 94˚C for 1 minute, 50˚C for 1 minute, and 72˚C for 1 minute, with a final extension of 10 minutes at 72˚C. The products were checked by agarose gel electrophoresis as explained in the next section.

2.3.5 Real-time polymerase chain reaction (RT-PCR)

Real-Time (RT)-PCR was set up with SYBR Green technology. RT-PCR reagents were provided by PrimerDesign (PrimerDesign Ltd., Southampton, UK). A SYBR Green 2x Master Mix containing low-concentration ROX as a reference dye was used in all experiments. RT-PCR mixes were set up in 20 µL composed of 10 µL of Master Mix, 1-3 µL of relevant primers at 6 µM depending on the necessary final concentration (300-900 nM), and 0-4 µL of distilled H_2O. The PCR reactions, set in triplicates, were made up in MicroAmp 96-well optical plates (Applied Biosystems, Foster City, California, USA), which were sealed with adhesive film.

The cycling and data acquisition were performed on an AB 7500 RT-PCR System (Applied Biosystems, Foster City, California, USA). The cycling protocol included an initial denaturation at 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and a data collection step at 60˚C for 1 minute. In addition to the cycling protocol, a dissociation curve step was added to each run in order to evaluate the purity and specificity of the products generated in each reaction. All RT-PCR fluorescence data were analysed with the AB 7500 SDS software (Applied Biosystems, Foster City, California, USA). ROX was used as the inter-well normalization dye. SYBR Green fluorescence was normalized against ROX (Rn), a baseline was deducted (ΔRn) and then transformed logarithmically to obtain a linear phase region for the determination of threshold cycle (Ct) values. Ct values and denaturation temperature (Tm) were determined automatically by the software, and were exported to the Excel software (Microsoft Corporation) for further analysis.

Target gene expression was determined semi-quantitatively by normalization to 2 reference genes using the ΔΔCt method: average Ct values for the target and reference gene were used to calculate a relative expression index using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

2.3.6 Agarose gel electrophoresis

Products of amplification of nucleic acids by polymerase chain reaction (PCR) were visualised by electrophoresis in 2% agarose (Seachem LE agarose, Lonza, Wokingham, UK) gels in order to confirm amplification efficiency and specificity. Agarose was resuspended in 1x Tris-Borate EDTA (TBE) buffer (from 10X concentrate, Lonza, Wokingham, UK). The mixture was heated until ebullition and complete dissolution of agarose. Subsequently, the agarose solution was cooled down in agitation until it reached approximately 60˚C, time at which it was stained with 500 ng/mL ethidium bromide (Sigma-Aldrich, Dorset, UK). The stained agarose suspension was then poured into the electrophoresis tanks and left to cool until polymerization occurred.

For the electrophoresis, a 2.5 µL aliquot from the amplicon to be assessed was mixed with 1.0 µL of 10x loading buffer (Invitrogen, Life Technologies, Paisley, UK) and 6.5 µL of distilled H_2O. This mixture was loaded onto the gel and run at an adequate voltage depending on the size of the tank so that the PCR products would separate in 30 minutes. TBE was used as running buffer. In order to check the correct size of the amplicon, electrophoresis of a molecular size marker was carried out in parallel for every run. The marker used was EasyLadder 1 (Bioline, London, UK). After the electrophoresis finished, the products were visualised by exposing the gel to ultra-violet light using the GelDoc-It system (UVP Ltd., Cambridge, UK). A photograph of the exposed gel was taken and recorded.

2.3.7 Molecular cloning

In some experiments, allele separation in order to define polymorphism phase was performed by molecular cloning and sequencing in isolation of the region of interest. This was achieved by following an in-house Anthony Nolan Research Institute protocol normally used for cloning of HLA class I PCR products. For this, an amplification of the region of interest was carried out with the relevant primers and using Finnzyme Phusion polymerase (Thermo Scientific, ABgene House, Surrey, UK), which generates blunt-end PCR fragments and has proofreading capacity and low mis-incorporation rate. All amplification reagents were obtained from Thermo Scientific, ABgene House, Surrey, UK, unless stated otherwise.

The amplification reaction was carried out in duplicate for each sample of interest and consisted of 4.0 µL of 5x Buffer GC, 0.4 µL of each relevant primer at 25 µM, 0.6 µL of 100% dimethyl sulfoxide, 0.4 µL of dNTP, 0.2 µL of the Phusion polymerase, and 50 ng of target DNA with the necessary amount of sterile distilled H_2O (usually 13 µL) to complete a 20 µL reaction. This PCR mix was subject to cycling on a Research PTC-200 thermal cycler (Alpha Laboratories Ltd., Hampshire, UK). The cycling program consisted on an initial activation at 98˚C for 30 seconds, followed by 10 cycles of 98˚C for 15 seconds, 70˚C for 30 seconds and 72˚C for 3 minutes, these in turn followed by 20 cycles of 98˚C for 15 seconds, 68 ˚C for 30 seconds and 72˚C for 3 minutes, and a final extension at 72 ˚C for 30 minutes. The PCR products were then checked for purity, yield and specificity by agarose gel electrophoresis, and were then purified by GFX merging of the twin amplifications in 30 µL of sterile H_2O.

The PCR products were cloned into a Blunt TOPO PCR vector (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Life Technologies, Paisley, UK). The selection for these vectors is based on kanamycin resistance conferred by the plasmid. The cloning reaction was

performed as instructed by the manufacturer. The plasmid with insert was then transfected to the competent *Escherichia coli* (supplied with the kit) by heat-shock.

The bacteria were then enriched in a short-term culture in S.O.C. medium, followed by plating of this medium on Luria-Bertrani agar with kanamycin (Sigma-Aldrich Ltd., Gillingham, Dorset, UK) and overnight incubation at 37˚C. The transformed, kanamycin-resistant *E. coli* that grew were then picked (typically 24 per sample) and cultured in 800 µL of a liquid culture in Luria-Bertrani broth culture media (Sigma-Aldrich Ltd., Gillingham, Dorset, UK) with kanamycin overnight at 37˚C. After this second culture, 200 µL of the broth were used to create a glycerol-preserved stock stored at -20˚C, while the remaining 600 µL was used for the plasmid DNA extraction. The extraction of plasmid DNA from each individual clone culture was done with the PureYield™ Plasmid Miniprep System (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions.

After the plasmid DNA was extracted for all clones, restriction enzyme digests for each were set up. EcoRI was used for the digestion. This enzyme generated 2 cuts on the vector, which allowed for a clear separation of the incorporated fragment and the vector itself. The restriction digests were set up mixing 0.2 µL of EcoRI enzyme, 0.5 µL EcoRI buffer (both from Invitrogen, Life Technologies, Paisley, UK), 3.3 µL of sterile H_2O and 1.0 µL of the relevant plasmid. The digestion was carried out on the PTC-200 thermalcycler and consisted of a 16-hour incubation at 37˚C, followed by a 30-minute inactivation at 65˚C, with a final cooling at 4˚C. The digested plasmids were run on an agarose gel and their digestion pattern recorded so that clones that had incorporated the fragment of interest would be identified.

Once the right clones had been identified, these were subjected to sequencing as described elsewhere (see below) in order to obtain the data of interest. The sequencing reaction was prepared with 2.5 µL of sterile H_2O, 1.0 µL of Ready Reaction mix, 0.5 µL of 5x buffer, 1.0 µL of the relevant primer at 1.6 µM, and 5.0 µL of plasmid DNA.

2.3.8 DNA sequencing

Sequencing of DNA was carried out following Sanger's method of fluorescently-labeled dideoxinucleotide extension termination.

2.3.8.1 Purification of templates

Initially, the PCR amplification products of the regions of interest were purified in order to eliminate excess primers and dNTP and to get templates for sequencing. Two strategies were employed for template purification: the column-based Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK), and the enzyme-based

illustra ExoStar PCR and Sequence Reaction Clean-Up Kit (GE Healthcare, Buckinghamshire, UK). The GFX method was used more commonly for cases of low-throughput or in cases where the amount of PCR product was reduced, allowing for combination and merging or various amplifications. The Exostar method was preferred when large numbers of samples had to be processed because of its one-step nature.

The GFX method was used according to the manufacturer's instructions except for the fact that the volumes for the capture buffer of the template and washes were reduced to 200 µL in order to allow for the use of a single collection tube and to eliminate the need of removal of the effluents of these stages. Briefly, 200 µL of capture buffer were added to each PCR tube and thoroughly mixed with the post-PCR mixture. All of the resulting mixture was then transferred to the assembled capture MicroSpin™ column and collection tube, and centrifuged for 30 seconds at 16,000 rpm. After this step, 200 µL of wash buffer were added to each column and another centrifugation with the same parameters ensued. After this washing step, the column containing the captured template was removed from the collection tube and transferred to a clean 1.5 mL microcentrifuge tube. Subsequently, 25-50 µL of sterile distilled H_2O (depending on the PCR product's band strength) was added to each column and incubated for 1 minute at room temperature. Finally, the hydrated column was spun for 1 minute at 16,000 rpm and the eluted template was collected in the microcentrifuge tube. The column was then discarded and the template was stored at -20˚C until used for sequencing.

The Exostar method was used according to the manufacturer's instructions, the only adaptation being the amount of PCR mixture and of Exostar reagent that were used per reaction depending on how much template was needed for down-stream applications. Briefly, a 5 µL aliquot of the PCR product was mixed with 2 µL of the Exostar reagent (or proportionally scaled higher or lower volumes of both substances depending on the amount of final purified template needed) in a PCR tube and incubated at 37˚C for 15 minutes (enzyme activation), and then at 80˚C for a further 15 minutes (enzyme inactivation) on a Perkin Elmer 9600 thermalcycler (Global Medical Instrumentation Inc. Minnesotta, USA). The reactions were then cooled to 4˚C for short-term storage or frozen at -20˚C if not used on the same day.

2.3.8.2 Cycle sequencing

The sequencing reactions were prepared with purified template and the BigDye Terminator sequencing technology v.3.1 (PE Applied Biosystems, Foster City, California, USA). The sequencing reactions were prepared according to each protocol's specific proportions of template, distilled H_2O, the BigDye chemistry Ready Reaction mix, 5x

buffer, and relevant primer (1.6 μM) in 96-well frosted subskirted thin-wall ABI-compatible plates (ABA17500, Bioplastics, Landgraaf, The Netherlands). All sequencing reactions had a final volume of 10 μL and the plates were sealed with adhesive PCR sealing foil sheets (Thermo Scientific, ABgene House, Surrey, UK).

The general cycle sequencing protocol was carried out on either a Research PTC-200 thermal cycler (Alpha Laboratories Ltd., Hampshire, UK), an AB GeneAmp® PCR System 2700 thermalcycler (PE Applied Biosystems, Foster City, CA, USA), or an Eppendorf Mastercycler gradient thermalcycler (Eppendorf, Stevenage, UK), and consisted on an initial denaturation at 96˚C for 1 minute, followed by 25 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 2 minutes, followed by a final cooling at 4˚C. After cycle sequencing, the plates were kept refrigerated until purification of the sequences. This was usually performed within 18 hours of cycling.

2.3.8.3 Purification of cycle sequencing products

After the template samples had been subject to cycle sequencing, the products were purified by alcohol precipitation. For this purpose, 2.5 μL of 125 mM EDTA were added to each reaction well and the plate was spun for a few seconds. Next, 30 μL of absolute ethanol were added to each reaction well. The plate was resealed and vortexed briefly to ensure the mixing of the ethanol and the reaction mix. The plate was then incubated at room temperature for 15 minutes in order to ensure that the entire DNA in the wells precipitates. After this incubation, the plates were centrifuged for 30 minutes at 3,200 g at room temperature. The supernatant was then removed by inversion and quick centrifugation of the plate onto absorbent paper. The plate containing the DNA pellet was then washed with 30 μL of 70% ethanol, and spun for 15 minutes at 3,200 g. After the centrifugation, the supernatant was removed as previously, and the plate was then left to dry for 5 minutes at room temperature so that evaporation of any ethanol occurred. After this incubation, the DNA pellets were resuspended by addition of 10 μL of HiDi Formamide solution (Applied Biosystems, Foster City, California, USA). The plate containing the resuspended stable cycle sequencing products was either frozen at -20˚C until microelectrophoresis, or immediately sealed with a rubber septum and loaded onto an ABI 3730XL genetic analyser-compatible cassette.

2.3.8.4 Microelectrophoresis of sequences

All sequencing data were generated by capillary electrophoresis on an ABI GA-3730xl genetic analyser (Applied Biosystems, Foster City, California, USA). The sequencing platform consisted on 96 coated glass capillaries (50 cm) filled with POP-7 polymer. The runs on the sequencer were performed at 60˚C with an injection time of 15 seconds, and

injection voltage of 1.5 kV and a run voltage of 8.5 kV. In some experiments, the run time was 3,600 seconds, while in others that were sequencing larger fragments this time was increased to 7,200 seconds. All other settings were kept as recommended by the manufacturer.

2.3.8.5 Analysis of sequencing data

The data generated after the capillary electrophoresis were transferred to a computer and analysed with either Sequencing Analysis 5.2 or Seq Scape 2.5.0 software (PE Applied Biosystems, Foster City, CA, USA). For HLA class I sequences, the data were analysed with AssignSBT v 3.6+ (Conexio-Genomics, Fremantle, Australia).

In the case of the *TGFB1* data, the sequences were analysed with the help of a purpose-built *TGFB1* polymorphism library in and Seq Scape 2.5.0 software. For this purpose the consensus sequences for the relevant regions were obtained from the USA National Centre for Biotecnology Information databases and were inserted as the target sequences. Known forms of polymorphism were located and recorded on the library. Sequencing data was then loaded onto the program and automatically aligned with the reference sequence and known and unknown polymorphisms were identified by the program. All previously known polymorphic positions were checked in order to confirm that the calls by the software were correct. If new polymorphisms were suggested by the software, these new positions were also checked for accuracy. Sequencing Analysis was used for manual analysis of samples in which in depth analysis of the raw data was required or when SeqScape was unable to align the sequences because of sequence quality or the presence of polymorphisms generating sequence misalignment, such as insertions or deletions. After checking the data, the genotypes for the relevant positions were recorded in an Excel database (v. 14.0.0, Microsoft Corporation, Redmond, Wahington, USA).

2.3.9 Human leukocyte antigen (HLA) class I typing by sequence-based typing (SBT)

Typing of HLA class I genotypes was performed by sequence-based methods with an in-house protocol developed at Anthony Nolan Research Institute. All reagents are from Bioline, London, UK, unless otherwise stated. This protocol is based on the sequencing of exons 2, 3 and 4 of the genes that code for HLA-A, HLA-B and HLA-C molecules. The amplification done on approximately 50 ng of genomic DNA generated products of 1.8 kb that were subsequently purified and sequenced as described previously using exon-specific primers.

The primers for the amplification of the respective genomic regions are listed in **Table 2.3**.

Table 2.3 Primers used for the amplification of HLA class I exons 2-4.

Primer specificity	Sequence 5'-3'	Product size
HLA-A		
Forward	5'-TGTAAAACGACGGCCAGTGAAACSGCCTCTGYGGGGAGAAGCAA	1,846 bp
Reverse 1	5'-CAGGAAACAGCTATGACCGGATGGTGGGCTGGGAAGACAGC	
Reverse 2	5'-CAGGAAACAGCTATGACCGGATGGTGGGCTGGGAAGACGGC	
Reverse 3	5'-CAGGAAACAGCTATGACCGGACGGTGGGCTGGGAAGATGGC	
HLA-B		
Forward	5'-TGTAAAACGACGGCCAGTGGGAGGAGCGAGGGGACCSCAG	1,876 bp
Reverse	5'-CAGGAAACAGCTATGACCAGGCCAGCAACAATGCCCACGATG	
HLA-C		
Forward	5'-TGTAAAACGACGGCCAGTAICGAGGIGCCCICCCGGCGA	1,890 bp
Reverse	5'-CAGGAAACAGCTATGACCGGGATGGTGGGCTGGGAAGAIGGC	

bp, basepairs; HLA, human leukocyte antigen.

All the primers were prepared at 25 mM concentrations. The amplification reactions were made by adding the necessary volume of genomic DNA to 2.5 µL of 10x NH_4 buffer, 0.4 µL (for *HLA-A*) or 0.35 µL (for *HLA-B* and *HLA-C*) of 50 mM $MgCl_2$, 0.2 µL of dNTP (12.5 mM each), 0.25 µL of forward and reverse primer mixtures, 0.5 µL of BioTaq polymerase and the necessary volume of sterile H_2O in order to complete a 25 µL reaction (usually 20.45 µL for *HLA-A* and 20.45 µL for *HLA-B* and *HLA-C*).

The amplification cycling was carried out on a Research PTC-200 thermal cycler (Alpha Laboratories Ltd., Hampshire, UK), and the amplification conditions were 95˚C for 5 minutes, followed by 37 cycles of 95˚C for 30 seconds, 69˚C for 1.5 minutes and 72˚C for 2 minutes, followed by a final extension at 72˚C for 8 minutes and cooling to 4˚C.

The amplification was confirmed by agarose gel electrophoresis, and this and the purification of the templates were carried out as previously described. The sequencing reaction was composed of 1.5 µL of purified template, in addition to 1.0 µL of 5x BDT buffer, 1.0 µL of Ready Reaction mix, 1.0 µL of the relevant primer and 5.5 µL of sterile H_2O. The sequencing primers were used at 1.6 µM, and were exon-specific. **Table 2.4** lists the sequencing primers for each exon in each gene.

Cycle sequencing, precipitation of the sequenced products and capillary electrophoresis were performed as stated above. A 3,600-second run time was used for this protocol, and

the sequences were examined in AssignSBT v 3.6+ (Conexio-Genomics, Fremantle, Australia) in order to define the correct high-resolution genotype (alleles that have the same sequence for exons 2-4) for each sample.

Table 2.4 Primers for the sequencing of HLA class I

Primer specificity	Sequence 5'-3'
HLA-A	
Exon 2 Forward	5'-AGCCGCGCCKGGASGAGGGTC
Exon 2 Reverse	5'-ATCTCGGACCCGGAGACTGT
Exon 3 Forward	5'-CCCACAGTCTCCGGGTCCGA
Exon 3 Reverse	5'-TGTTGGTCCCAATTGTCTCCCCTC
Exon 4 Forward	5'-GGTGTCCTGTCCITTCTCA
Exon 4 Reverse	5'-AGGCTCCTGCTTTCCCTA
HLA-B	
Exon 2 Forward	5'-GGGCGCAGGACCYGRGGA
Exon 2 Reverse	5'-CGACCCIGGCCGTICGTG
Exon 3 Forward	5'-GGGGCCAGGGTCTCACA
Exon 3 Reverse	5'-GCGAICTATAGGAGATGGGG
Exon 4 Forward	5'-GGTGTCCTGTCCITTCTCA
Exon 4 Reverse	5'-GGGCTCCTGCTTTCCCTG
HLA-C	
Exon 2 Forward	5'-AGCGAGGKGCCCGCCCGGCGA
Exon 2 Reverse	5'-GGAGGGGTCGTGACCTGCGC
Exon 3 Forward	5'-GGGGCCAGGGTCTCACA
Exon 3 Reverse	5'-AGGCCATICCGGGAGATCTA
Exon 4 Forward	5'-GGTGTCCTGTCCITTCTCA
Exon 4 Reverse	5'-GGGCTCCTGCTTTCCCTG

HLA, human leukocyte antigen.

2.3.10 Molecular genotyping of *TGFB1* codon 10 polymorphisms

Two approaches were used in order to genotype the blood donors' *TGFB*1 +29 T>C: 1) a sequence-specific primer (SSP) PCR and 2) sequencing of *TGFB1* signal peptide region, encompassing codon 10. Both the SSP-PCR and the sequencing reaction were validated using a panel of well-characterised in-house maintained B-lymphoblastoid cell lines (Marsh, 1997). All PCR reagents were obtained from Bioline Ltd. (London, UK) unless stated otherwise.

2.3.10.1 PCR-sequence-specific primer (SSP)

A PCR-SSP reaction based on that described by Perrey C. *et al*. (1999) (Perrey *et al.*, 1999) was optimised in house with annealing temperature and $MgCl_2$ gradients. Sequence specific primers were: sense 5'-TCCGTGGGATACTGAGACAC and antisense 5'-AGCAGCGGTAGCAGCAGCA (T allele) and 5'-GCAGCGGTAGCAGCAGCG (C allele). Primers were obtained from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK).

The gene encoding human growth hormone was used as an internal control and its primers were as follows: sense 5'-GCCTTCCCAACCATTCCCTTA and antisense 5'-TCACGGATTTCTGTTGTGTTTC.

The 12.5 µL optimised allele-specific reaction mix contained 0.1 µL of DNA polymerase (5 U/mL), 0.25 µL of each primer (50 µM), 1.25 µL of 10x buffer, 0.375 µL of $MgCl_2$ (50 µM), 0.2 µL of dNTP (12.5 µM each), 9.1 µL of water and 0.5 µL of test DNA (100 ng/µL).

After optimisation, the thermal cycling procedure was set up to an initial denaturation at 95˚C for 2 minutes followed by 35 cycles of 95˚C for 30 seconds and 63.6˚C for 40 seconds (C allele) or 66.5˚C for 40 seconds (T allele), plus a final extension at 72˚C for 30 seconds. Amplifications were performed on an MJ Research PTC-200 thermal cycler (Alpha Laboratories Ltd., Hampshire, UK). The products were analysed by electrophoresis in 2% agarose gels and visualised with ethidium bromide staining. The *TGFB1* target and internal control product bands were 241 and 429 bp long, respectively.

2.3.10.2 Sequencing

As a means of adding an additional confirmatory typing strategy for *TGFB1* +29 T>C polymorphism a sequencing reaction encompassing this region of the gene was optimised. The M13 tail-tagged primers for the amplification of this region were described by Berro M. *et al*. (2010) (Berro *et al.*, 2010): sense 5'-GTAAAACGACGGCCAGTTCCGTGGGATACTGAGACAC and antisense 5'-CAGGAAACAGCTATGACCCAGTTTCTTCTGCCAGTCA. The sequencing reaction was performed by primers for the M13 tails: forward 5'-GTAAAACGACGGCCAGT and reverse 5'-CAGGAAACAGCTATGAC. Both primers and M13 tails were obtained from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK)..

The amplification reaction was performed in a final volume of 25 µL including 2.5 µL of 10x buffer, 0.5 µL of each primer (50 µM), 0.5 µL of $MgCl_2$ (5 µM), 0.4 µL dNTP (12.5 µM each), 0.2 µL of DNA polymerase, 1 µL of test DNA (100 ng/µL) and 19.4 µL of water. Subsequently, the mix was amplified with the following optimised protocol: an initial denaturation at 95˚C for 2 minutes, followed by 37 cycles of 95˚C for 30 seconds, 59.5˚C for 30 seconds, and 72˚C for 30 seconds, and a final extension at 72˚C for 1.5 minutes.

Amplifications were performed on an MJ Research PTC-200 thermal cycler (Alpha Laboratories Ltd., Hampshire, UK). The final 644 bp product was assessed by electrophoresis in a 2% agarose gel and purified with an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK).

The sequencing reaction was performed with the BigDye Terminator sequencing technology v.3.1 (PE Applied Biosystems, Foster City, CA, USA). Forward and reverse 10 µL reaction mixtures included 1µL of Ready Reaction mix, 0.5 µL of 5x buffer, 1 µL of primer (1.6 µM), 1 µL of DNA template and 6.5 µL of water. The sequencing was performed on an AB GeneAmp® PCR System 2700 (PE Applied Biosystems, Foster City, CA, USA) with the following program: an initial denaturation at 96˚C for 1 minute, and 24 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 2 minutes.

The forward and reverse sequencing products were purified by alcohol precipitation and assessed by capillary electrophoresis on an ABI Sequencer (GA-3730xL). The sequences were analysed with the help of a purpose-built *TGFB1* SNP library in Sequencing Analysis 5.2 and Seq Scape 2.5.0 software (PE Applied Biosystems, Foster City, CA, USA).

2.3.11 Sequencing of the regulatory region of *TGFB1*

A strategy for the comprehensive typing of polymorphisms in *TGFB1*'s regulatory region and exon 1 as described by Shah *et al*. (Shah *et al.*, 2006; Shah *et al.*, 2009) was designed in this thesis in order to be applied to DNA from UD-HSCT patient and donor samples so that these genetic data could be correlated with the outcome and complications of transplantation. Since this was one of the objectives of this thesis, and the strategy was conceived, designed and tested by myself, the complete details of its development are included in Chapter 3 of this thesis. Moreover, its application on clinical UD-HSCT samples is described in Chapter 6. Hence, only a brief description will be given here.

The 3.0 kb genomic region of the genome known to exert regulatory functions over *TGFB1* expression was analysed for polymorphism by sequencing. The region extending from -2,664 to +423 according to this gene's translation start site was divided into 4 subregions that were amplified separately by PCR. The basic PCR mix included 2.5 µL of 10x NH_4 buffer, 0.4 µL of 50 mM dNTPs, 0.5 µL of 50 mM $MgCl_2$, 0.5 µL of each relevant primer at 25 µM, 0.2 µL of Taq polymerase, 19.4 µL of distilled H_2O, and 1.0 µL of the target DNA (50-100 ng). The products of these amplifications were then purified by any of the methods stated above, and were then sequenced using M13 tail-targeted primers in two directions and Sanger sequencing chemistry. The sequencing reaction was set up with 5.5 µL of distilled H_2O, 1 µL of 5x BDT buffer, 1 µL of BDT Ready Reaction mix, 1 µL of either M13 forward or reverse primers at 1.6 µM, and 1.5 µL of the purified template. The sequenced

fragments were then aligned onto a template sequence of the complete genomic region set up on SeqScape software, and the polymorphic positions, both known and unknown, were recorded. The analysis protocol used base caller KB.bcp and KB_3730_POP7_BDTv3.mob as the DyeSet/Primer. The mixed bases were called with more than 25% peak height threshold. Clear range was defined with both quality values (bases were removed from the end until less than 4 in 20 bases had quality values of less than 20) and reference trimming. The filtering settings were set to maximum 20% mixed bases, maximum 10% undefined bases, a minimum clear length of 50 bp, and a minimum sample score of 20. All other parameters were kept as recommended by the manufacturer.

Based on the genotypes for 18 known polymorphic positions and on previous work by Shah *et al*. (2006), in which the authors defined the phase of these polymorphisms into 17 alleles, the sequencing data were used to assign a *TGFB1* regulatory region and exon 1 allelic genotype. This allowed for the covering of the variation in a large region and the identification of allelic blocks associated with this variation present in healthy volunteer donors, cell lines, and HSCT patients and their stem cell donors. Following the convention in histocompatibility and immunogenetics, in this thesis, the term 'allele' will refer to specific sequences for *TGFB1*'s regulatory region and exon 1 characterised by a unique combination of variants at the 18 polymorphic positions described by Shah and collaborators (2006 and 2009) rather than the term 'haplotype', considered to refer to specific alleles of different genes that are inherited together.

2.4 Statistical methods

Statistical analyses were performed using Prism software (v. 5.0, GraphPad Software Inc., San Diego, USA), Microsoft Excel (v. 14.0.0, Microsoft Corporation, Redmond, Wahington, USA), EpiTools (http://epitools.ausvet.com.au/content.php?page=home, AusVet Animal Health Services, Australia), R (v. 3.0.3. 2014-03-06, The R Foundation for Statistical Computing) and SPSS (v. 21.0.0, Chicago, Illinois, USA).

Kruskal-Wallis and Mann-Whitney tests were used to compare Treg LAP expression levels between *TGFB1* +29 T>C genotype groups. A probability (*p*) under 0.05 was considered significant, whereas *p* between 0.05 and 0.1 were considered trends.

In the clinical study, the Z-test was used to compare *TGFB1* regulatory region and exon 1 allele and specific variant and genotype frequencies between the HSCT patient and donor cohorts. Probability (p) values were calculated for two-tailed comparisons using a significance level of 0.05. Deviation from Hardy-Weinberg equilibrium in the whole cohort was assessed with Fisher's exact test or Chi^2 test.

The outcome of HSCT is usually analysed by measuring several time-dependent variables. The key events that take place after an HSCT are neutrophil and platelet engraftment, aGVHD, relapse or progression, death, and cGVHD. These events are quantified in order to calculate the following HSCT outcomes (Szydlo, 2012):

- Neutrophil engraftment: in the first of 3 consecutive days when the neutrophil count is ≥500/μL.
- Platelet engraftment: in the first of 3 consecutive days after HSCT when the platelet count is ≥50,000/μL without platelet support.
- GVHD: probability of developing a specific degree of GVHD.
- Transplant-related mortality (TRM): the probability of dying from a cause that is not disease relapse.
- Overall survival (OS): the probability of survival irrespective of disease status.
- Disease-free survival (DFS) or leukaemia-free survival (LFS): the probability of being alive and without disease.
- Relapse: the probability of disease recurrence.
- Progression-free survival (PFS): the probability of being alive and without changes in disease status from the time of transplant.
- Event-free survival (EFS): the probability of being alive and without a specific event such as relapse, or progression or any other.
- Non-relapse mortality (NRM): the probability of dying without relapse or progression of disease at any time post transplant
- Time to progression (TTP): time to first event related to the disease.

Primary graft failure is defined as failure to achieve neutrophil and platelet cut-offs for engraftment after 28 days post-HSCT. Secondary failure occurs in patients that have previously achieved engraftment but lose this developing at least two cytopenic lines (Lowsky, 2010).

GVHD after allo-HSCT occurs in acute and chronic forms, whose cut-off was somehow arbitrarily set at before or after 100 days post- transplant, respectively. However, this notion has recently changed due to the recognition of occurrence of acute-like symptoms after 100 days in RIC-HSCT and after DLI, thus prompting the use of the term late-onset aGVHD. Of note, there is also an overlap syndrome, in which aGVHD and cGVHD manifestations can coexist (Filipovich *et al.*, 2005; Apperley, 2012).

For analysis of time-to-event data, the Kaplan-Meier method (Kaplan, 1958) was used to estimate the probabilities of OS and EFS. EFS was defined as survival without relapse. Log-

rank statistics were used to compare OS and EFS probabilities between groups of interest. The probabilities of NRM and relapse were estimated by the cumulative incidence method, and different probabilities between groups were compared using Gray's test. Disease relapse and death without relapse were considered competing events for the cumulative incidence analysis of NRM and relapse, respectively. aGVHD incidences were compared by means of the Chi^2 test, or by Fisher's exact test if numbers were insufficient for the former. Multivariate analyses involving genetic and clinical factors and their effect on HSCT outcome or complications were performed using Cox-regression analysis, except for aGVHD, for which logistic regression was used. The variables selected for multivariate analyses were those that suggested relevant association ($p \leq 0.2$) with transplant outcome in univariate models.

Chapter 3. Development of molecular tools to characterise *TGFB1 and its* polymorphisms

3.1 Introduction and aims

The evidence of the presence of significant polymorphism in the *TGFB1* gene has led to the study of its influence on the regulation of TGF-β1 production by human cells. Moreover, it has prompted interest in the role of these polymorphisms in the modification of the susceptibility, evolution and severity of diseases in which this cytokine is thought to play a role. Consequently, a significant number of scientific projects have tried to address these questions by associating specific genotypes of *TGFB1* polymorphisms with differing risk of disease complications. Likewise, interest has also come from the HSCT field taking into account the potentially very relevant role of TGF-β1 in almost any pathophysiologic process related to this modality of transplantation.

Normally, the criteria for the selection of polymorphisms of interest include a plausible functional consequence, as well as a reasonable minor allele frequency (MAF) that would ensure the feasibility of any study. Thus, attention has focused on selected polymorphisms in *TGFB1*.

One of the most studied polymorphisms in disease and transplantation association studies is *TGFB1* +29 T>C. This SNP is located at codon 10 of the TGF-β1 precursor, thus part of its signal peptide, and causes an amino acid change from (T) Leu to (C) Pro, and potentially affects the way in which TGF-β1 production develops. As explained in Chapter 1, this SNP has been associated with differences in TGF-β1 production in *in vitro* experiments, and has also been related to differing susceptibility to disease and complications in transplantation, including HSCT. The +29T/C has been reported to have a MAF (C) of 35-42% in European Caucasians, 40-45% in Sub-Saharan African-derived populations, 40-50% in Hispanic populations, and actually to predominate in Asian (Oriental) individuals at 61% (Hoffmann *et al.*, 2002; Delaney *et al.*, 2004; Amani *et al.*, 2008; Visentainer *et al.*, 2008). Consequently, this polymorphism is both interesting and feasible for genetic association studies.

One of the first steps towards the characterisation of the influence of *TGFB1* polymorphisms on Treg behavior was to develop molecular tools for the typing of the polymorphisms of interest. Initially, I chose *TGFB1* +29T>C due to the aforementioned

characteristics and also because of its precedent association with HSCT outcome found by our group (Berro *et al.*, 2010).

Polymorphisms can be typed for at the genome level in diverse ways. When working with a SNP, one of the simplest ways to type it is by sequence-specific primer (SSP) amplification. In this system, allele-specific primers ending or starting on the SNP are used in combination with a common forward or reverse primer to perform PCR. Consequently, samples that are homozygous for one of the alleles will have a negative reaction for the other allele. Usually, an additional non-polymorphic primer pair targeted to another gene is used as internal control. The advantages of SSP are that its design is simple, it setup requires little equipment and is hence cheap, as well as being a quick genotyping technique. Another way of examining the genotype of a given SNP in unknown samples is using sequencing of a gene fragment containing the relevant SNP. In this case, primers are designed for regions that lack polymorphism, and amplify a region spanning the SNP of interest. This amplification is sequenced by Sanger's technique using either sequence-targeted primers or by primers targeted to labels adapted to the amplicon during PCR (e.g. M13 tails). In general, sequence-based typing (SBT) is considered to be more reliable, but it requires complicated equipment and is more laborious and expensive unless several SNPs are covered in one reaction.

Another research question I decided to address was whether *TGFB1* +29T>C was directly responsible for the associations found for it, or if it was rather a marker for another functional SNP. Also, since there are conflicting results on the effect of this and other *TGFB1* polymorphisms on the outcome of HSCT and diseases, I hypothesised that the fact that different studies focused on different polymorphisms that may or may not be associated and/or have antagonistic or synergistic effects on HSCT outcome could be confounding the real effect of genetic variation within this gene. Consequently, I also designed an SBT protocol to comprehensively type all known and potentially unknown polymorphisms in *TGFB1*'s regulatory region and exon 1. The design and results from the optimisation of this protocol are also presented in this chapter.

Finally, in order to further evaluate the kinetics of TGF-β1 production by Treg, I also optimised a real-time (RT)-PCR protocol for the semi-quantitative assessment of the levels of *TGFB1* mRNA in resting and stimulated cells that would complement and contrast the evaluation of protein levels. The results of the optimisation process are presented in this chapter, while the application of this technique is presented in Chapter 4.

3.2 Molecular tools for the typing of *TGFB1* +29T>C

Molecular typing techniques for the *TGFB1* +29 T>C polymorphism were designed and optimised with a gradient thermal cycler and different annealing temperatures and $MgCl_2$ concentrations in order to get a reliable and non-laborious means of characterising this polymorphism in both HSCT patient-donor pairs and healthy volunteer donors using DNA of variable quality. Next, both techniques were applied to type healthy volunteer donors for the subsequent cellular experiments. **Figure 3.1** shows the region of interest and the *TGFB1* primers used for these experiments.

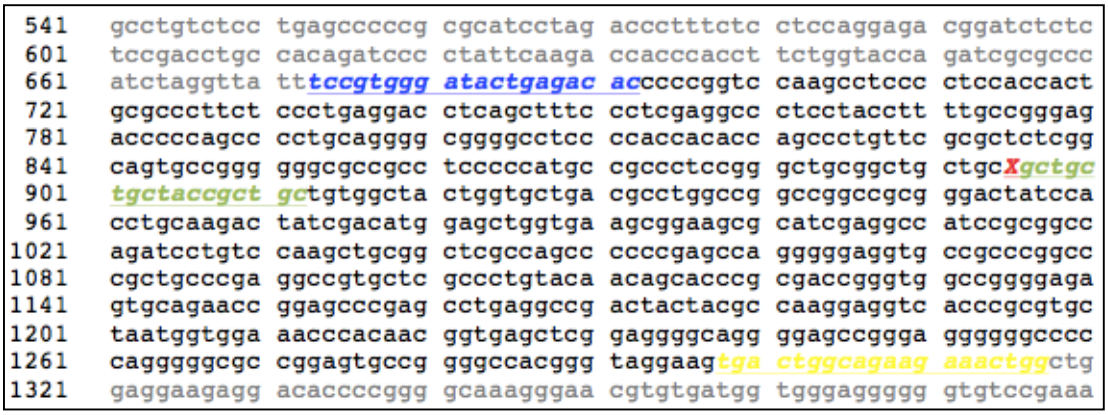


Figure 3.1 Genomic region of interest for *TGFB1* +29T>C typing. *Homo sapiens TGFB1* RefSeqGene on chromosome 19 (negative strand) showing the sequences and positions of the oligonucleotides used for the sequence-specific primer (SSP) and sequencing-based typing (SBT) of the *TGFB1* codon 10 SNP. The common generic forward primer is shown in blue, while the complementary sequences for the reverse primers for the SSP are shown in green with the polymorphic position in red. The SBT reverse primer complementary sequence is shown in yellow.

3.2.1 Optimisation of a sequence-specific primer (SSP) protocol for *TGFB1* +29T>C

As SSP relies on specific amplification relying on a single base difference between two strands of DNA, which is potentially not enough to confer specificity, an initial attempt for the setup of the +29T>C SSP was made by using an amplification-refractory mutation system (ARMS) in order to try to increase specificity. In this system, the primers are designed so that their 3' end is at the polymorphic position, but it also includes a mutation further upstream in the primer. With this modification, it is expected that 2 mismatches (i.e. the allele-specific one and the artificial one) will remove non-specificity during amplification. I used a common sense primer (5'-TCCGTGGGATACTGAGACACA) and two SNP-specific mutated primers: 5'-GCAGCGGTAGCACCAGC**G** and 5'-GCAGCGGTAGCACCAGC**A**, where the base in red is the artificially mutated position, and

the final base in bold is the SNP-specific position. These primers were tested in PCR mixtures that were subjected to annealing temperature gradients in order to determine an optimal temperature in a two-stage amplification cycling protocol as reported by Berro *et al*. (2010). However, these experiments repeatedly resulted in inefficient amplification of the target DNA fragment, and were consequently abandoned.

I then chose to continue the attempts of optimizing a +29T>C SSP with non-mutated primers. I used the same generic primer and non-mutated versions of the ARMS primers (5'-AGCAGCGGTAGCAGCAGC**A** and 5'-GCAGCGGTAGCAGCAGC**G**) as anti-sense primers, and included primers for the amplification of a section of the gene encoding human growth hormone as an internal control, as described by Perrey *et al*. (1999). Also, I changed the two-step cycling to a three-step one having 95˚C and 72˚C as denaturation and extension temperatures, respectively. These primer combinations were tested in a 12-µL reaction that was subject to annealing temperature gradient experiments, with temperatures ranging from 60 to 72˚C. The annealing experiments were repeated several times, narrowing the temperature ranges aiming for maximal amplification of the target band while keeping the desired degree of specificity.

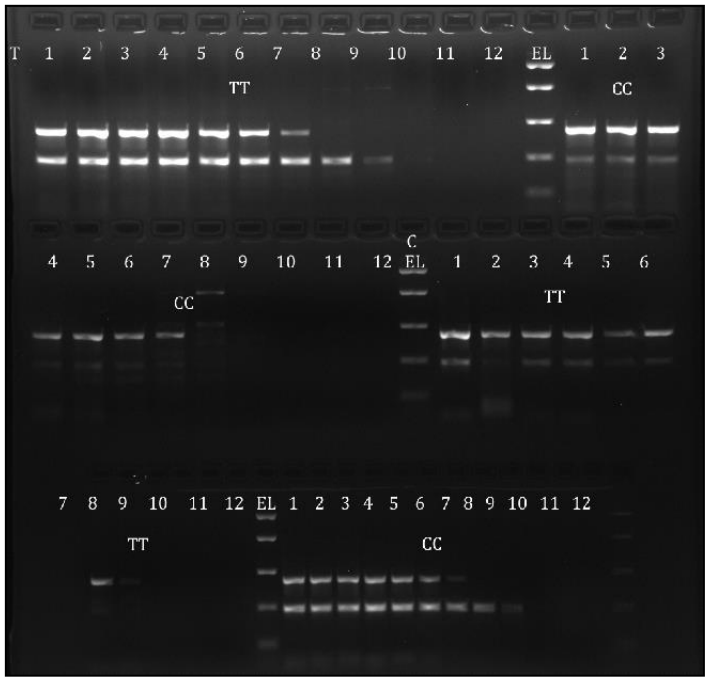
When the optimal annealing temperatures were determined, the PCR reaction was tested along a range of Mg^{++} concentrations, still with the aim of maximizing amplification efficiency while keeping specificity. For this purpose, PCR mixes were prepared in which a final $MgCl_2$ concentration ranged from 0.8 to 2.4 mM. **Figure 3.2** shows an example of the optimisation experiments that were carried out for this particular technique.

The SNP-specific reactions were tested with cell lines with known types for *TGFB1* +29T>C. Specifically, cell line RSH was used as a +29TT cell, JBUSH as a +29CC cell, and DOP-ND as a heterozygous sample (see Chapter 2 for further details on these cell lines). The typings for these cell lines were confirmed by the optimised SSP.

Also, in order to determine the range of DNA quantities along which the SSP protocol worked, I tested this protocol with DNA quantities ranging from 2.5 to 50 ng per reaction. **Figure 3.3** shows an example of these experiments, which confirmed that the SSP protocol worked with good efficiency along the whole range of concentrations in most cases.

Once the correct annealing temperatures and $MgCl_2$ concentrations for the amplification reactions were determined (as described in Chapter 2) and their results validated with a number of cell lines, the optimised PCR protocol was used to type healthy volunteer donors. DNA extracted from peripheral blood was tested with the SSP-PCR protocol and cell line DNA and blank reactions were used as controls. **Figure 3.4** shows an example of a healthy blood donor cohort typing experiment. A total of 32 healthy donors with unknown *TGFB1* +29T>C types were tested and successful typing was obtained.

(A)



(B)

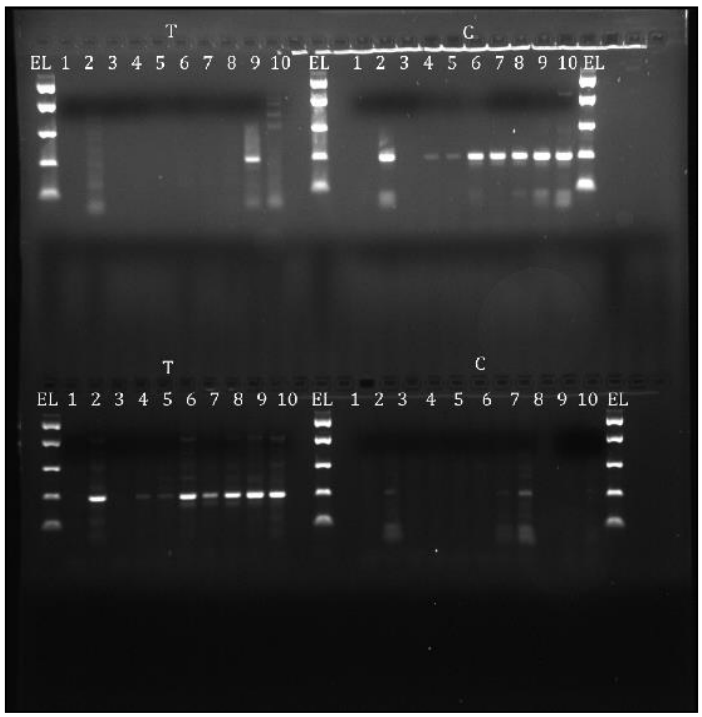


Figure 3.2 Optimisation of *TGFB1* +29T>C sequence-specific primer-polymerase chain reaction (SSP-PCR). (A) Annealing temperature gradient (top row: T reaction for TT and CC cell lines; middle row: T reaction for CC cell line (cont.) and C reaction for TT cell line; bottom row: C reaction for TT cell line (cont.) and C reaction for a CC cell line). Numbers 1-12 indicate reactions with annealing temperatures from 60.0 to 72.0˚C. (B) $MgCl_2$ titration (top row: T and C reactions for a CC cell line; bottom row: T and C reactions for a TT cell line). Numbers 1-10 indicate reactions with 50 mM $MgCl_2$ volumes ranging from 0.2-0.6 µL. Target band: 241 bp, Internal control band: 429 bp. EL=EasyLadder I (2 kb, Bioline Ltd., London UK).

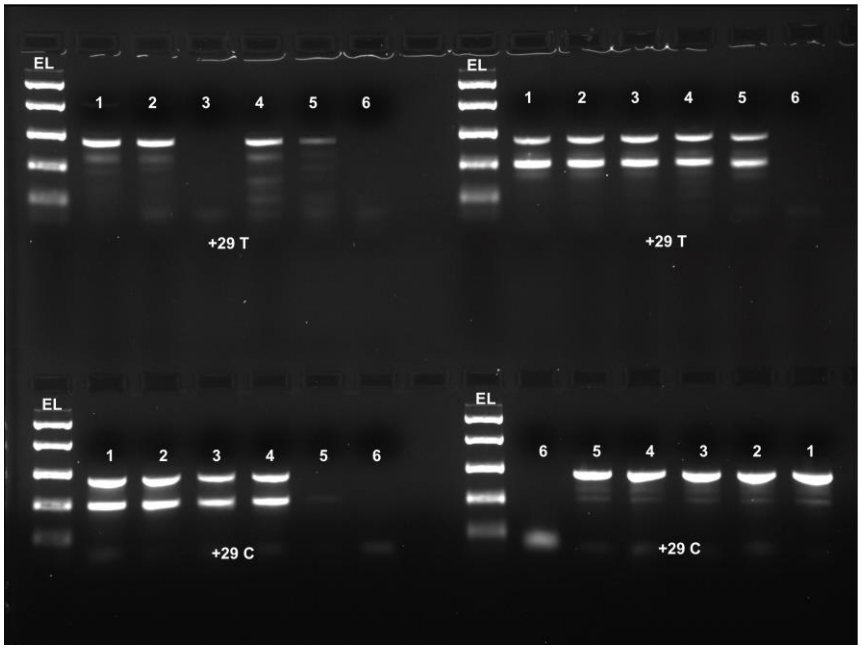


Figure 3.3 Test of the *TGFB1* +29 T>C sequence-specific primer (SSP) protocol with different quantities of target genomic DNA. The *TGFB1* +29T>C was used to amplify DNA from TT and CC cells using 2.5 to 50 ng of genomic DNA. Top row: T reaction, bottom row: C reaction. Left rows: +29 CC sample, right rows: +29 TT cells. DNA quantities: (1) 50 ng, (2) 25 ng, (3) 10 ng, (4) 5 ng, (5) 2.5 ng, (6) 0 ng. (3) for the T reaction of the CC cell failed.

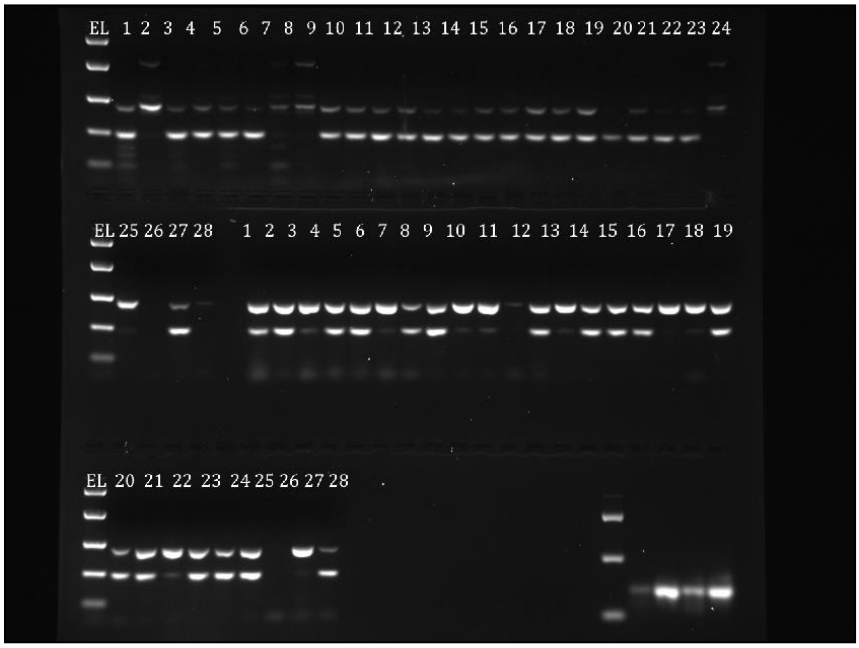


Figure 3.4 Example of a *TGFB1* +29T>C sequence-specific primer (SSP) typing experiment with healthy donors. Top row: T reaction for test DNA samples 1-24; Middle row: continuation of T reaction (test DNA samples 25-28) and C reaction for test DNA samples 1-19; Bottom row: C reaction for test DNA samples 20-28. Test DNA 26 is negative control, and 27 and 28 are TT and CC cell lines, respectively. Target band: 241 bp, Internal control band: 429 bp. EL=EasyLadder I (2 kb, Bioline Ltd., London UK).

3.2.2 Optimisation of a sequence-based typing (SBT) protocol for *TGFB1* +29T>C

After having optimised the PCR-SSP reaction, a sequencing protocol was devised in order to use it as a second typing option and as a way to verify the PCR-SSP results. The amplification reaction for SBT used the same generic forward (sense) primer as the SSP, but including M13 tags (5'-GTAAAACGACGGCCAGT-forward primer). The reverse primer (5'-CCAGTTTCTTCTGCCAGTCA), as reported by Berro *et al*. (2010), also included M13-phage tags (reverse 5'-CAGGAAACAGCTATGAC-reverse primer), but was located further downstream as compared to the SSP antisense primers (see **Figure 3.1**). The M13 tails were used to target the amplicon at the cycle sequencing stage (forward 5'-GTAAAACGACGGCCAGT and reverse 5'-CAGGAAACAGCTATGAC).

The amplification primers were tested in a 25-µL PCR mixture that was subjected to a PCR cycling protocol devised by myself following the annealing temperature reported by Berro *et al*. (2010). I used a 95˚C denaturation temperature and a 72˚C extension temperature, and the protocol included an initial 2-minute denaturation, 37 cycles of 30-second incubation at the three temperatures, and a final 1.5-minute extension. Since this protocol proved successful straight away by producing a strong and specific target band, and sufficient DNA template from most of the samples tested, it was consequently selected for further experiments. An example of the *TGFB1* signal peptide region amplification for sequencing is presented in **Figure 3.5**.

Subsequently, bi-directional cycle sequencing of the purified DNA template was carried out. Purification was done as explained in Chapter 2. The cycle sequencing and subsequent purification and microelectrophoresis of the product were performed as explained in Chapter 2. The sequence data were analysed with Sequencing Analysis software, by direct examination of the polymorphic position. **Figure 3.6** shows 3 examples of the sequencing results obtained with this protocol as seen with the sequence data analysis software, corresponding to each of the possible *TGFB1* +29T>C genotypes. Again, the results for this sequencing protocol were validated with cell lines of known types, as done for the SSP protocol. All typings were in agreement with previous data.

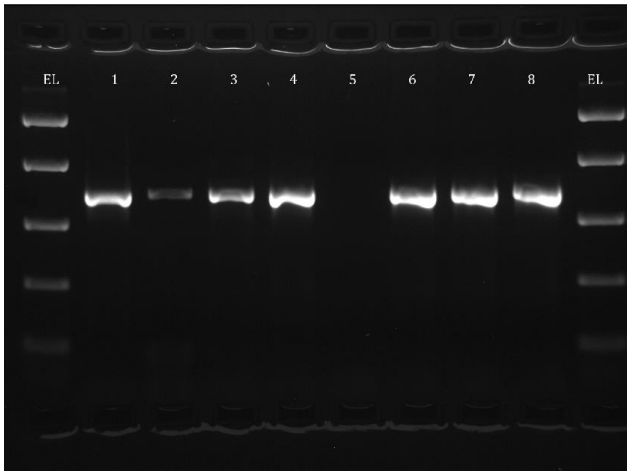
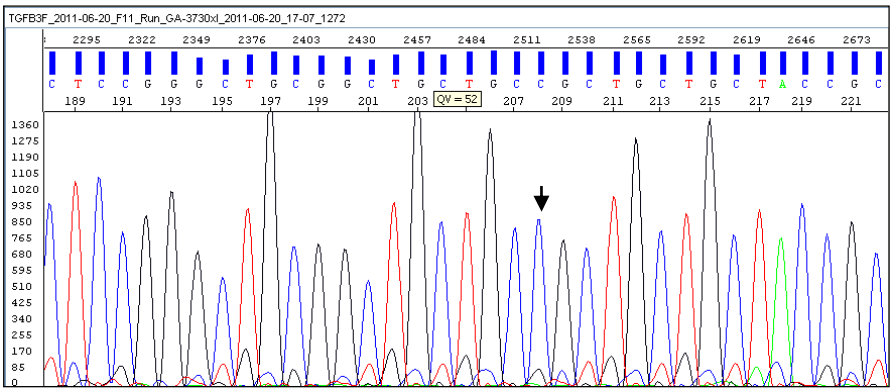


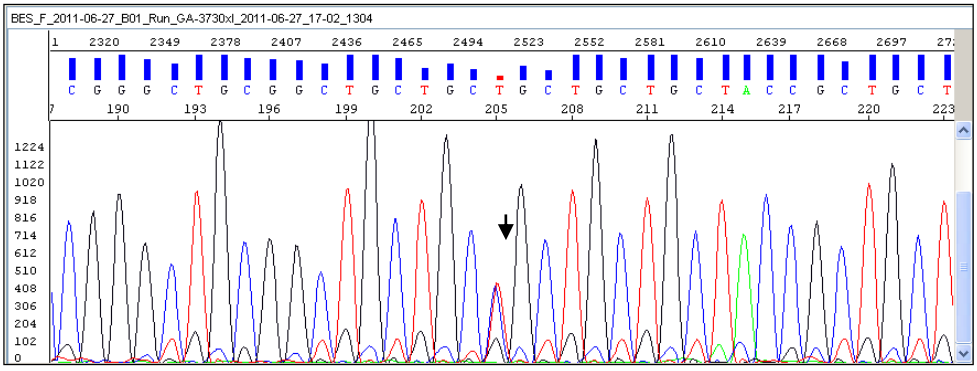
Figure 3.5 Amplification reaction for sequencing of the *TGFB1* signal peptide region. Numbers 1-8 indicate samples tested. Number 5 designates a negative control. Target band: 644 bp. EL=EasyLadder I (2 kb, Bioline Ltd., London UK).

Once the optimisation of the SSP and the SBT was completed, both protocols were applied to the typing of a cohort of healthy donors that would serve as a source of cells for functional experiments. A total of 31 healthy volunteer blood donors were recruited for functional experiments. All of them were initially typed by PCR-SSP before any cellular experiments were carried out, and these results were confirmed by sequencing using a second sample at the time of cell collection for functional experiments. Thirteen individuals bore a TT genotype (41.9%), 10 a TC genotype (32.3%), and 8 a CC genotype (25.8%). Allelic (T=0.58, C=0.42) and genotype frequencies agree with what had been previously reported for this polymorphism (Shah *et al.*, 2006; Amani *et al.*, 2008). When ambiguous results were obtained by SSP-PCR, results from the sequencing reaction were considered as definitive.

(A)



(B)



(C)

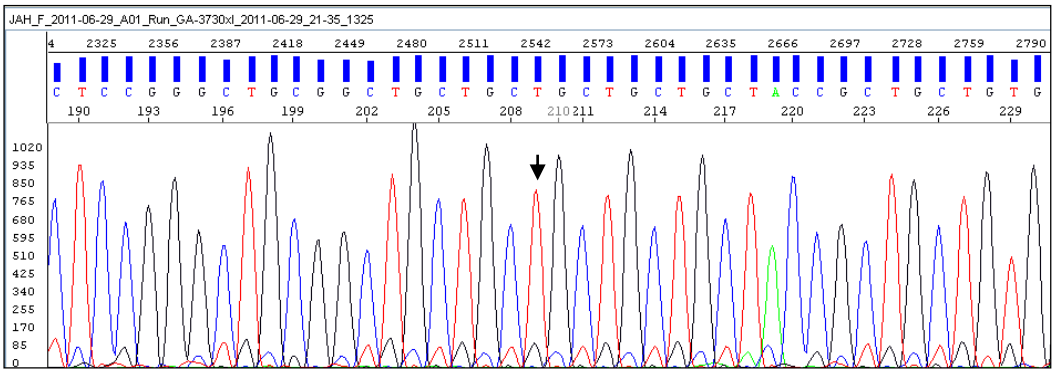


Figure 3.6 Three *TGFB1* signal peptide region sequencing results showing +29T>C. (A) CC individual. (B) TC individual. (C) TT individual. Sequences analysed with Sequencing Analysis 5.2 (PE Applied Biosystems, Foster City, CA, USA).

3.3 Development of a typing strategy for 20 polymorphisms within *TGFB1*'s upstream regulatory region and exon 1, and the definition of *TGFB1* regulatory region and exon 1 alleles

The upstream regulatory region of *TGFB1* is known to extend from -2,665 to +423 (Kim *et al.*, 1989; Park *et al.*, 2003). Shah *et al*. (2006 and 2009) studied the polymorphisms in this regulatory region of *TGFB1* and reported the presence of 17 alleles (see **Table 3.1**) formed by the combination of 18 of 20 previously reported polymorphisms. Two previously reported polymorphic positions (-1826 and -1740) did not show variability in their studies. These studies prompted my interest in analysing the role of the combined effect of the polymorphisms in these alleles in HSCT, in an attempt of comprehensively analyse the effect of genetic variation in this gene. Consequently, I developed a protocol for this aim, as well as a tool for the assignment of *TGFB1* alleles based on the typing of these 18 positions.

3.3.1 Typing strategy for *TGFB1*'s upstream regulatory region and exon 1

Based on the studies by Shah *et al*. (2006 and 2009), I decided to base the protocol on the amplification of 4 sub-regions (RI, RII, RIII, RIV) within *TGFB1* upstream regulatory region and exon 1, followed by the sequencing of these sub-regions. **Figure 3.7** shows a schematic representation of *TGFB1*'s upstream regulatory region and exon one, and how it was divided into the four regions for this protocol. My approach was to design primers that would amplify regions of 600-800 bp, so that they could be efficiently sequenced. I also designed the 'interior' primers so that they bound at the same position in *TGFB1*, in an attempt to ensure that all the primer combinations could be used to amplify the relevant regions with the same PCR conditions. This meant that, for example, the reverse primer for RI (RI-R) and the forward primer for RII (RII-R) were placed at the same position within the regulatory region, albeit with different extension directions. Consequently, I designed primers for RI, RII, and RIII, while for RIV I used the primers already employed in the sequencing protocol for *TGFB1* +29T>C. The RIV primer characteristics were used as a base for the design of the other primers, so that amplification conditions would be shared with this primer pair. The ideal criteria for primer design were: (1) primers that would generate fragments between 600-800 bp, (2) 18-20 bp in length, (3) having 55-65% GC content, (4) having similar melting temperatures (Tm) between the pair (usually within 5°C), and close to the other primer pairs, (5) the presence of at least 2 G and/or C within the last 5 bases of the 3' end, (6) absence of mono- or di-nucleotide repeats (up to three were tolerated for mono-nucleotide repeats), (7) not overlapping any known polymorphic

positions (with one exception explained below), and (8) allowing for approximately 50 bases between the 3' end of the primer and the next known polymorphic position.

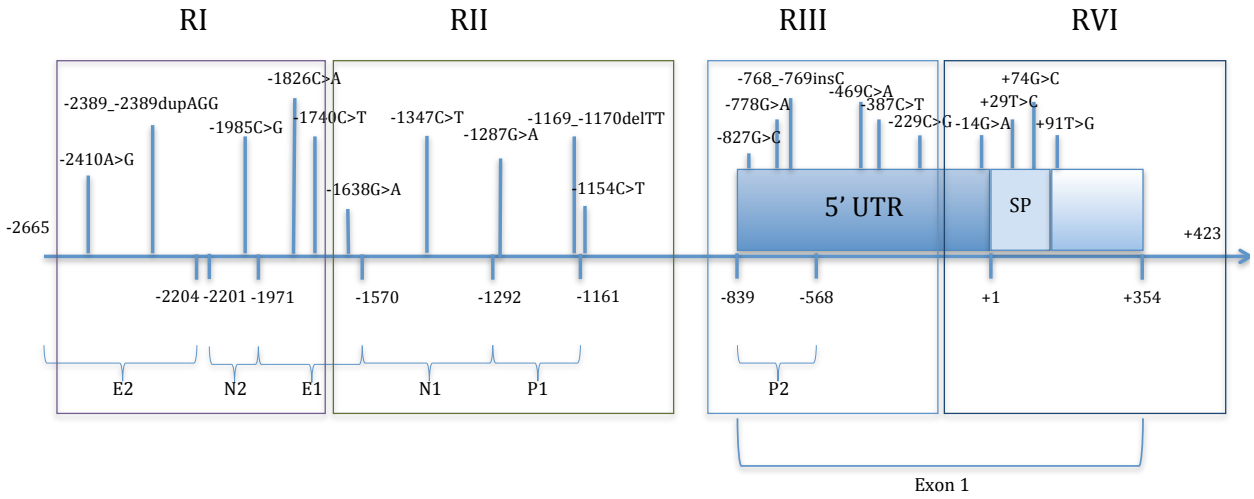


Figure 3.7 *TGFB1*'s upstream regulatory region and exon 1 showing the sub-regions established for the allele-typing strategy. Each box represents one of the sub-regions and the known polymorphic positions enclosed (vertical bars) are their targets. The gap between RII and RIII is due to primers RII-R and RIII-F-alt not lying at the same position (explained below). E, enhancer; N, negative regulatory region; P, promoter; SP, signal peptide; UTR, untranslated region. Figure not to scale. Positions given according to the translation start site (+1).

The GenBank accession number AY871232 (*Homo sapiens* transforming growth factor beta 1 precursor (*TGFB1*) gene, *TGFB1**p001 allele, promoter regions, exon 1 and partial cds) was used as the reference sequence for all the designs and in silico testing of the primers. The selected candidate oligonucleotide pairs were evaluated for specificity, biochemical characteristics, and presence of secondary structures using in silico analysis with the US National Center for Biotecnology Information (NCBI)'s Primer-BLAST online tool, and the results were confirmed by running in silico PCR with AmplifX v.1.5.4 software (by Nicolas Jullien ; CNRS, Aix-Marseille Université - http://crn2m.univ-mrs.fr/pub/amplifx-dist).

Table 3.1 ***TGFB1*** **promoter alleles based on 18 polymorphic positions within its upstream regulatory region and exon 1.**

Allele[b]	Position[a]																	
	-2410	-2389dupAGG[c]	-1985	-1638	-1347	-1287	-1169delTT	-1154	-827	-778	-768insC[c]	-469	-387	-229	-14	+29	+74	+91
p001	A	-	C	G	T	G	TT	C	G	G	-	C	C	C	G	C	G	T
p002	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p003	G	AGG	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p004	G	AGG	G	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p005	-	AGG	-	-	C	A	-	T	C	-	-	-	T	-	A	-	-	-
p006	G	AGG	-	A	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p007	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p008	G	AGG	-	-	C	-	-	-	-	-	-	A	-	-	-	T	-	-
p009	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
p010	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-
p011	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p012	-	-	-	-	-	-	del	-	-	-	-	-	-	-	-	-	-	-
p013	G	AGG	-	-	C	-	del	-	-	-	-	-	-	-	-	T	-	-
p014	-	AGG	-	-	C	-	-	-	-	-	C	-	-	-	-	-	C	-
p015	G	AGG	-	-	-	-	-	-	-	-	-	-	-	G	-	T	-	-
p016	G	AGG	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-
p017	G	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	G

a. Nucleotide position relative to the major translation start site (+1). Only positions that are polymorphic are included. A dash (-) indicates no difference from allele p001 at that position.
b. Promoter allele defined by the sequence of the regulatory region and exon 1 as described by Shah *et al*. (2006 and 2009).. Sequences are compared to allele p001 (GenBank accession no. AY871232).
c. A dash (-) means no duplication or no insertion.
del, deletion; dup, duplication; ins, insertion. Modified from Shah *et al*. (2006 and 2009).

Six basic primers for RI-RIII were designed and tested, in addition to two potentially useful primers for the -2389 AGG duplication (one reported by Shah *et al*. (2006), RI-AGG-POS, and the other, RI-AGG-NEG, designed by myself). **Table 3.2** shows all the primers designed, as well as those used for the SBT assay for *TGFB1* +29T>C, henceforward called RIV-F and RIV-R, and their basic characteristics. All the primer sequences were found to be specific exclusively for *Homo sapiens* chromosome 19 genomic contig, GRCh37.p5 Primary Assembly (NT_011109.16). M13 phage tags (detailed in the previous section) were added to the forward and reverse primers accordingly. As will be explained below, the design of an alternative forward primer for RIII was necessary and, thus is also included in the list.

Table 3.2 Primers used for the amplification of the 4 sub-regions of *TGFB1*'s upstream regulatory region and exon 1.

Primer name	Sequence (5'-3')	GC content (%)	Tm (˚C)
RI-F	M13F-GATGCTTCCAGATGCCAGGT	55	56.7
RI-R	M13R-TCGAAGTTGCGGAGCAGCAG	60	60.1
RII-F	M13F-CTGCTGCTCCGCAACTTCGA	60	60.1
RII-R	M13R-CACCGTCCTCATCTCGCGT	63.16	58.9
RIII-F	M13F-ACGCGAGATGAGGACGGTG	63.16	58.9
RIII-F-alt	M13F-ATCTCCCTCCCACCTCCCT	63.16	57.5
RIII-R	M13R-GTGTCTCAGTATCCCACGGA	55	55.2
RIV-F	M13F-TCCGTGGGATACTGAGACAC	55	55.2
RIV-R	M13R-CCAGTTTCTTCTGCCAGTCA	50	54.4
RI-AGG-POS	M13F-AGGGCAGGGACATG**AGGAGG**	65	59.7
RI-AGG-NEG	M13F-AGGGCAGGGACATG**AGG**GAA	60	59.3

F, forward; R, reverse; Tm, melting temperature.

Figure 3.8 shows the annealing sites of the primers along the complementary sequence for the for *Homo sapiens* chromosome 19 genomic contig, GRCh37.p5 Primary Assembly.

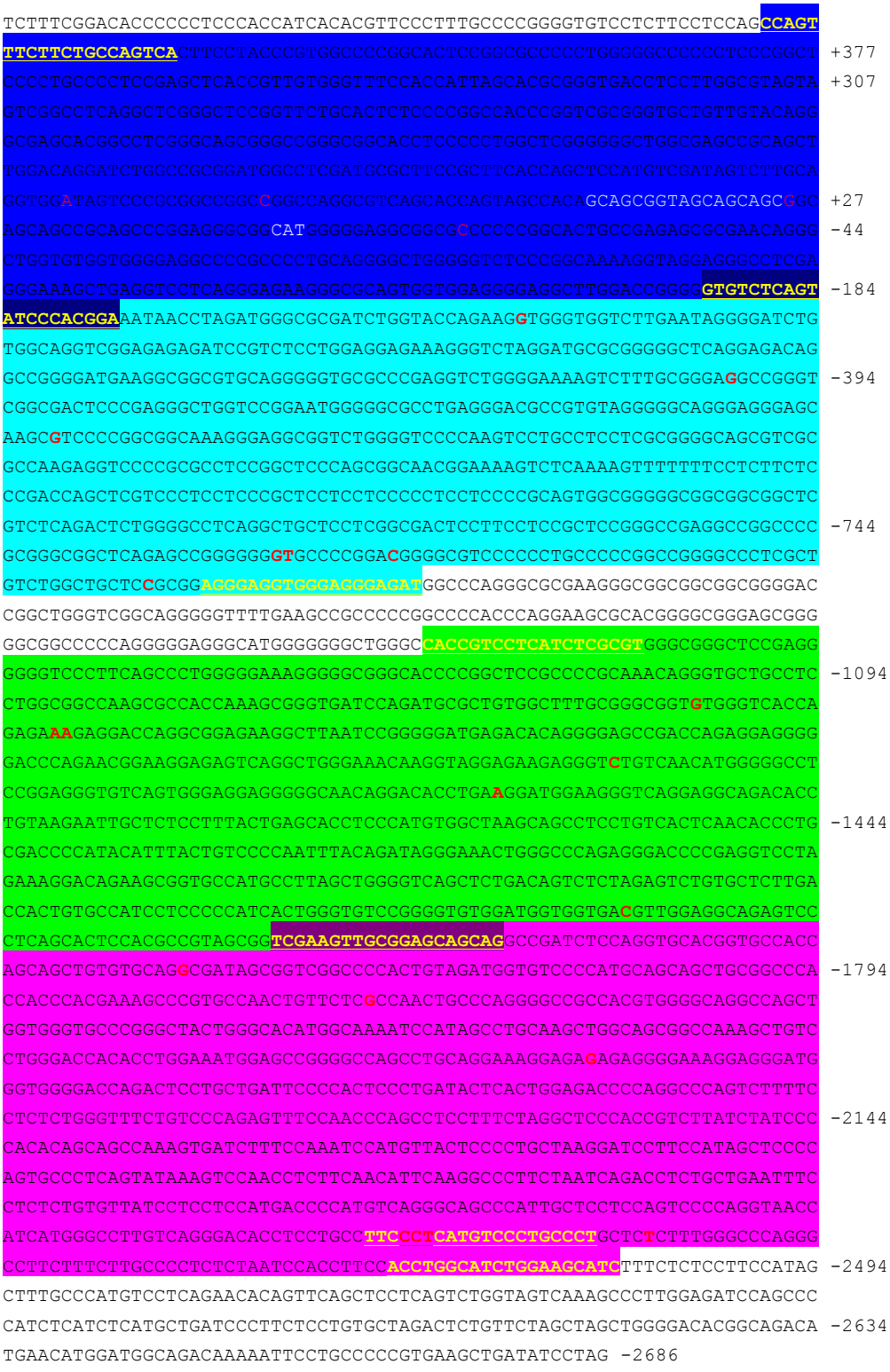


Figure 3.8 Complementary genomic sequence of the *TGFB1* regulatory region and exon 1 showing the annealing sites of the primers used for the SBT protocol, known polymorphic sites and sequencing sub-regions. The upstream regulatory region and exon 1 was divided in sub-regions RI (purple), RII (green), RIII (aquamarine) and RIV (blue). Known polymorphic positions are shown in red, while primer binding sites are shown in yellow. Numbering of the right-end position refers to the translation start site (codon 1, shown in white). For illustration, the binding site for the +29T>C SSP reverse primers is also shown in white.

Initially, I tested the primers in different combinations aiming to potentially reduce the number of amplifications required from 4 to two, or even one. However, the attempts to amplify two sub-regions or a single amplification of the whole region of interest (i.e. by using primer combinations such as RI-F+RII-R or RI-F+RIV-R, respectively) proved unsuccessful with normal PCR reagents (i.e. a basic Taq polymerase from Bioline). Consequently, taking into account the fact that a simple, quick and cheap method was my aim, and that the samples on which I was eventually going to apply this protocol might have a range of DNA quality and concentrations (and possibly limited material), all of which would hamper the efficiency of longer amplifications, I decided not to pursue this and therefore to go forward with the amplification of 4 sub-regions.

Based on the protocol for *TGFB1* +29T>C SBT and on the annealing temperatures suggested by the in silico PCR, the first experiments were set up using an initial cycling protocol with annealing temperature gradients. The amplification mixtures were prepared as presented in Chapter 2. The initial experiments were done with cell line DNA from cells with different +29T>C genotypes (RSH, JBUSH, DOP-ND). **Figure 3.9** shows an example of the annealing temperature optimisation experiments done for the basic 4-sub-region and 2-sub-region primer combinations, as well as for the -2389 AGG duplication-specific primers. The annealing temperature optimisation experiments suggested that a temperature of 59 to 61˚C could amplify all of the sub-regions using the same PCR mixes with acceptable efficiency, and 60˚C was then chosen for further experiments. These amplicons were purified and used as templates for sequencing. The cycle sequencing, precipitation of the products and microelectrophoresis were performed as described in Chapter 2 with the 7,200-second run time.

The amplification of RIII with the basic primer combination (i.e. RIII-F+RIII-R) proved erratic, less efficient and showed inter-sample variations. I thus conducted $MgCl_2$ titrations for these primer combinations, but these experiments did not show significant improvements in amplification stability and efficiency. After repeated unsuccessful attempts to use this primer combination and suspecting the presence of some kind of detrimental interaction between the two primers was affecting the amplification, I decided to design a different forward primer in order to see if the problem with this region could be solved. I chose a sequence lying 140 bp downstream of the end of the original RIII-F because the region between these two sequences (and all of RIII) had many single-base repeats and a very high GC content, thus making primer design difficult (see **Figure 3.8**).

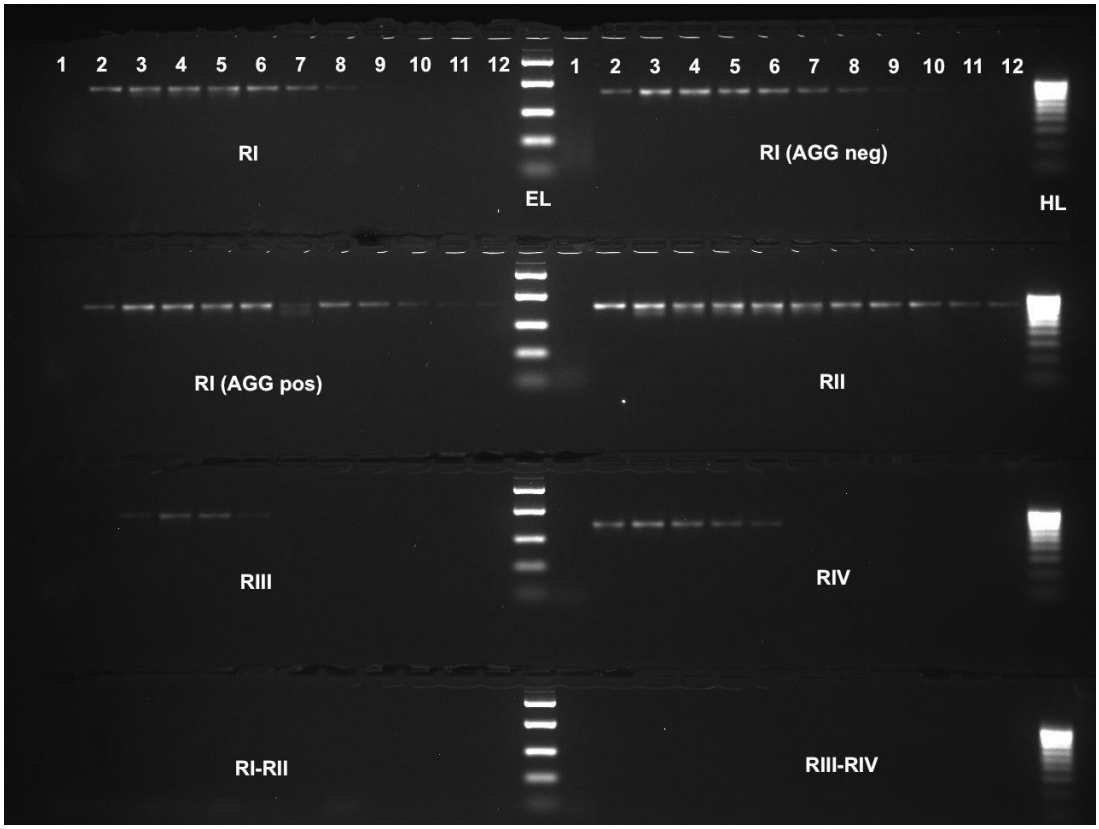


Figure 3.9 Annealing temperature optimisation experiment for different *TGFB1* regulatory region and exon 1 fragments. Twelve annealing temperatures from 58 to 70˚C (lanes 1-12) were tested for 8 primer combinations for RI, RII, RIII and RIV, as well as for the -2389 AGG duplication-specific ones and for amplicons spanning RI+RII and RIII+RIV using DNA from the JBUSH cell line as template. The optimal temperature, which showed good amplification for all the single-region amplicons was deemed to be between lanes 3 and 4 (i.e. 59.1 and 60˚C). Target bands range between 800 bp for RI to 644 bp for RIV. EL=EasyLadder I (2 kb), HL= Hyperladder IV, (both from Bioline Ltd., London UK).

The use of this alternative RIII forward primer (RIII-F-alt) in combination with the RIII-R proved successful and good amplifications were obtained with the PCR conditions in place for the other primer pairs (i.e. an annealing temperature of 60˚C and a 1 mM final Mg^{++} concentration) for all samples tested. This primer was then included as the primer of choice for RIII amplification in subsequent experiments. **Figure 3.10** shows an example of an amplification experiment using the RIII-F-alt+RIII-R primer combination.

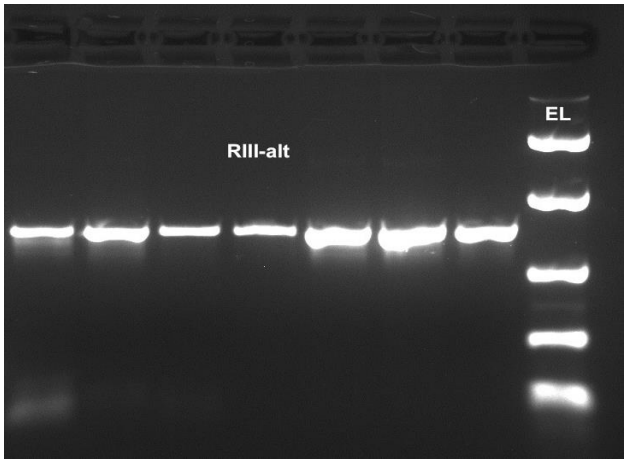


Figure 3.10 Example of the amplification of RIII in 7 cell line DNA samples with the RIII-F-alt primer. The combination of primers RIII-F-alt and RIII-R solved the problem of RIII amplification giving strong and specific bands for this fragment consistently among different samples. EL=EasyLadder I (2 kb), (Bioline Ltd., London UK).

For the analysis of the sequencing data, a project template was designed in the SeqScape software. I used the complementary sequence for GeneBank accession number AY871232 as shown in **Figure 3.8** as the reference sequence.

As mentioned before, the optimisation experiments were carried out using cell lines RSH, JBUSH and DOP-ND, which showed genotypes compatible with the presence of alleles p003/p003, p001/p014, and p001/p003, respectively. This meant that polymorphism among these cell lines was seen only for 6 positions (-2410, -2389, -1347, -768, +29, +74) out of the 18 used by Shah *et al*. to define *TGFB1* regulatory region and exon 1 alleles. Consequently, in order to further validate the protocol and to see polymorphism at other positions, I selected cell lines tested by Shah *et al*. that would present other allele combinations. Six IHWS cell lines reported to have allele combinations that included polymorphism at other positions, and for which DNA was available at our laboratory, were tested with my protocol and the optimised conditions: HHKB (p003/p006), T7527 (p001/p008), BOB (p002/p003), MOU (p012/p013), OMW (p003/p005), and RML (p001/p007) (Shah *et al.*, 2006). The results of these experiments confirmed most of the allelic genotypes reported by Shah *et al*. (2006) (MOU was confirmed to be a p013 homozygous rather than a p012/p013), and allowed for the observation of polymorphism at positions -14, -387, -469, -827, -1154, -1169, -1287 and -1638. **Figure 3.11** shows a selection of sequencing data showing some of the polymorphisms that were identified.

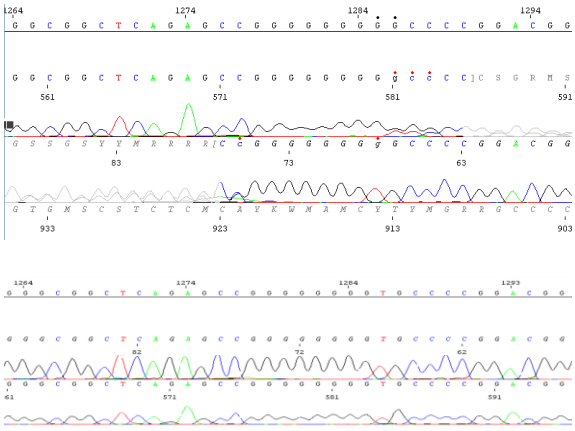
Having optimised and validated the protocol for *TGFB1*'s upstream regulatory region and exon 1 SBT, I decided to type all of the healthy donors recruited for previous experiments in order to test the consolidated protocol with a higher number of samples with different

DNA qualities. There was available DNA for 31 of the original 32 healthy donors. All of the samples were processed for amplification and sequencing with the same conditions for all primer pairs, and all were successfully typed. The results for the typing of *TGFB1*'s upstream regulatory region and exon 1 alleles among healthy donors are presented in **Table 3.3**, and correlate with previous *TGFB1* +29T>C data. Among these samples, 3 *TGFB1* alleles were seen: p001 (30% allele frequency), p003 (60% allele frequency) and p014 (10% allele frequency), accounting for polymorphism at six positions within the genomic region. Moreover, the most common genotypes were, in descending order, p003/p003 (43%), p001/p003 (30%), p001/p001 and p001/p014 (10% each), and p014/p014 and p003/p014 (3.3% each). All genotype assignments were done manually and were inferred from the polymorphic sites' genotypes base on **Table 3.1**. However, one of the samples (HVD28) showed a genotype that did not fit with any of the possible allele combinations. This sample showed results agreeing with a p001/p014 combination for RI and RII, but its RIII and RIV seemed to come from a p001-related allele.

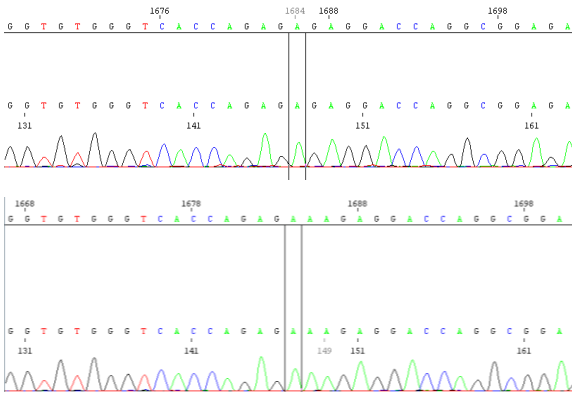
(A)


Analysis DBSearch Window Help
Active Layer: Layer 1
Tab jumps to next: Multiple...
TGFB1_promoter+exon1_PD_2781-3296 / 3079 / TGFB1 -2686–+516
Layout Assembly
Sample Name | Orientation | Ref.Start | Ref.End | CR-Length | CR-Start | CR-End | %N's | Sample Score | %Mixed Bases
3079-IVF_201 | reverse | 64 | 667 | 604 | 636 | 33 | 0.0 | 51 | 0.0
3079-IVR_201 | forward | 105 | 713 | 609 | 29 | 637 | 0.0 | 46 | 0.0
3079-IIIF_201 | reverse | 691 | 1331 | 642 | 662 | 21 | 0.0 | 48 | 0.0
Legend: Clear Range | Known Variants | Unknown Variants | Algorithm Edited | Unknown Variant

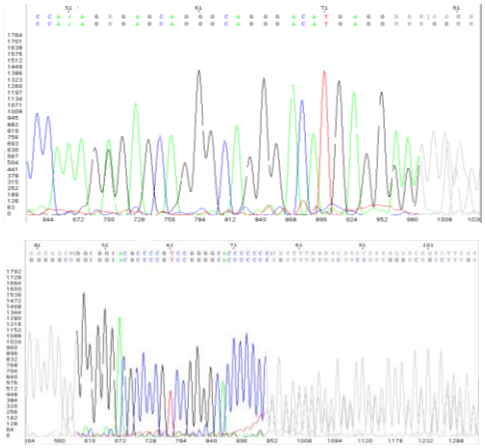
(B)



(C)



(D)



(E)

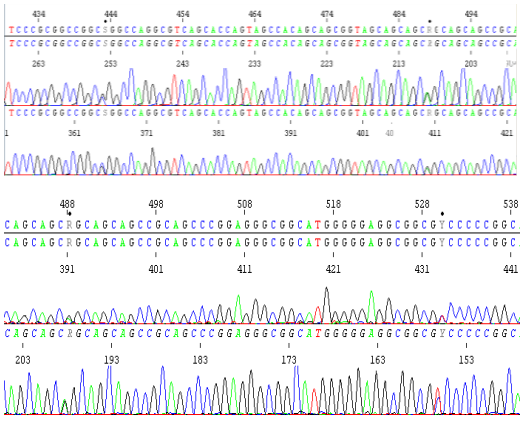


Figure 3.11 Example of the analysis layout for the *TGFB1* upstream regulatory region and exon 1 sequencing protocol and a selection of target polymorphisms. (A) Example of the analysis of a sample with the SeqScape analysis layout. Known polymorphisms are marked in red by the software, while potential new polymorphic positions appear as blue markings. The sequence from RI shows a -2410 AA (position 2926 on the template) and -2389 AGG homozygous duplication (positions 2905 to 2907 on the template). (B) Example of a heterozygous (top) and of a homozygous (bottom) insertion of a C at -768 (position 1285 on the template). (C) Example of a homozygous -1169 AA deletion (positions 1685 and 1686 on the template) (top) and of a sample without this deletion (bottom). (D) Electropherogram view of a non-assembled RI fragment (top, positive strand) showing heterozygosity at -2410 and -2389 (position 54 and 76 of the sequence fragment), and non-assembled positive strand RIII fragment (bottom) showing a heterozygous -768 C insertion. (E) Heterozygosity in RIV at signal peptide positions +29 and +74 (top, positions 488 and 443 on the template, respectively), and at -14 (bottom, position 530 on the template).

The typing was repeated with the same DNA sample from the beginning and the original results were confirmed. This suggested the possibility that this sample could be carrying a novel recombinant allele.

In order to confirm this, I decided to use 3 strategies to try to identify this potentially novel allele. First, samples from relatives from HVD28 were sought, as well as for HVD28 themself (in order to rule out sample contamination and as a confirmation). It was possible to obtain samples from HVD28's father and mother, as well as from a sibling. DNA was extracted from these samples, and this genetic material was typed for *TGFB1*'s upstream regulatory region and exon 1 alleles with this protocol. The typing results for these experiments are presented in **Table 3.4**. As is evident from the results, the mother bears a p001/p001 genotype, while HVD28's father bears what appears to be the novel allele and, potentially, a p003 allele. Their sibling had the same genotype as HVD28, further confirming the suspicion of the presence of this novel allele. All the family samples were typed for HLA class I as explained in Chapter 2, in order to confirm their relationship.

Table 3.3 Results for the typing of *TGFB1* regulatory region and exon I alleles among healthy volunteer donors by the sequencing protocol.

SAMPLE	-2410A>G	-2389dupAGG	-1347C>T	-768_-769insC	+29T>C	+74G>C	Alleles
HVD1	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD2	AA	-/-	TT	-/-	CC	GG	**p001/p001**
HVD3	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD4	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD5	AA	-/-	TT	-/-	CC	GG	**p001/p001**
HVD6	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD7	AA	AGG/-	C/T	C/-	CC	G/C	**p001/p014**
HVD8	AA	AGG/AGG	CC	C/C	CC	CC	**p014/p014**
HVD9	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD10	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD11	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD12	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD13	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD14	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD15	A/G	AGG/AGG	CC	C/-	T/C	G/C	**p003/p014**
HVD16	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD17	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD18	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD19	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD21	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD22	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD23	A/G	AGG/-	C/T	-/-	T/C	GG	**p001/p003**
HVD24	AA	AGG/-	C/T	C/-	CC	G/C	**p001/p014**
HVD25	AA	AGG/-	C/T	C/-	CC	G/C	**p001/p014**
HVD26	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD27	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD28	AA	AGG/-	C/T	-/-	CC	GG	**?**
HVD29	GG	AGG/AGG	CC	-/-	TT	GG	**p003/003**
HVD30	AA	-/-	TT	-/-	CC	GG	**p001/p001**
HVD31	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**
HVD32	GG	AGG/AGG	CC	-/-	TT	GG	**p003/p003**

Only positions at which polymorphism was seen are reported. dup, duplication; ins, insertion.

Secondly, the samples from HVD28 and their father were subjected to molecular cloning in order to try to isolate the recombinant allele and to be able to generate sequence from a sole fragment of this genomic region.

Table 3.4 Results for the typing of *TGFB1* regulatory region and exon 1 polymorphisms for HVD28 and their relatives.

Sample	-2410A>G	-2389dupAGG	-1347C>T	-768_-769insC	+29T>C	+74G>C	Alleles
HVD28	AA	AGG/-	C/T	-/-	CC	GG	**p001/N**
Father	GA	AGG/AGG	CC	-/-	T/C	GG	**p003/N**
Mother	AA	-/-	TT	-/-	CC	GG	**p001/p001**
Sibling	AA	AGG/-	C/T	-/-	CC	GG	**p001/N**

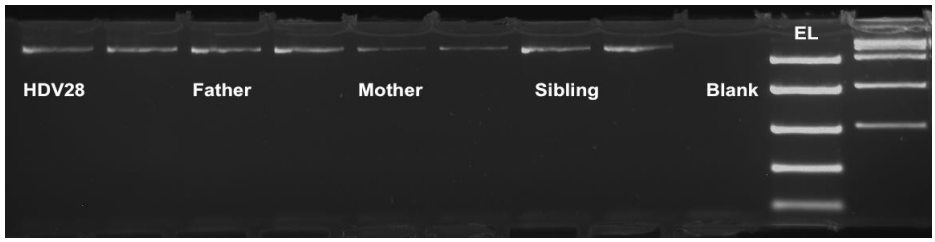
All other positions were not polymorphic. dup, duplication; ins, insertion; N, novel allele.

For this, I used an in house protocol originally used for the isolation and sequencing of novel alleles of the HLA system, whose details are given in Chapter 2. In order to ensure that sequence from a single DNA amplicon could be generated, I amplified the genetic region using primers RI-F and RIV-R, thus spanning all of the sub-regions (see **Figure 3.12**). Sequencing was done with a different set of primers, which were versions of the primers included in **Table 3.2** that lacked M13 tags. However, the original RIII-F without M13 tag and not the alternative primer was used in order to maximize coverage.

The analysis of the sequencing data allowed the confirmation of the presence of the new recombinant allele in a number of clones. The allele was then shown to be similar to the p001, but having the -2389 AGG duplication and also a C at position -1347, and thus differing from all other previously reported alleles.

Finally, due to the success in amplification of a RI-RIV fragment for cloning, I also decided to test if this amplification could be performed with the -2389 AGG duplication-specific primers, aiming at developing another strategy to segregate alleles by allele-specific amplifications in samples that are heterozygous for this polymorphism. Consequently, I set up amplifications similar to the ones set up for the cloning protocol, but using the RI-AGG-POS and RI-AGG-NEG primers in combination with primer RIV-R for HVD28. These amplifications were successful, and both amplicons were purified and sequenced with the same sequencing primers used for the cloning protocol. This strategy proved successful, giving good allele-specific sequencing data and thus providing a less laborious protocol for the sequencing of isolated *TGFB1* regulatory region and exon 1 alleles, especially for novel alleles that might potentially be encountered.

(A)



(B)

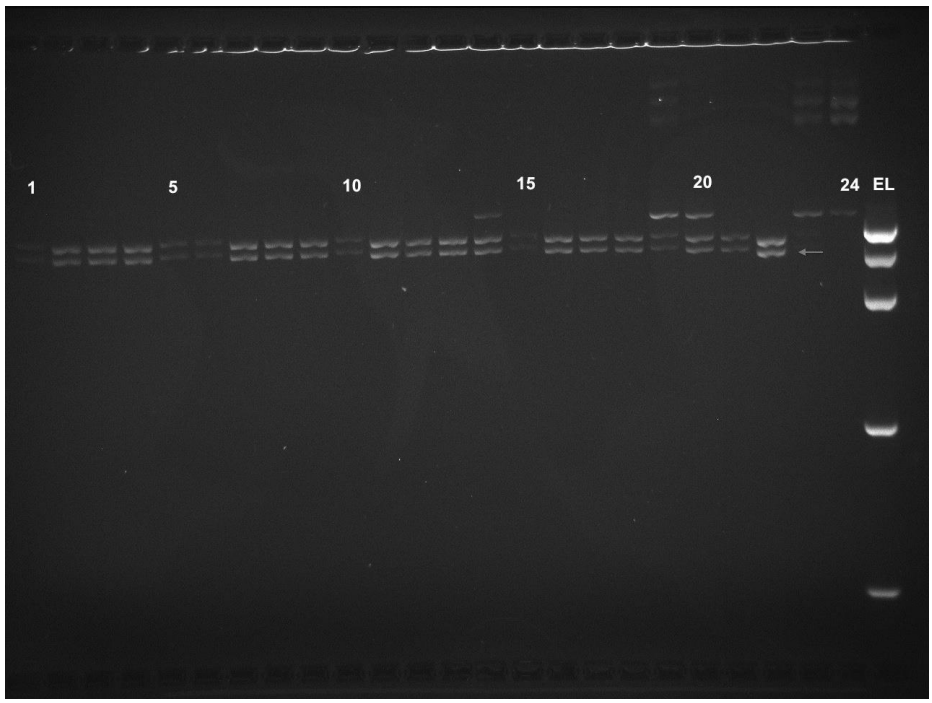


Figure 3.12 Cloning of *TGFB1*'s regulatory region and exon 1 novel allele discovered in sample HVD28. (A) Results of the amplification of the RI-RIV fragment (2.9 kb) using the Phusion polymerase amplification protocol. Duplicate amplifications were set up for each sample in order to increase the amount of template for further steps in the cloning process. DNA ladders (EL) are EasyLadder I (2 kb) (left) and EasyLadder II (5 kb) (right) (Bioline Ltd., London UK). (B) Restriction enzyme digests of HVD28 plasmid DNA clones (lanes 1-24). Extracted plasmid DNA was digested with enzyme EcoRI as explained in Chapter 2. All clones for HVD28 incorporated the *TGFB1* fragment (2,928 bp, lighter band marked by an arrow) into the vector (3,519 bp, heavier band). A heavier band (~6.5 kb in lanes 14, 19, 20, 23 and 24) seems to be undigested *TGFB1* fragment+vector. Heavier fainter bands (lanes 19, 23, 24) could be supercoiled plasmid. DNA ladder is EasyLadder II (5 kb) (right).

3.3.2 Development of a bioinformatics tool for the calling of *TGFB1* regulatory region and exon 1 alleles

In order to facilitate the definition of *TGFB1* regulatory region and exon 1 allelic genotypes in unknown samples, I collaborated with our research institute's Bioinformatics group to create a tool that would help in this respect. The definition of the allelic genotype would be based on the evaluation of the individual genotypes at the 18 polymorphic positions on which Shah and collaborators defined these alleles.

With the aim of developing the tool for identifying *TGFB1* regulatory region and exon 1 alleles, a simple imputation algorithm using existing allele sequences was created. A custom Perl (http://www.perl.org/) script was written to impute *TGFB1* promoter allele pairs given a genotype with eighteen nucleotide positions; nucleotide positions are relative to the major translation start site (+1). *TGFB1* allele p001 was used as the reference allele sequence; only nucleotide positions that are polymorphic were used. Currently seventeen alleles are used for imputation.

Users input a nucleotide for each position as showing either consensus with p001, an alternate base according to existing allele nucleotide content or using IUPAC/custom nucleotide codes. Missing values are not permitted. The script will then iterate over each position translating any nucleotide codes and performing a search for possible allelic combination matches across the full allele sequence. Where possible, genotypes will be resolved into a single allele pair, and potential ambiguities are considered and reported.

This tool will be made available as a web tool allowing allelic imputation from a single genotype. It will be accessible through the Immuno Polymorphism Database (http://www.ebi.ac.uk/ipd/). An example of its interface and output is presented in **Appendix C**.

3.4 Optimisation of a quantitative real-time PCR for the assessment of *TGFB1* expression

As a tool for understanding the kinetics of TGF-β1 in human cells to be applied in functional experiments, I optimised a protocol for the semi-quantitative measurement of *TGFB1* mRNA expression by real-time (RT)-PCR. For this purpose, I used primers for *TGFB1* and β-Actin mRNA reported by White et al. (White *et al.*, 2010), and also primers for FOXP3 as reported by Morgan et al. (Morgan *et al.*, 2005). As internal controls, I used a panel of primers for reference genes, all obtained from PrimerDesign (PrimerDesign Ltd., Southampton, UK). These reference genes included *B2M*, *TOP1*, *UBC*, *ATP5B*, and *ACTB*. The primer sequences are shown in **Table 3.5**. No sequence is available for primers obtained from PrimerDesign, as these are private. However, confirmation from the manufacturer that these were splice-site-spanning was sought and received. The rest of the primers were also designed to span splicing sites in order to make them mRNA-specific. This was confirmed by in silico and *in vitro* experiments. The optimisation process was done following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin *et al.*, 2009) recommendations as closely as possible.

Table 3.5 Sequences for the RT-PCR primers used in this thesis.

mRNA target	Primer sequence (5'-3')	Product size (bp)
β-Actin	F: GGATGCAGAAGGAGATCACTG	90
	R: CGATCCACACGGAGTACTTG	
TGF-β1	F: GACTACTACGCCAAGGAGGTCA	88
	R: TGCTGTGTGTACTCTGCTTGAAC	
FoxP3	F: GCACCTTCCCAAATCCCAGT	100
	R: GGCCACTTGCAGACACCA	

In silico mRNA specificity testing was done by blasting the primer sequences on both mRNA and DNA templates for the target genes using the NCBI's Primer-BLAST online tool. All the primers were confirmed to produce amplicons of the expected size only when an mRNA template was used. **Figure 3.13** shows an example of the in silico testing of the mRNA PCR primers for *TGFB1*.

(A)

Primer-BLAST Primer-Blast results

NCBI/ Primer-BLAST : results: Job id=JSID_01_95557_130.14.22.10_9001 more...

Input PCR template

NG_013364.1 Homo sapiens transforming growth factor, beta 1 (TGFB1), RefSeqGene on chromosome 19

Range

1 - 30020

Specificity of primers

Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

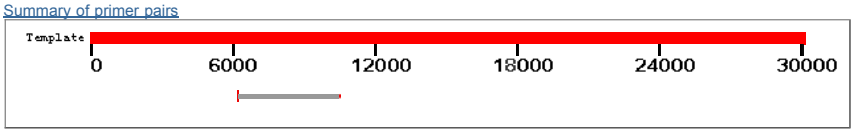
Other reports

Search Summary

Search parameters and other details

Search parameter name	Search parameter value
Number of Blast hits analyzed	30054
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Misprimed product size deviation	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	1000
Min PCR product size	70
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes

Summary of primer pairs

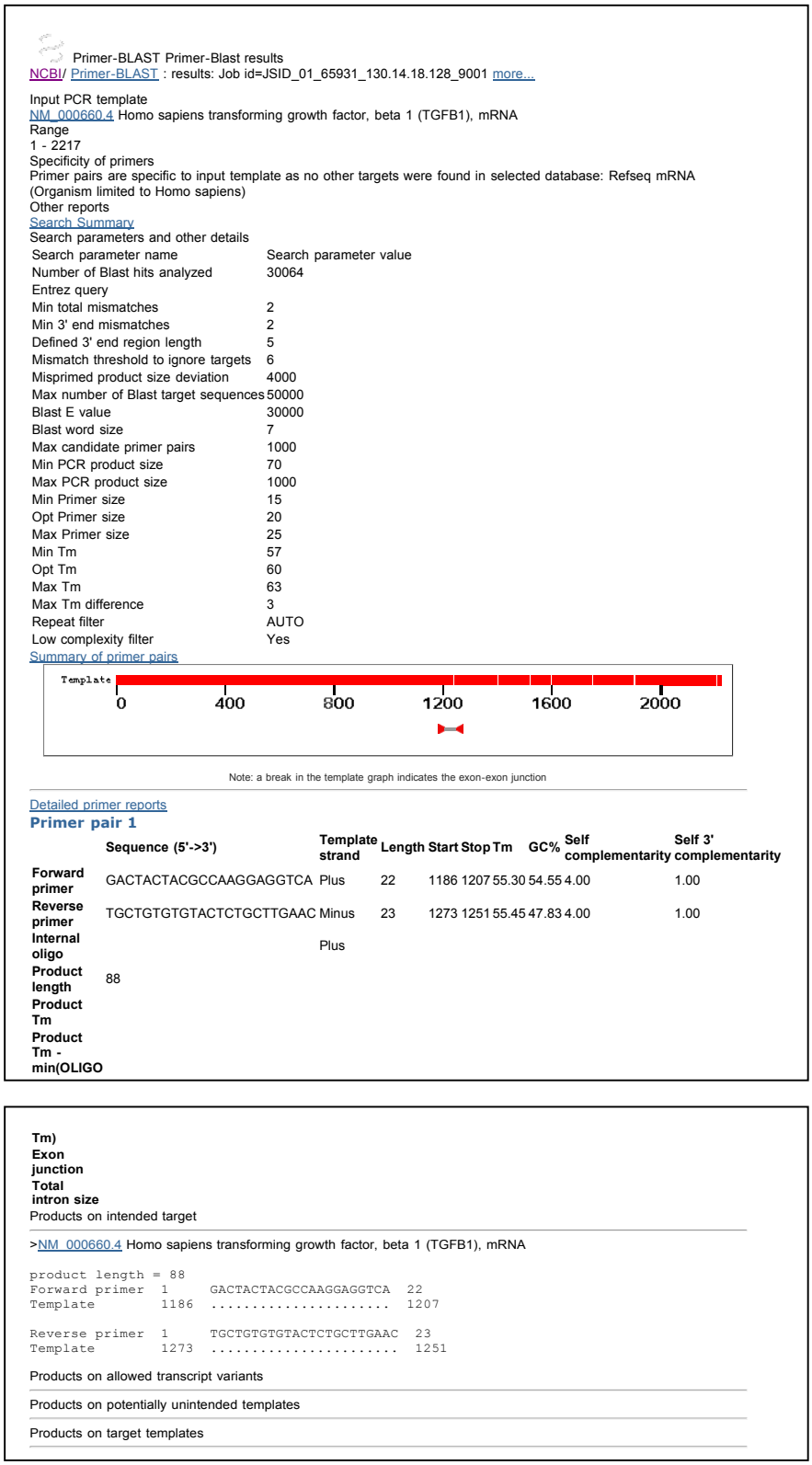


Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACTACTACGCCAAGGAGGTCA	Plus	22	6186	6207	55.30	54.55	4.00	1.00
Reverse primer	TGCTGTGTGTACTCTGCTTGAAC	Minus	23	10507	10485	55.45	47.83	4.00	1.00
Internal oligo		Plus							
Product length	4322								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									

(B)



Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=JSID_01_65931_130.14.18.128_9001 more...

Input PCR template
NM_000660.4 Homo sapiens transforming growth factor, beta 1 (TGFB1), mRNA
Range
1 - 2217
Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports
Search Summary
Search parameters and other details

Search parameter name	Search parameter value
Number of Blast hits analyzed	30064
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Misprimed product size deviation	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	1000
Min PCR product size	70
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes

Summary of primer pairs

Note: a break in the template graph indicates the exon-exon junction

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACTACTACGCCAAGGAGGTCA	Plus	22	1186	1207	55.30	54.55	4.00	1.00
Reverse primer	TGCTGTGTGTACTCTGCTTGAAC	Minus	23	1273	1251	55.45	47.83	4.00	1.00
Internal oligo		Plus							
Product length	88								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									
Total intron size									

Products on intended target

>NM_000660.4 Homo sapiens transforming growth factor, beta 1 (TGFB1), mRNA

```
product length = 88
Forward primer  1     GACTACTACGCCAAGGAGGTCA  22
Template        1186  ......................  1207

Reverse primer  1     TGCTGTGTGTACTCTGCTTGAAC  23
Template        1273  ......................  1251
```

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

Figure 3.13 Primer-Blast results for *TGFB1* real-time polymerase chain reaction (RT-PCR) primers. (A) Blasting results for the *TGFB1* RT-PCR primers on the genomic DNA sequence for *TGFB1*. An irrelevant 4,322 product is shown. (B) Blasting results for the *TGFB1* RT-PCR primers on the sequence for mature *TGFB1* mRNA. The single relevant 88-bp product is shown to span an exon-exon junction.

The *in vitro* testing of the specificity of the RT-PCR primers was done by conventional PCR following the conditions stated in the relevant paper (White *et al.*, 2010). The primers were tested with cDNA produced from RNA extracted from PBMC of healthy donors, as well as with genomic DNA, according to the procedures explained in Chapter 2. **Figure 3.14** shows the results for examples of these *in vitro* tests.

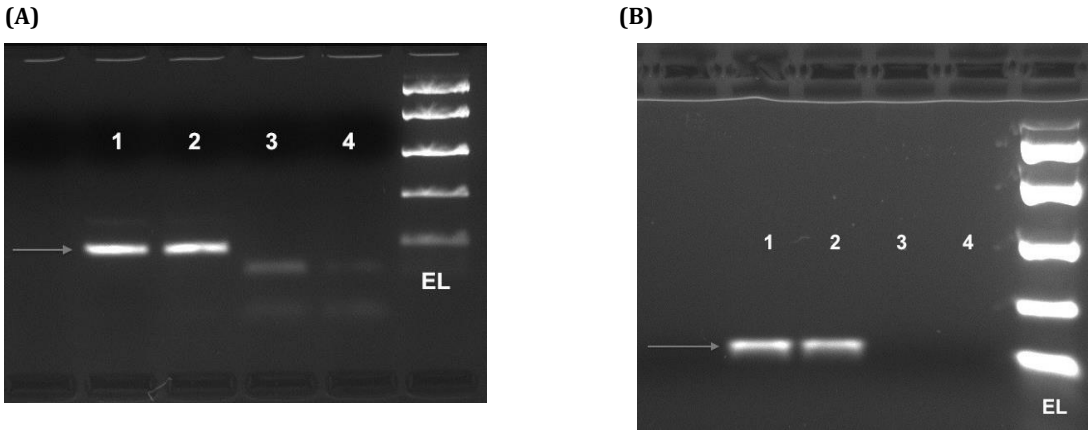
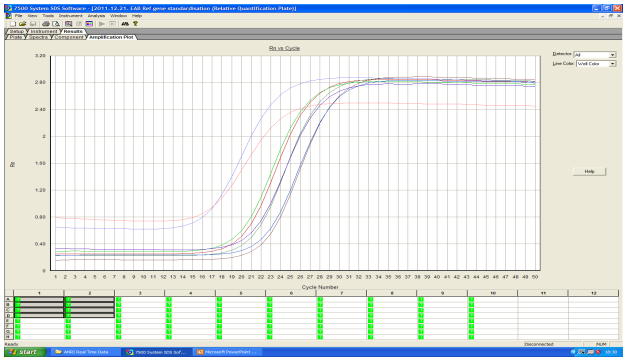


Figure 3.14 In vitro testing of real-time (RT)-PCR primers. (A) Conventional PCR results for primers for *TGFB1*. 88-bp target band is marked by the arrow. (B) Conventional PCR results for primers for *ATP5B*. Target band is marked by the arrow Primers were tested with two cDNA samples (lanes 1 and 2), genomic DNA (lane 3) and H_2O (lane 4). EL=EasyLadder I (2 kb), (Bioline Ltd., London UK).

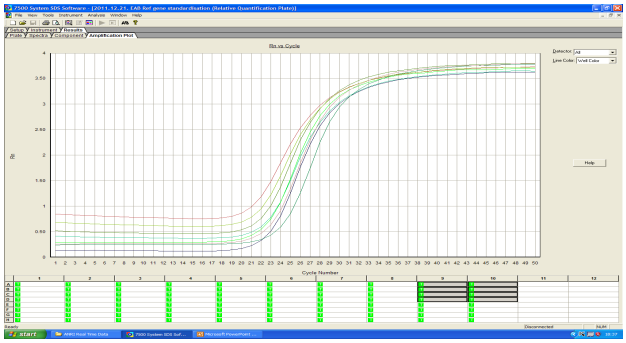
The next step in the optimisation process was to standardize and select the reference genes that were going to be used for the assay. As mentioned previously, I tested a panel of commercially available reference genes produced by PrimerDesign. For these experiments, Treg and effector CD4+ cells were isolated from healthy donors and a fraction of them were activated with soluble and plate-bound antibodies against CD3 and CD28 for 16 hours following the procedures explained in Chapter 2. The resting and activated cells were harvested and RNA was extracted and used for cDNA production as explained in Chapter 2. These conditions were selected because this assay would be used to assess *TGFB1* mRNA expression in different CD4+ subsets and at different activation stages.

RT-PCR was set up as explained in Chapter 2. The reactions for the two cell subsets and 4 culture conditions (i.e. freshly isolated, 16-hour soluble or PB-activated, and 16-hour non-stimulated) were set up in triplicates following PrimerDesign's geNorm Hosekeeping Gene Selection Kit. The data were analysed for stability in both cell subsets across different culture conditions. **Figure 3.15** shows an example of the amplification and denaturation curves obtained in these experiments. **Table 3.6** shows the complete Ct value data and the variation found for each of the 5 candidate genes across the various culture conditions.

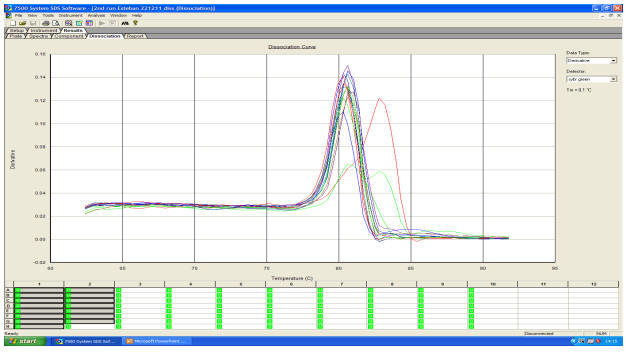
(A)



(B)



(C)



(D)

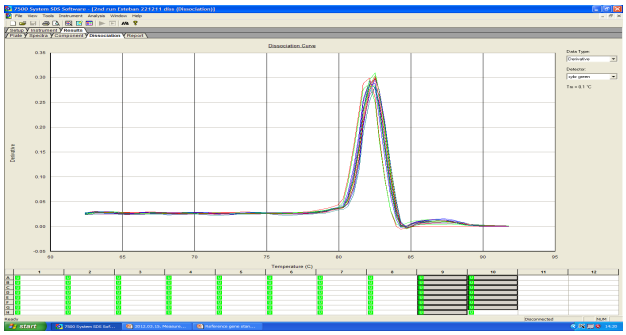


Figure 3.15 Reference gene selection experiments. RT-PCR results of representative candidate reference genes as analysed in SDS software. Amplification and dissociation curves for *B2M* (A, C, respectively) and *TOP1* (B, D) in Treg in different culture conditions. Results for *B2M* are unsatisfactory because of variation in amplification curves in certain conditions (i.e. changes in the quantity of message) and in dissociation curves. On the contrary, *TOP1* shows low variation across culture conditions and a sharp dissociation curve.

Table 3.6 Results (Ct values) for the reference gene RT-PCR on cDNA from mRNA extracted from CD4 subsets in different culture conditions.

	Treg (Ct)					Effector (Ct)				
Condition	*B2M*	*ATP5B*	*UBC*	*ACTB*	*TOP1*	*B2M*	*ATP5B*	*UBC*	*ACTB*	*TOP1*
Fresh 0 h	19.127	21.506	19.235	16.631	20.823	17.255	20.953	16.015	16.905	22.321
16 h NS	20.852	22.172	18.284	17.904	21.673	17.060	20.509	18.022	16.528	21.146
16 h sol	20.677	21.168	19.034	17.464	20.735	18.407	22.004	17.357	15.666	21.627
16 h PB	15.862	19.648	16.108	15.764	21.361	18.771	20.618	18.087	16.337	21.281
CV (%)	12.09	5.06	7.88	5.58	2.11	4.72	3.25	5.54	3.17	2.43

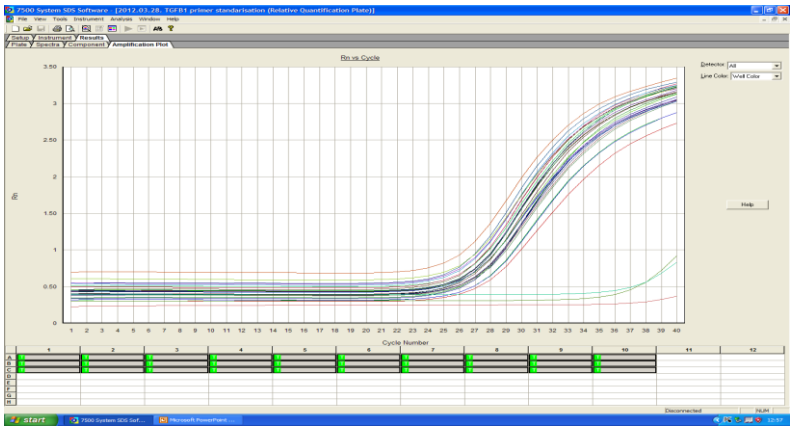
CV, coefficient of variation; PB, plate-bound; NS, non-stimulated; sol, soluble.

In view of their lower variation, I selected primers for *TOP1* and *ATP5B* as reference genes for the rest of the RT-PCR experiments.

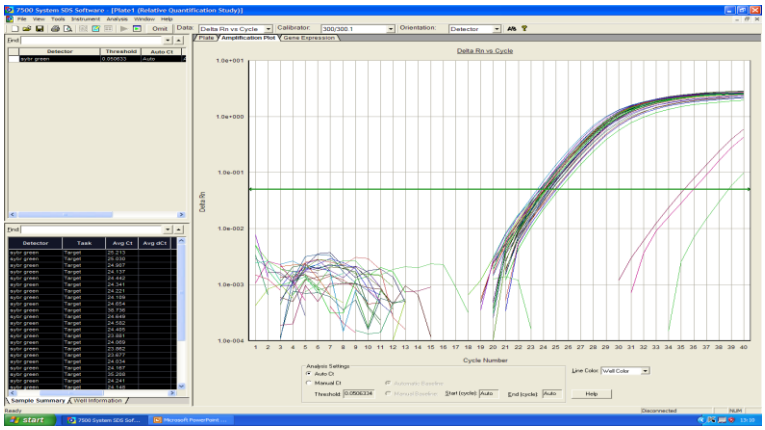
The next step in the optimisation process was to determine the right concentrations for the main target gene, *TGFB1*. Primers for *FOXP3* were used as reported in the relevant paper (Morgan *et al.*, 2005) since these primers were not going to be used for any quantitative experiments, but only as qualitative option for cell characterisation in a few experiments. Likewise, β-actin primers were only used for quality control of the cDNA production procedure in conventional PCR as explained before, and thus underwent no further optimisation.

The right concentrations for the *TGFB1* primers in this system were determined by evaluating amplification efficiency. For this purpose, I conducted an experiment in which a pool of cDNA produced from RNA extracted from resting PBMC of healthy donors was subjected to RT-PCR with different concentrations of forward and reverse primers for *TGFB1*. A 96-well optical plate was used to set up triplicate amplifications of the same amount of cDNA but with final primer concentrations ranging from 300 to 900 nM for each primer. The primer concentration combinations were selected depending on their average Ct value: the lowest concentrations that would produce the lowest Ct values and the steepest slopes in amplification plots, and with stable melting temperature in a dissociation curve were selected (Stordeur *et al.*, 2002). RT-PCR reactions for *TGFB1* primers were done as explained in Chapter 2. After cycling, Ct values were determined for each well, and average Ct values from triplicates having the same primer concentrations were recorded. **Figure 3.16** shows the amplification and dissociation curves for all the primer combinations. The results show the specificity of the PCR product (only one peak in the dissociation curves) and decreasing Ct values depending on the concentration of each primer. The data from these experiments is summarized in **Table 3.7**.

(A)



(B)



(C)

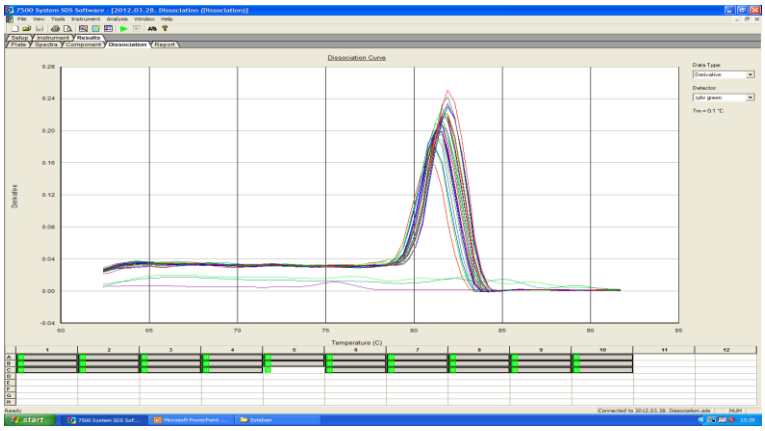


Figure 3.16 Results for the optimisation of the primers for *TGFB1* real-time polymerase chain reaction (RT-PCR). (A) Amplification curves (fluorescence vs. cycle) for all the primer combinations and blank wells. (B) Ct determination by logarithmic transformation of the fluorescence data. The green bar indicates the automatic cutoff for the determination of the Ct value. (C) Dissociation curves for the primer combinations showing the melting temperature (Tm) for each primer combination.

Table 3.7 Results for the optimisation of the *TGFB1* RT-PCR primer combinations.

F/R (nM)	Avg Ct	SD	CV	Tm (°C)
300/300	25.0500	0.1540	0.6147	81.0667
300/600	24.3067	0.1554	0.6392	81.3333
300/900	24.3280	0.2878	1.1831	81.6000
600/300	24.5453	0.1261	0.5136	81.3333
600/600	23.9373	0.1144	0.4780	81.7000
600/900	23.9593	0.2534	1.0576	81.7000
900/300	24.2000	0.0475	0.1961	81.3333
900/600	23.7913	0.3252	1.3669	81.9000
900/900	23.6943	0.0474	0.1999	81.9000

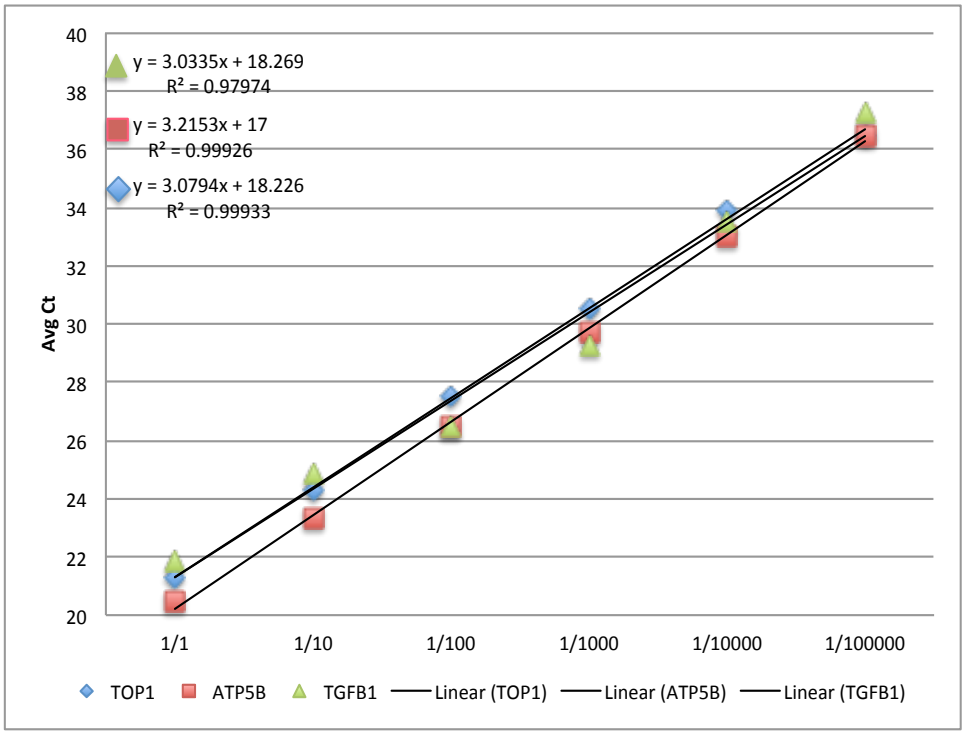
Avg, average; CV, variation coefficient; F, forward; R, reverse; SD, standard deviation; Tm, melting temperature

Both the 900/600 nM (forward / reverse) and the 900/900 nM combinations gave the earliest Ct values with a highest and stable Tm. For ease of set up of the RT-PCR experiments (3+2 µL at 6 µM), and taking into account that these were the original concentrations employed in the paper by White *et al*. (2010), the 900/600 nM combination was selected for further experiments.

The next step in the optimisation process was the determination of the efficiencies of the PCR reactions for *TGFB1* and the two reference genes. This was done to ensure that RT-PCR reactions have high efficiencies, but also that the efficiencies of the target gene reaction and the reference genes reactions are comparable if the ΔΔCt method is to be used (Bustin *et al.*, 2009).

For this experiment, pooled cDNA produced from RNA extracted from resting PBMC from healthy donors was diluted serially ten-fold from 1/10 to 1/100,000. The original cDNA and its 5 dilutions were used to set up triplicate amplification reactions. The RT-PCR cycling was done as previously explained, with the exception that the number of cycles was increased to 50 because of expected late exponential phases when low cDNA concentrations were used. The Ct values for each of the RT-PCR runs for each cDNA dilution were determined and examined for linearity and slope. **Figure 3.17** shows the results for this experiment.

(A)



(B)

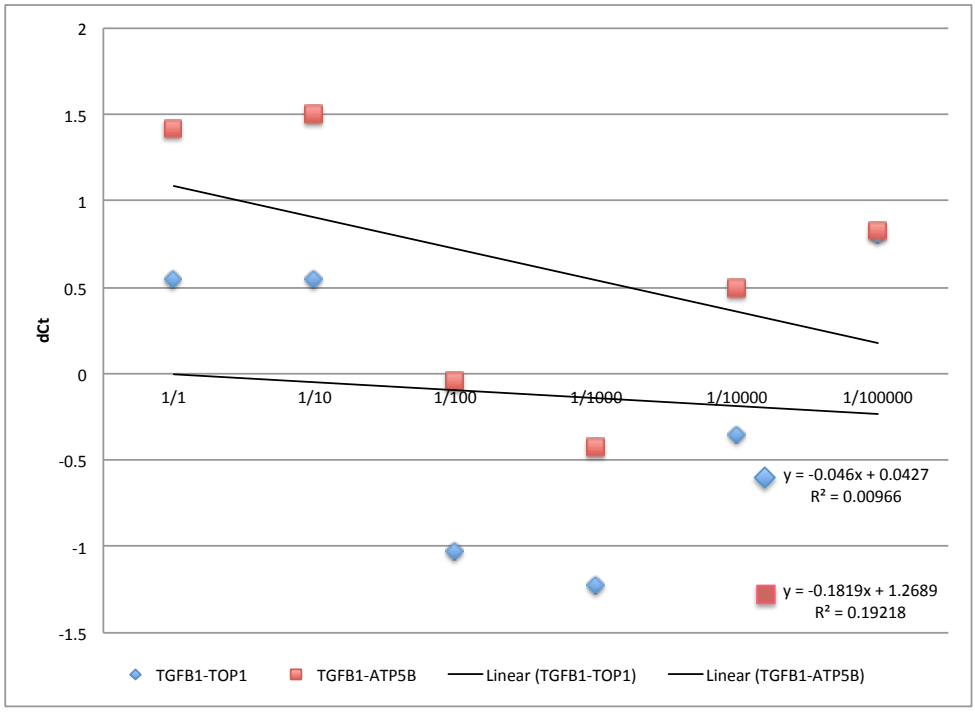


Figure 3.17 Results for the assessment of the efficiency of real-time polymerase chain reaction (RT-PCR) for *TGFB1*, *ATP5B* and *TOP1* cDNA. (A) Plot depicting the average (Avg) Ct values determined for each of the cDNA dilutions for each of the 3 genes. Best-fit linear curves are given, as well as their equations (top: *TGFB1*, bottom *TOP1*). (B) Plot showing the stability of the ΔCt between *TGFB1* and each of the reference genes across the different dilutions. Linear best-fit curves and their equations are given.

The data generated in this experiment were used to calculate the efficiency (E) of the three reactions according to the following formula (Stordeur *et al.*, 2002; Bustin *et al.*, 2009):

$$E = 10^{(1 / \text{slope})} - 1$$

The efficiencies for the reactions for *TGFB1*, *ATP5B* and *TOP1* were thus calculated to be 114%, 104% and 111%, respectively. Efficiencies were found to be thus comparable, and slopes are within the recommended 3.3 ± 10%.

3.5 Discussion

In this chapter, I have presented the work I carried out in order to conceive, design, develop and validate molecular strategies for the assessment and analysis of polymorphism in *TGFB1* and its expression in terms of mRNA. These protocols were either based on previous studies and optimised by myself in the conditions available to me, or were designed and developed wholly by my own initiative.

3.5.1 Optimisation of typing strategies for the typing of *TGFB1* +29T>C

In the first instance, I optimised molecular strategies for the typing of *TGFB1*'s +29T>C polymorphism. As was mentioned before, I was interested in this polymorphism because of previous evidence of its involvement in the outcome of HSCT (Berro *et al.*, 2010). This role in HSCT, as well as its expected functional consequences and the evidence for its involvement in other clinical settings prompted my interest in testing the effect of this polymorphism in functional studies with live human lymphocytes. So, in order to select healthy donors for functional studies, I optimised an SSP and an SBT protocol so that I could type these donors.

The SSP gives a quick, low-cost and non equipment-complicated system for the typing of this SNP. In my experiments, the SSP worked fairly well, and the typing of cell-line DNA with known results validated its performance satisfactorily. Moreover, when this SSP was used to type unknown healthy donor DNA samples, all of the results that were produced by the SSP were confirmed to be correct by sequencing. However, limitations of SSP are that, because it bases it specificity on a single-base difference between one allele and the other, there is always a chance for amplification of the one allele with primers for the other allele, depending on the conditions of that particular PCR. This is the explanation for the occasional presence of faint bands (meaning low-level, inefficient amplification) for example in +29C reaction for a +29TT sample. Even though the faint bands are clearly not evidence of the presence of the allele in that sample when they are compared with the strong bands seen for the allele that is indeed present, and all of the interpretations of the SSP were confirmed by sequencing, it could prove misleading for the untrained eye. Despite several attempts to improve this by means of [Mg^{++}] titrations, annealing temperature gradients, template DNA concentration titrations, a number of proven homozygous samples kept presenting with these faint bands for the allele they did not bear. This was the case of only some samples in some experiment, and was not observed as a systematic result. This means that this phenomenon is most likely caused by random variations in sample and reagent concentration between wells and experiments, and possibly also due to temperature variations between machines and even within the same

machines, from well to well. Consequently, it appears that this situation is very hard to eliminate, at least with these +29 T>C-specific primers. For this reason, I decided to use the SSP for the initial typing and selection of a high number of potential subjects for functional experiments, but would consider these results as preliminary, and confirmatory typing by sequencing was deemed to be definitive. Another limitation of the SSP is that it only allows for the typing of a known mutation, and thus cannot accommodate the presence of novel polymorphism. This is an aspect in which sequencing outperforms SSP and why SBT is a good complement to it.

3.5.2 Optimisation of a sequence-based typing (SBT) strategy for the typing of *TGFB1* regulatory region and exon 1 alleles

The good performance of the *TGFB1* +29T>C SBT coupled with my interest in performing a comprehensive analysis of the genetic variation in this gene and its potential role in HSCT prompted the idea of developing an SBT for the whole upstream regulatory region and exon 1. As explained before, the aim was to cover a 2.9 kb region of chromosome 19 in which at least 17 alleles had been described (Shah *et al.*, 2006; Shah *et al.*, 2009). The analysis of these alleles reveals that, for example, an individual with a +29 CC genotype could have different genotypes at the other main *TGFB1* target polymorphism, -1347 C>T. Consequently, conflicting results in the literature looking at the effect of these and other polymorphisms in *TGFB1* individually could be caused by missing information regarding other points of variation. For this reason, I decided to set up a protocol allowing for the comprehensive typing of this region. The rationale that I employed was that the protocol should be amenable to efficient sequencing of DNA of varying qualities (such as the one you might encounter when working with clinical HSCT samples). This meant that a relatively big region of the genome had to be split in sub-regions that could be more manageable and that would increase the chances of successful typing in potentially degraded old DNA samples. Moreover, if several regions were to be amplified and sequenced, it was desirable that a consolidated protocol could be developed for all of these regions. This effort for unique conditions was based on the design of the amplification primers. As explained before, the whole RI-RIV region was split in 4 sub-regions, flanked by primer-binding sites that would serve both for the reverse primer for one region as well as for the forward primer for the next region. This was to ensure that the thermodynamic characteristics of the primer molecules would resemble each other since both came from the same genomic sequence, albeit in different directions. This approach proved successful in most cases. The only case in which complementary primers could not be use was for RIII-F. The result for the RIII-F and RIII-R primer pair experiments

suggested the presence of some interaction that impaired amplifications. This suspicion was further intensified since the use of an alternative forward primer, whose binding site was further downstream, worked very well when used with primer RIII-R. Moreover, the fact that this alternative primer gave very good amplification with the amplification conditions used for the other sub-regions' primers, resulted in this primer to be selected as the routine one for the rest of the experiments. The selection of a forward primer for RIII whose sequence was not complementary to RII-R meant that a gap in the coverage for the complete region was created. This 140 bp gap did not, however, contain any known polymorphic positions, and was thus deemed dispensable in favour of the use of the alternative forward primer for RIII. It is also worth noting that primer design in RIII is complicated by the fact that this is a region with very high GC content and several one-base repetitions. In summary, the optimisation process for the *TGFB1* regulatory region and exon 1 SBT produced a very robust protocol capable of generating good sequencing data for 20 known polymorphic positions within this region with a single amplification, purification, sequencing and analysis protocol, set up with low-cost PCR reagents and thoroughly validated in terms of specificity and capacity of polymorphism coverage. Compared with the strategy used by Shah *et al*. (2006, 2009), which involved a complicated set of long amplifications coupled with very laborious cloning and possibly requiring high quality DNA for it to be successful, the protocol I developed can be easily applied in any molecular biology laboratory with sequencing capabilities, and can be taken from amplification to sequence analysis and report production in approximately 8 hours. The only drawback is that in this protocol we are not defining the phase of the polymorphisms, thus impairing definitive allele combination determination, with the added complexity of the potential presence of allele pair ambiguities. Despite this, in the majority of cases the allele combination can be determined empirically by genotype analysis producing a single possible allele combination based on the 17 previously identified alleles. In cases where two or more allele combinations agree with the genotype for the relevant 18 polymorphic positions, one possibility is to conduct further tests to define the phase of key polymorphic positions. For example, a sample bearing a p001/p006 genotype could also be resolved as a p002/p003 genotype. However, if the -2389 AGG duplication-specific primers are used to differentially amplify and segregate the alleles as was done for the characterisation of the new HVD28-borne allele, one can determine if the -1687 A is associated with the p001-like allele p002, or if it is instead associated with the p003-like allele p006. In this way the right combination of alleles present in any given sample that has heterozygosity for the -2389 AGG duplication could be determined. In cases where both alleles were homozygous for the -2389 AGG

duplication (or its absence), other polymorphic positions could be used to segregate the alleles. More details on these additional strategies will be given in Chapter 6. Overall, the fact that samples such as cell line JBUSH and HVD7, 24 and 35 bore a p001/p014 genotype means that all of them would be classified as +29 CC homozygous along with samples bearing p001/p001 in studies looking solely at this polymorphism. However, they would be classified as -1347 T/C samples as opposed to samples bearing p001/p001 genotypes, which would be -1347 TT, and potentially considered in a different group in studies focusing on this upstream SNP. This highlights the potential relevance of assessing alleles spanning a relatively long region with regulatory functions rather than individual polymorphisms. However, it is also conceivable that a single polymorphism (or a marker for it) is the sole responsible for an effect. This less likely scenario must nevertheless be proven against a combined effect in well-designed experiments.

The generation of a bioinformatics tool for the easy determination of the allele combinations present in a given sample based on the genotypes at the relevant 18 polymorphic positions represents a useful spinoff of undertaking the development of this typing protocol. The tool, which will allow for the quick assignment of *TGFB1* regulatory region and exon 1 genotypes in unknown samples, is to be made available freely in the context of the Immuno Polymorphism Database website. With this, it is hoped that the comprehensive typing of *TGFB1* polymorphism and the use of these data in clinical studies could be encouraged and facilitated, hopefully resulting in finer genetic risk associations as compared to those made upon single SNPs.

3.5.3 Characterisation of novel alleles of *TGFB1* regulatory region and exon 1

The identification of a novel recombinant allele in one healthy donor allowed for the development of strategies to characterise this situation. The identification of a genotype that could not be explained by any combination of the previously discovered alleles prompted the study of this sample. Analysis of samples of the relatives of this healthy donor added evidence to the predicted sequence of this novel allele. Final proof came from sequencing in isolation of the allele, but also, a novel strategy was devised: allele-specific full-length amplification of the genomic region with primers for the -2399 AGG duplication. This useful method would provide a much less laborious way to characterise new alleles involving heterozygosity at this position. The discovery of this novel allele, as well as other recombinant alleles discovered during the typing of an UD-HSCT patient-donor cohort presented in Chapter 6, will be reported in a manuscript that is being prepared for publication.

Furthermore, the fact that among a reduced number of samples typed a novel allele could be identified suggests that more polymorphism for *TGFB1*'s upstream regulatory region and exon 1 might be present. In fact, in the previous reports by Shah et al., few subjects were also typed. In their first publication, the authors found 14 alleles in 76 samples (Shah *et al.*, 2006), while in the second one they found 3 extra alleles in 38 patient-donor pairs (Shah *et al.*, 2009). This would suggest that more research is obviously needed in order to characterise the diversity in this genes regulatory region, highlighting even more the relevance of developing tools to assess this variation. However, it must be stressed that both in Shah *et al.*'s publications and in my own healthy donor cohort there is a predominance of certain alleles. Specifically, 3 alleles dominated this cohort: p003 (60%), p001 (30%) and p014 (10%). These alleles were also dominant in Shah *et al.*'s reports. This could mean that, despite a significant amount of polymorphism yet to be discovered, it is likely that for this gene the vast majority of the individuals bear common alleles, in a situation that could resemble, albeit in a reduced way, the situation found for the HLA system (Cano *et al.*, 2007).

3.5.4 Optimisation of a semi-quantitative real-time (RT)-PCR assay for *TGFB1* mRNA

Regarding the development of an RT-PCR assay for the measurement of *TGFB1* mRNA expression, the assay was optimised and validated successfully. The optimisation process included the determination of primer specificity, reference gene selection, target gene primer concentration determination, and efficiency determination. The specificity of the target gene primers was confirmed both in silico and *in vitro* by conventional and RT-PCR. The primers had been designed so that they spanned exon-exon junctions, thus making their amplification products mRNA-specific. This was confirmed by the analysis, as well as by the lack of amplification of genomic DNA in a conventional PCR.

Two reference genes were selected from a panel of 5. This selection was based upon stability of these genes in cells that were either freshly isolated or cultured with or without activation. This procedure intended the identification of genes that would not alter their expression in functional assays where activation and exposure to cytokines would be present, such as those that I intended to use and that are detailed in the next chapter. Genes *TOP1* and *ATP5B* showed stability across the various culture conditions, and thus were selected. Two reference genes as opposed to one were chosen in order to reduce the chances of variations in the Ct analyses and to increase the soundness of any semi-quantitative result.

Regarding the efficiency studies for the RT-PCR for *TGFB1* and the two reference genes, it is clear from the data that the three reactions have high efficiencies. Moreover, these efficiencies are comparable, something that is clear from the slopes of the best-fit linear curves for the template dilution experiments. The *TGFB1* assay is closer to the reaction for *TOP1*, having almost the same slope. Of note, two points in the *TGFB1* curve (1/100 and 1/1000) were slightly off, causing a reduction of the correlation coefficient (R^2=0.97). This is unlikely to affect the efficiency analysis since the slope with or without those points remains very similar.

Apart from high efficiency, the assessment of this parameter is of high importance in semi-quantitative assays based on comparison of Ct values of a target gene to those of reference genes. If the efficiencies of the target gene PCR and those of the reference genes' assays are dissimilar, then the comparison between Ct values loses strength and can generate misleading results that are proportional to this difference. More specifically, the amplification of a higher quantity of template will give an earlier Ct under low efficiency conditions compared to a high efficiency reaction. In turn, with a lower target quantity there is an inversion of this relationship, and the low efficiency reaction gives a later Ct than the reaction with high efficiency. Since the semi-quantitative –ΔΔCt method is based on the comparison of the Ct values of a target gene and of various reference genes, then significant differences in efficiency are bound to affect the calculations at higher or lower gene message conditions.

Overall, the attempts at optimizing molecular tools for the characterisation of *TGFB1* expression and the detection of its polymorphism proved successful and produced robust assays that were implemented in combination with the functional studies that are included in subsequent chapters. Of note, a completely novel technique for the thorough characterisation of *TGFB1*'s upstream regulatory region and exon 1 has been developed and validated, and was applied to the study of the effect of this gene's variation in HSCT outcome as will be detailed in Chapter 6. The discovery of a novel allele of the *TGFB1* regulatory region and exon 1 and the generation of a tool for the easy assignment of allelic genotypes are additional products of these experiments.

Chapter 4. The production of TGF-β1 by regulatory T cells and the effect of *TGFB1* +29T>C polymorphism

4.1 Introduction and aim

As explained in Chapter 1, transforming growth factor (TGF)-β1 and regulatory T cells (Treg) share an intricate functional relationship. Treg are major producers of TGF-β1, and this cytokine has been identified as one of these cells' mechanisms for controlling the immune response (Chen & Wahl, 2003). Moreover, Treg were identified as cells that were able to express TGF-β1 as a surface molecule early after their discovery (Nakamura *et al.*, 2001). TGF-β1's latency-associated peptide (LAP) was found to be expressed on Treg upon TCR stimulation, which in most studies was a phenomenon specific to this cell type among lymphocytes, and was even proposed as a marker for Treg purification from expansion cultures (Tran *et al.*, 2009).

Indeed, surface LAP was found to play an important role in the suppression of the proliferation of activated T cells, but it was not required for the suppression of naive T cell activation (Andersson *et al.*, 2008). Moreover, Treg cell–derived TGF-β1 could generate de novo suppressive CD4+FoxP3+ T cells *in vitro* from naive precursors in a cell contact–dependent, APC–independent and integrin–independent manner. These observations suggested that surface LAP was a central mechanism by which Treg suppressed immune responses and expanded their lineage among naïve precursors. More specifically among murine Treg, CD4+CD25+LAP+ cells were found to express elevated levels of FOXP3 and Treg-associated molecules such as CTLA-4 and GITR, to secrete TGF-β1, and to express surface receptors for TGF-β1 (Chen *et al.*, 2008). A similar phenotype for this cell subset in humans was also later confirmed (Gandhi *et al.*, 2010).

The close relationship between TGF-β1 and Treg function makes it interesting to determine if the *TGFB1* +29T>C gene could have a functional effect on the way these cells behaved *in vitro*. Consequently, based on the fact that these cells specifically express surface LAP (or the SLC) upon activation by means of TCR stimulation, I decided to test this hypothesis and also to study the kinetics of TGF-β1 production by these cells in *in vitro* activation assays. In this Chapter, I present the data obtained for the surface,

mRNA and intracellular expression of TGF-β1 by Treg and for the role of this polymorphism in LAP expression by Treg.

4.2 Surface expression of transforming growth factor (TGF)-β1 on regulatory T cells (Treg) and the effect of *TGFB1* +29T>C

In order to observe the production of TGF-β1 by Treg, the expression of LAP on the cell surface was assessed by flow-cytometric staining of isolated CD4+CD25+ cells. In parallel, isolated effector CD4 cells (CD4+CD25-) were also assessed for LAP production. The isolation of both cell subsets from healthy donor PBMC was performed as described in Chapter 2, achieving purities of typically 85-90% of the CD4+ fraction. An example of a Treg isolation process is presented in **Figure 4.1**. The expression of LAP on the cell surface was measured in non-activated and activated cells. The activation of the cells was carried out by means of TCR stimulation with antibodies against CD3 and CD28, both at a final concentration of 10 μg/mL. Non-stimulated and plate-bound antibody-stimulated cells were also used as controls.

LAP production was assessed by flow-cytometric staining with anti-LAP antibodies or an isotype control. The staining protocols and acquisition and analyses were carried out as described in Chapter 2. The cells were stained at different time points during culture (0-96h), and culture supernatants (as well as plain culture media) were collected and stored at -80˚C for future studies. The assessment of LAP was done by gating on the different populations identified by markers (i.e. CD4, CD25, CD127, and, in some experiments, FOXP3), and determining the percentage of LAP+ cells and the intensity of the fluorescence they exhibited.

Once isolated, CD4+CD25- and CD4+CD25+ cell subsets were cultured and activated by means of soluble antibody-mediated TCR stimulation. Surface expression of LAP was assessed on a single-cell basis using flow cytometry. Isolated cells were stained with anti-LAP antibodies at the time of isolation and subsequently at different incubation time points. **Figure 4.2** shows an example of anti-LAP staining on the different cellular fractions at the time of isolation. As seen in **Figure 4.2**, LAP staining is detected on both CD4+ and CD4- cells, and, when isolated subsets are evaluated, LAP is detected mainly on CD4+ cells.

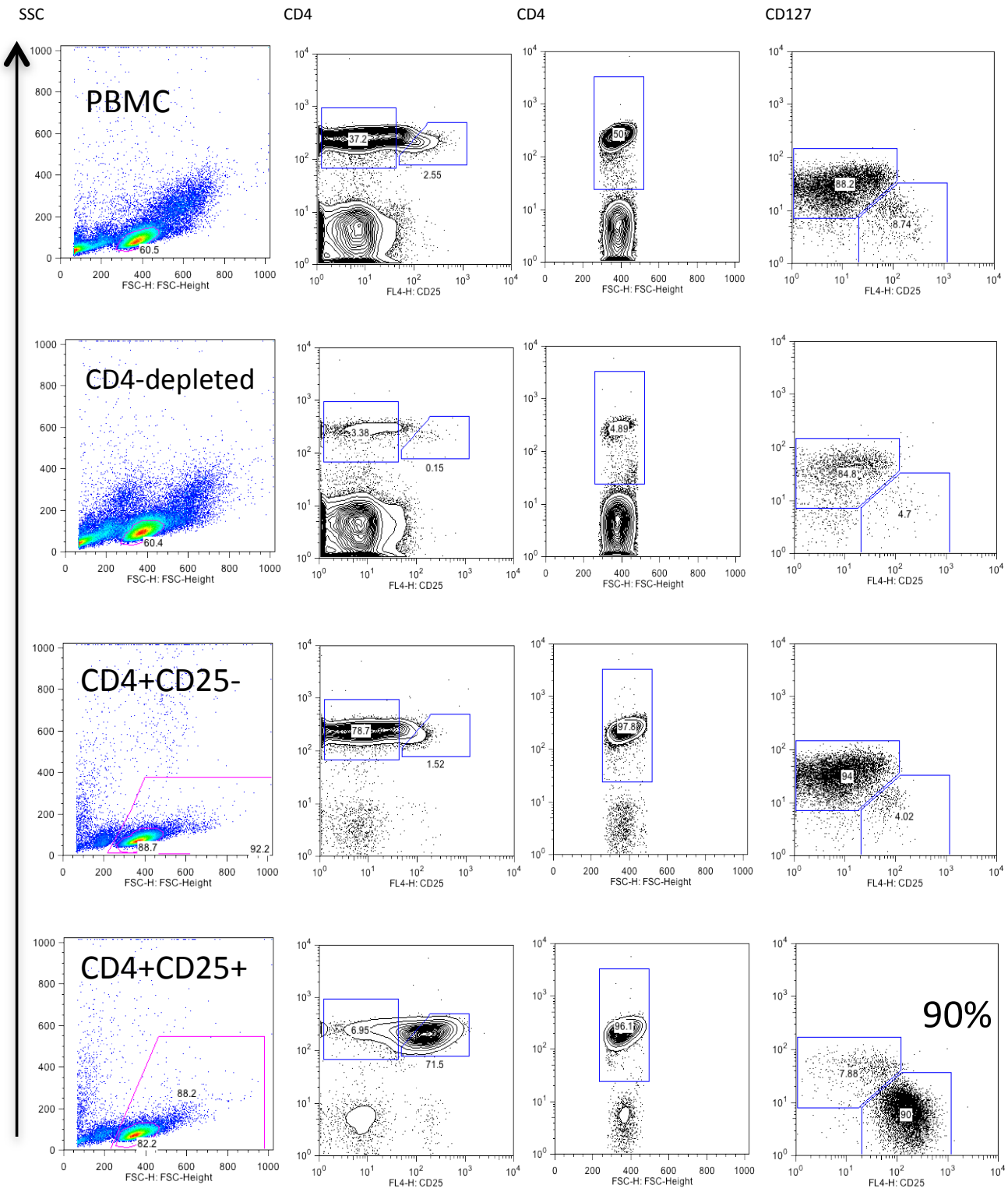


Figure 4.1 Example of a regulatory T cell isolation procedure. Peripheral blood mononuclear cells (PBMC), top row) obtained from healthy donors were used to isolate CD4+CD25- (third row) and CD4+CD25+ (bottom row) cells. The second row shows the CD4-depleted fraction. The first column shows the forward (FSC) and side scatter (SSC) plots (*y* axis) and lymphocyte gate. The second and third columns show scatter plots for CD4 (*y* axis) vs. CD25 expression and forward scatter on the lymphocytes, respectively. The fourth column shows CD127 (*y* axis) vs. CD25 expression scatter plots gated on the CD4+ lymphocytes. Plots generated with Flojo software (v. 6.4.7, Tree Star Inc., Ashland, USA).

When CD4+CD25+ cells were stimulated with antibodies against CD3 and CD28 as described in Materials and Methods, an upregulation of surface LAP was detected. A shown in **Figure 4.3**, this upregulation was observed only on the CD4+ cells. Induced LAP was seen exclusively on CD127lo cells and CD25+ cells. In fact, LAP+ cells after TCR stimulation are those that upregulate CD25. Contaminating CD4-, CD127hi, or CD25- cells did not show any surface LAP expression in response to the polyclonal stimulus.

In view of these observations, which are consistent with what has been previously reported by others (Chen *et al.*, 2008), the following gating strategy for the assessment of LAP expression upon TCR stimulation of Treg was used: live cells -> CD4+ -> CD25+CD127lo. Similarly, for comparison purposes, LAP expression was also measured on effector CD4 cells with the following gating strategy: live cells -> CD4+ -> CD25-CD127hi. A live cell gate was chosen instead of a more stringent lymphocyte gate in order to allow for changes in cell volume and internal complexity upon TCR-mediated activation. **Figure 4.4** shows the gating strategy and its correlation with FOXP3 staining, where 98-99% of the CD127lo cells are FOXP3+ (data from 3 independent experiments).

For both Treg and effector cells, LAP positivity as a percentage of the gated cells, and their median fluorescence intensities (MFI) were calculated by setting a 0.5% cutoff on the histogram for non-stained lymphocytes in each experiment. This cut-off was validated with both isotype control and fluorescence-1 methods, obtaining congruent results.

In 3 independent experiments (1 per genotype), FOXP3 staining was performed on resting and TCR-activated Treg and effector CD4 cells. In keeping with the previous observations, LAP arose exclusively on FOXP3+ cells. **Figure 4.5** shows a representative experiment where FOXP3+ cells are shown to become LAP+ after activation. These results further validate the gating strategy selected for the assessment of LAP expression on Treg upon TCR stimulation.

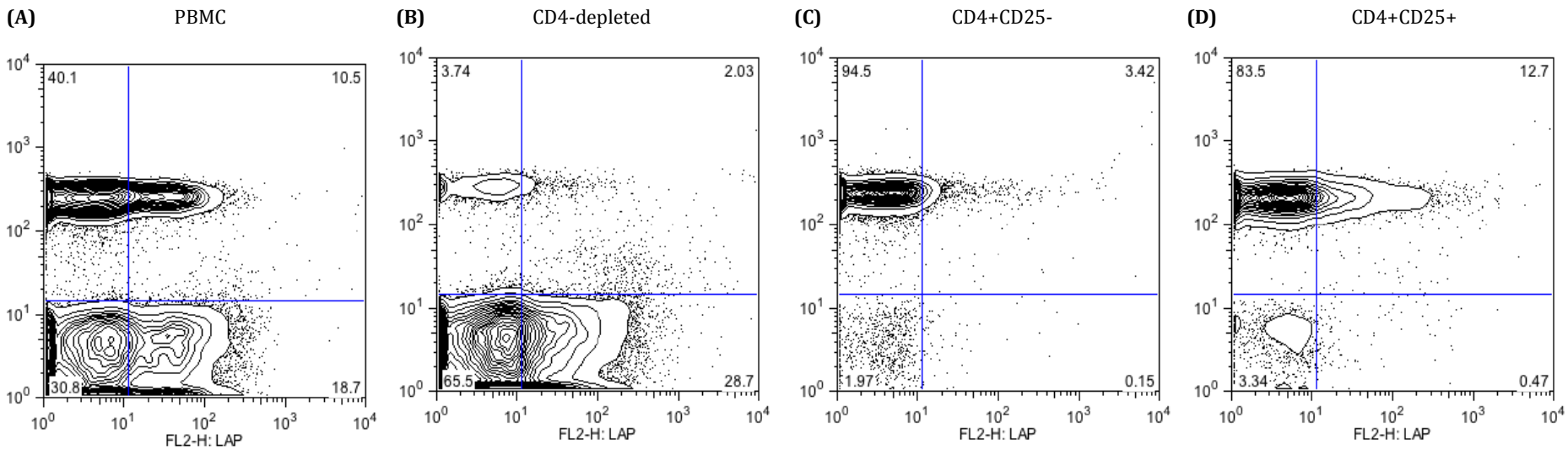


Figure 4.2 Latency-associated peptide (LAP) staining on cellular subsets at the moment of isolation. (A) Unmanipulated peripheral blood mononuclear cells (PBMC), (B) CD4-depleted PBMC, (C) effector CD4+, and (D) CD4+CD25+ regulatory T cell (Treg) subsets were stained with anti-LAP-PE antibodies. Scatter plots show CD4 (*y* axis) vs. LAP expression on gated lymphocytes.

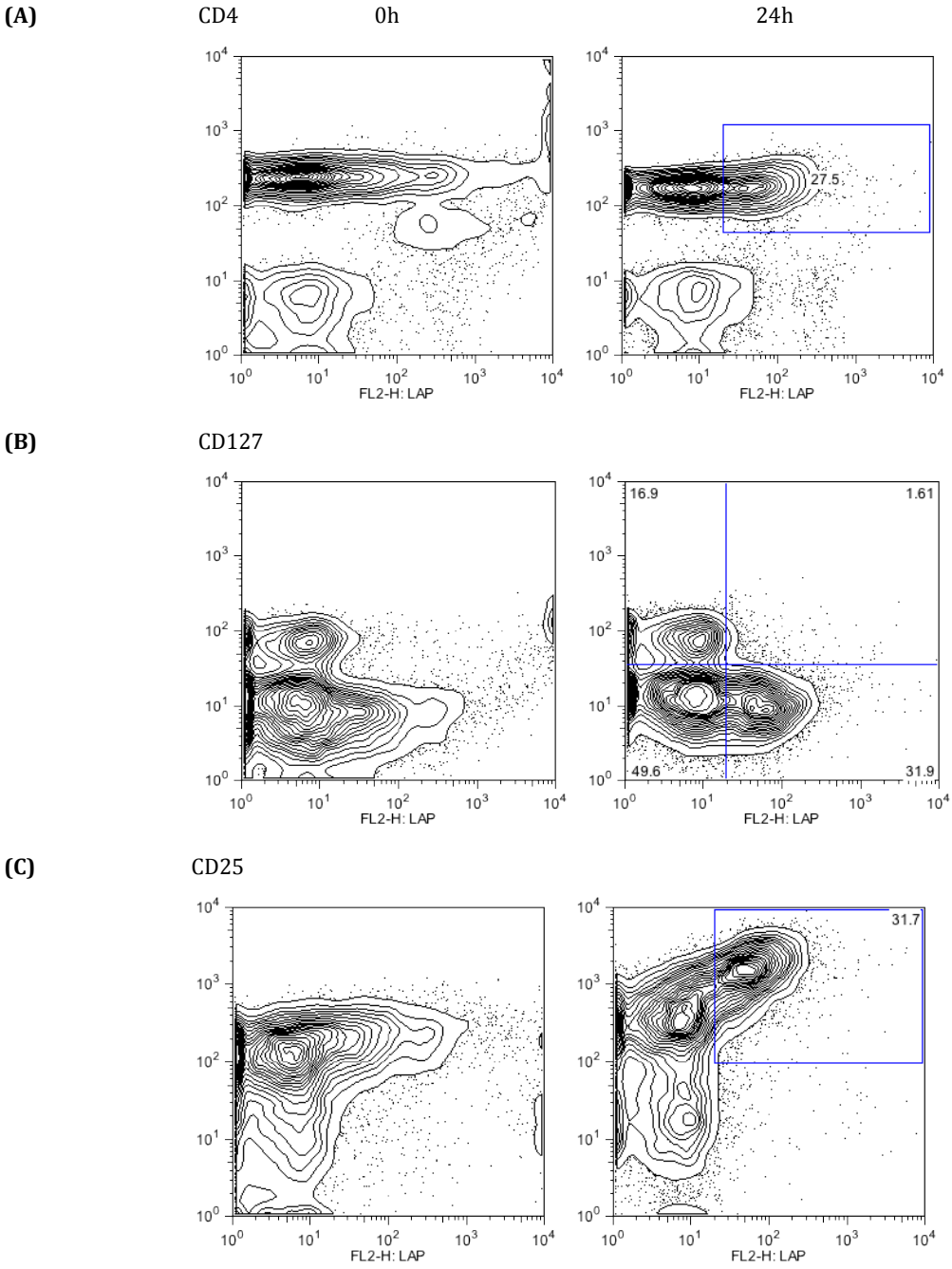


Figure 4.3 Surface latency-associated peptide (LAP) is upregulated solely on CD4+, CD127lo, and CD25++ stimulated lymphocytes. Isolated Treg were stimulated with anti-CD3 and anti-CD28 and cultured for 24h in the presence of IL-2. LAP staining levels at the moment of isolation (t=0, left column) and at 24h of culture (right column) are shown. LAP staining on gated lymphocytes is shown against CD4 (A), CD127 (B), and CD25(C) staining on the *y* axis.

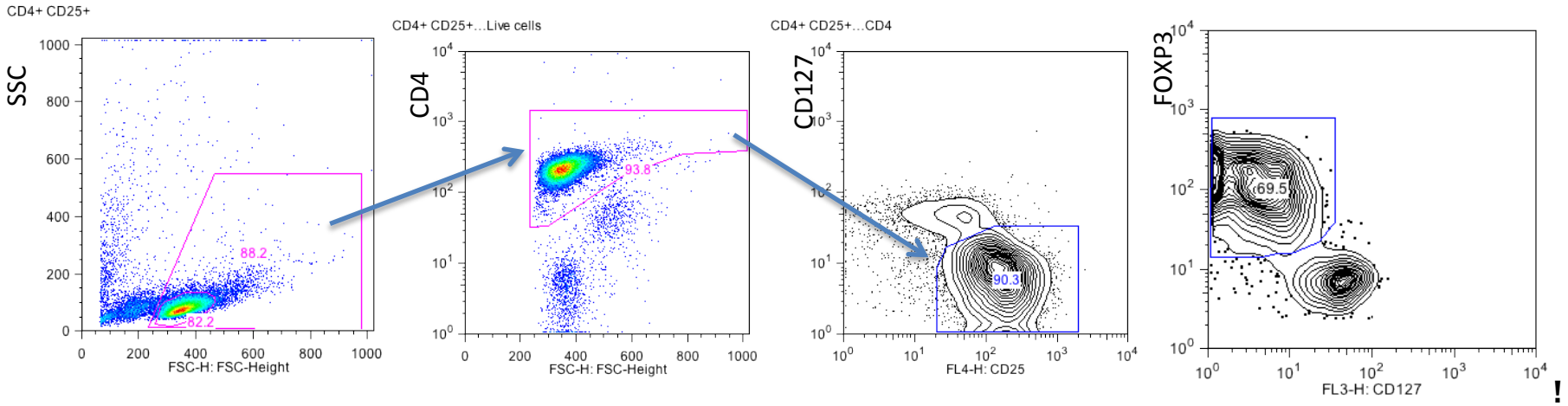


Figure 4.4 Gating strategy for the assessment of latency-associated peptide (LAP) expression on regulatory T cells (Treg) and effector CD4 cells. The first plot shows the ungated forward vs. side scatter (SSC, *y* axis), and the live cells gate. The second panel is gated on the live cells and shows CD4 staining (*y* axis) vs. forward scatter, and the CD4+ gate. The third panel is gated on the CD4+ cells and shows CD127 (*y* axis) vs. CD25 staining, and the Treg (CD25+CD127lo) gate. The fourth panel shows the correlation between CD127 and FOXP3 (*y* axis) staining in Treg cells.

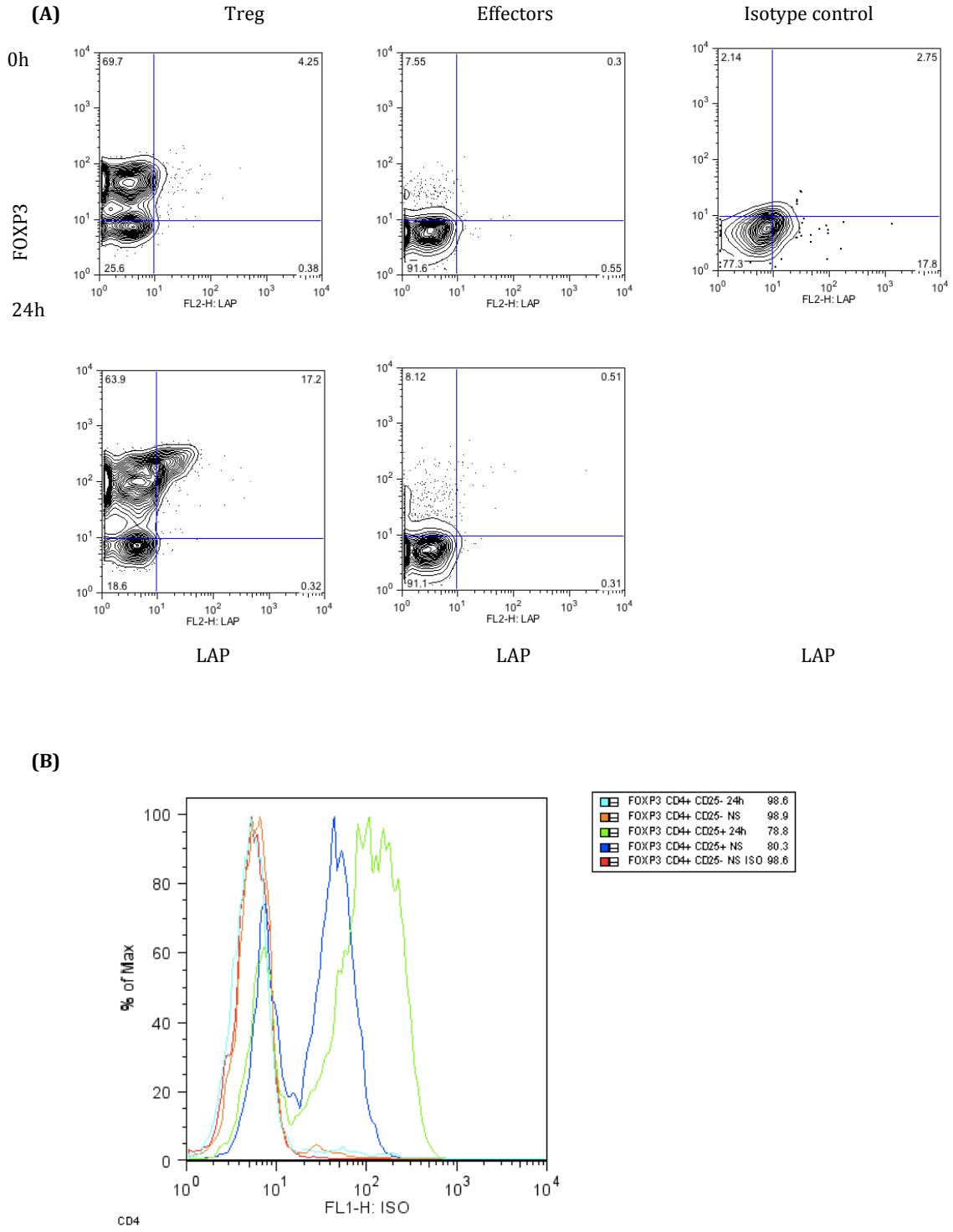


Figure 4.5 Latency-associated peptide (LAP) is induced on FOXP3+ and not on FOXP3- cells upon activation. Isolated regulatory T cells (Treg) and effector CD4 cells were activated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and subsequently stained with fluorescent antibodies against CD4, CD127, LAP and FOXP3 at t=0h and t=24h. (A) FOXP3 (*y* axis) vs. LAP staining on Treg (left column) and effectors CD4 cells (central column). Right column shows isotype control (*y* axis) vs. LAP staining on effector CD4 cells. Cells at the moment of isolation (t=0h, top row) and after 24h of culture in stimulating conditions (bottom row) are shown. (B) Histogram of FOXP3 staining on freshly isolated (t=0h) and activated (24h) Treg and effector CD4 cells. All plots and histograms are gated on CD4+ live cells.

To further characterize the kinetics of LAP expression on Treg after TCR stimulation with soluble anti-CD3 and anti-CD28, experiments with additional time points were carried out. Activated Treg and effector CD4 cells were stained at 0, 24, 48, 72 and 96 h of incubation. LAP staining on Treg peaked at ~24h of stimulation. **Figure 4.6** shows the short-term kinetics of LAP production by activated Treg and effector cells.

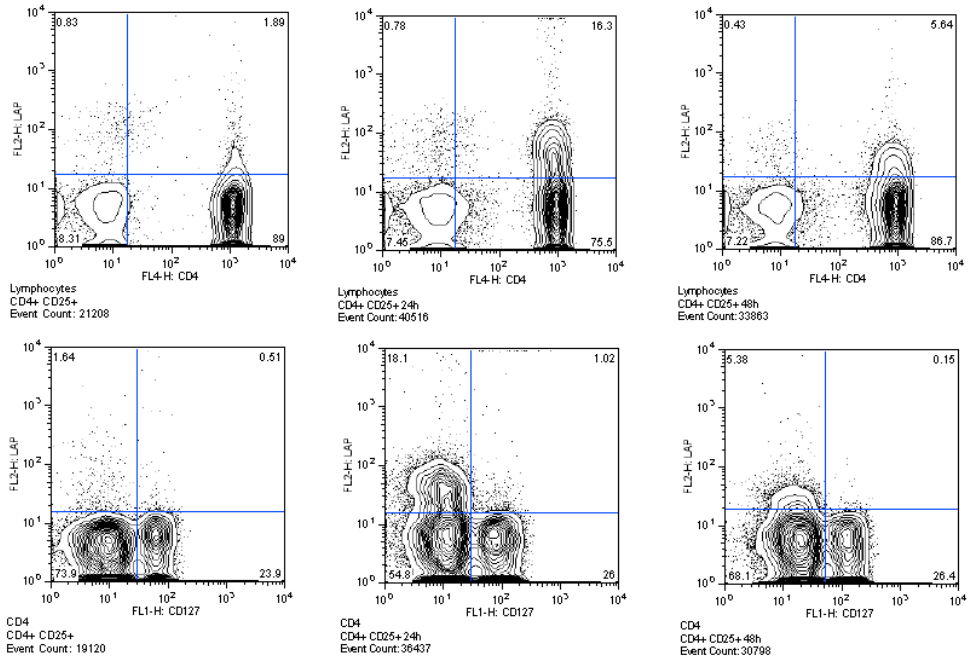
In 2 experiments, extra time points at 6h and 30h were included in order to better define the kinetics of LAP production by Treg (**Figure 4.7**). LAP levels at t=6h were found to be very similar to those at t=0h. Additionally, LAP expression on Treg at 30h of incubation was lower than that at 24h, confirming the previous observations of a peak expression around 24h.

When isolated cells were incubated for longer periods, a further reduction of LAP levels on Treg after stimulation was observed after 48h of incubation. **Figure 4.8** shows the extended characterization of LAP expression in a series of activation experiments. The percentage of LAP+ Treg levels from 72 to up to 96h remained constant and relatively low. This observation was consistent in all the experiments that included these time points.

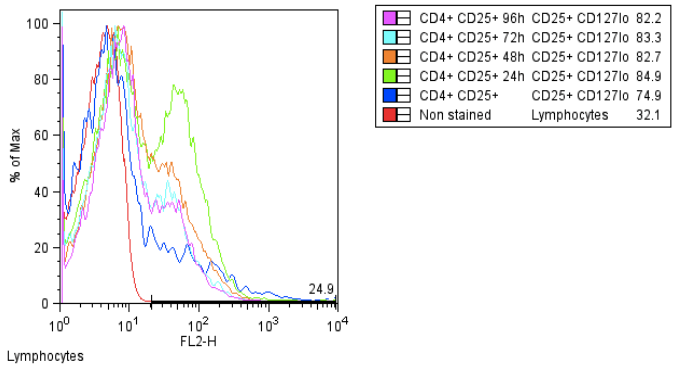
As a positive control for the activation experiments, both isolated Treg and effector CD4 cells were activated with plate-bound anti-CD3 and anti-CD28 antibodies as described in Materials and Methods. This procedure was chosen because of the increased strength of this type of stimulus in order to confirm that the cells were functional and capable of producing LAP. The kinetics of LAP production by the isolated subsets was also studied. As previously seen with soluble stimulating antibodies, LAP arose only on Treg and not on effector cells. As opposed to soluble activation, plate-bound TCR stimulus on Treg caused a sustained and maximal LAP expression. Thus, LAP levels were upregulated as early as 24h, but expression was held and augmented at 48 and 72h while the cells turned blastic. **Figure 4.9** shows a representative experiment.

The observation that LAP expression peaked on Treg at 24h after TCR stimulation with soluble antibodies prompted the selection of this time point for additional experiments in order to compare levels of LAP on cells from individual bearing different *TGFB1* +29 T>C genotypes. Thus, subsequent independent experiments were carried out to acquire data from 15 previously typed healthy blood donors (5 donors per genotype) to determine if there were differences in LAP production. As shown in **Figure 4.10**, *TGFB1* +29 T>C genotype appears to influence the levels of LAP produced by Treg upon TCR stimulation.

(A) LAP 0h 24h 48h

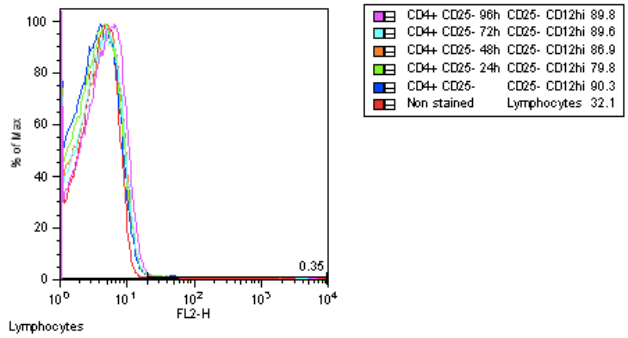


(B)



LAP

(C)



LAP

Figure 4.6 Latency-associated pepide (LAP) expression on activated regulatory T cells (Treg) peaks at 24h of incubation. Isolated Treg and effector CD4 cells were activated with soluble anti-CD3 and anti-CD28 in the presence of IL-2. LAP positivity was determined at t=0, 24 and 48h by flow cytometry. (A) LAP (*y* axis) vs. CD4 (top row) and CD127 (bottom row) staining on isolated Treg after 0, 24 and 48h of

incubation. Gating on live cells (top row) and on CD4+ cells (bottom row). Histograms for LAP staining on Treg (B) and effector CD4 cells (C) at different time points after activation. Cells gated on lymphocytes.

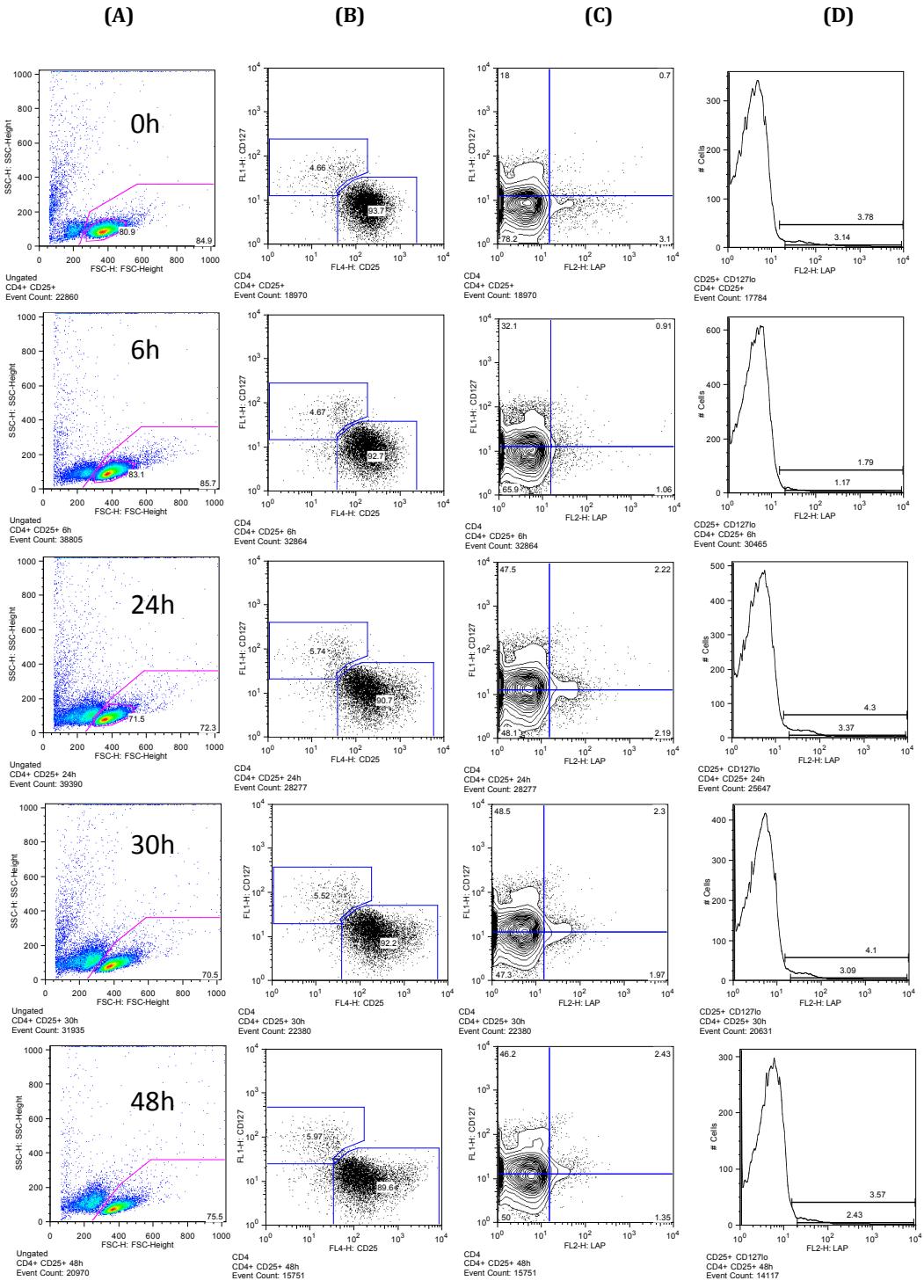


Figure 4.7 Latency-associated peptide (LAP) expression on activated regulatory T cells (Treg) peaks at 24h of incubation. Isolated Treg were activated with soluble anti-CD3 and anti-CD28 in the presence of IL-2. LAP positivity was determined at t=0, 6, 24, 30 and 48 h of incubation by flow cytometry. Columns represent (A) forward (FSC) vs. side (SSC) scatter, (B) CD25 vs CD127 in CD4+ cells, (C) LAP vs CD127 in

CD4+ cells, and (D) LAP staining on CD4+CD25+CD127lo cells. Rows represent each time point (top, 0 h, bottom 48 h).

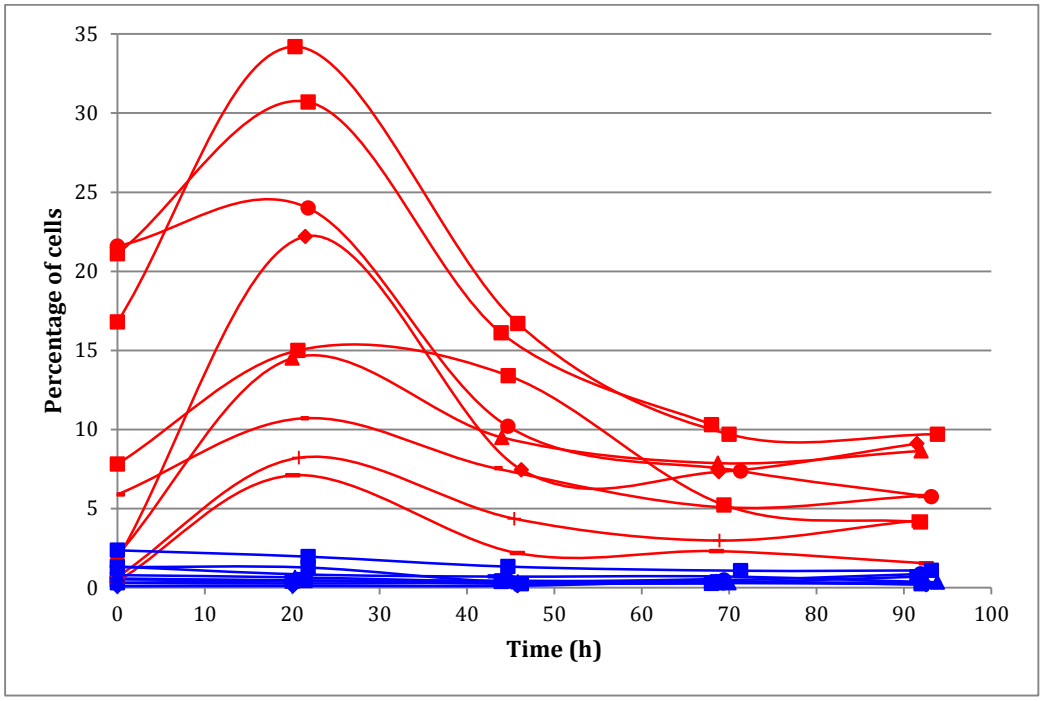


Figure 4.8 Latency-associated peptide (LAP)+ cells after stimulation of isolated regulatory T cells (Treg) (CD4+CD25+CD127lo) and Effector (CD4+CD25-CD127hi) lymphocytes. In a series of independent experiments, isolated Treg (red) and effector (blue) cells were activated with soluble anti-CD3 and anti-CD28 in the presence of IL-2 and cultured for different periods. The cells were harvested at specific time points and surface LAP expression was assessed by flow cytometry.

Although visually both TC and CC individuals appeared to generate higher percentages of LAP+ Treg after 24h of activation, this effect did not reach statistical significance when the three genotypes were assessed by Kruskal-Wallis test (p=0.17). Individual comparisons between genotypes were also not significantly different (Mann-Whitney test, p>0.05). However, when the experiments were grouped according to the presence or absence of the +29C allele assuming a dominant effect model, a trend towards higher LAP+ levels was seen (Mann-Whitney test; p=0.07). Conversely, when median fluorescence intensity was compared between the three genotypes or the grouped samples, no difference was seen. Of note, the frequency of Treg (CD4+CD25+CD127lo) in both total PBMC and CD4+ cells did not differ significantly among the *TGFB1* +29T>C genotypes at the time of cell procurement (**Figure 4.11**).

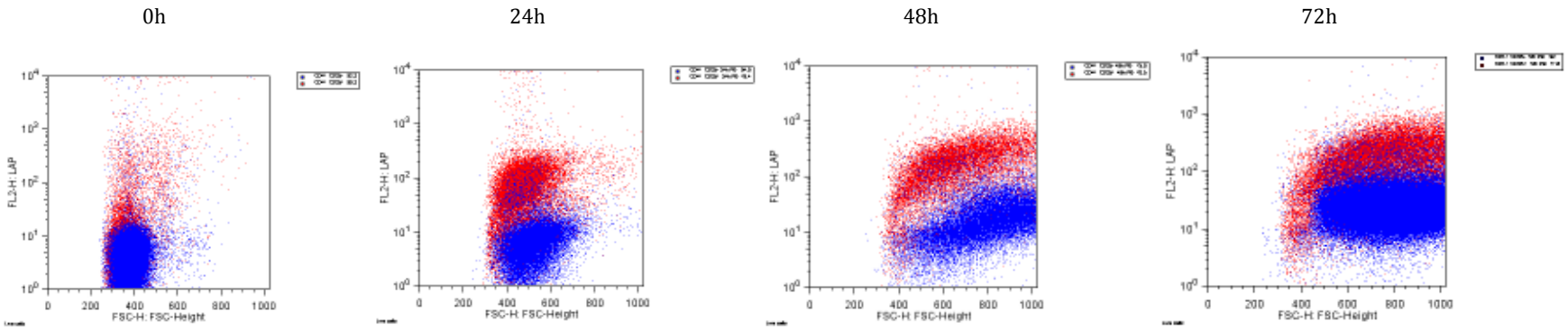


Figure 4.9 Latency-associated peptide (LAP) expression on regulatory T cells (Treg) is maximal and sustained after plate-bound stimulation. Isolated Treg (red) and effector CD4 (blue) cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2. LAP staining (*y* axis) was assessed at different time points after incubation. *X* axis: FSC. Gated on live cells.

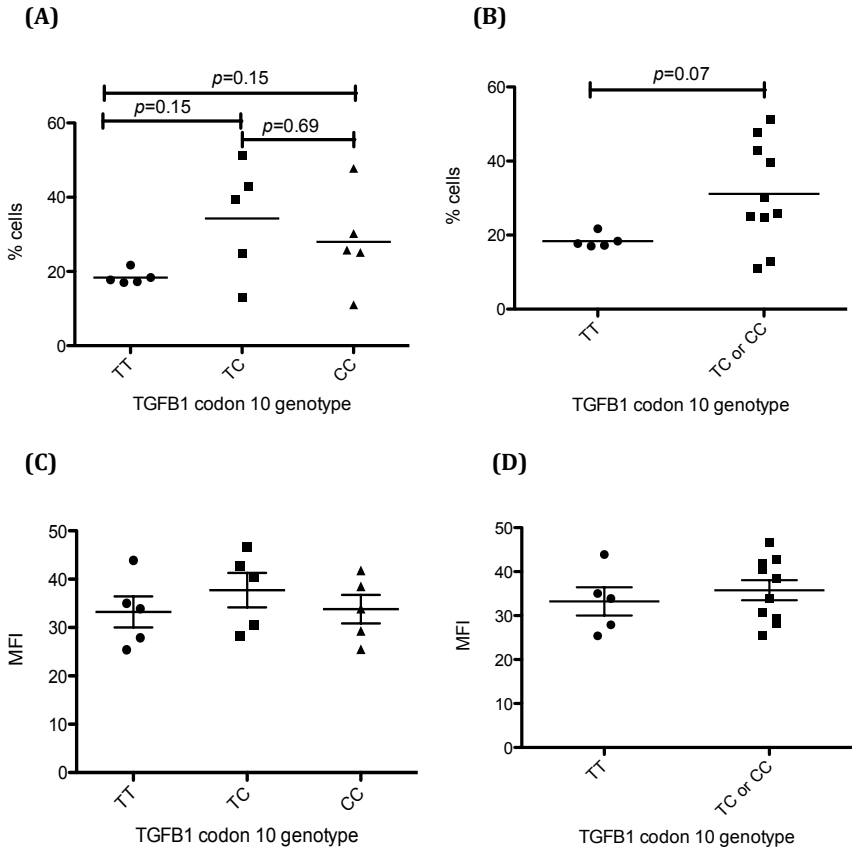


Figure 4.10 Latency-associated peptide (LAP) expression on regulatory T cells (Treg) upon stimulation differs according to *TGFB1* codon 10 +29T>C genotype. A total of 15 independent Treg activation experiments (5 per genotype) were performed and LAP expression was assessed at 24h of incubation with soluble anti-CD3 and anti-CD28. (A+B) Percentage of LAP+ cells within the CD4+CD25+CD127lo gate according to their *TGFB1* +29 T>C genotype. (C+D) Median fluorescence intensity (MFI) of the LAP+ cells according to their *TGFB1* +29 T>C genotype.

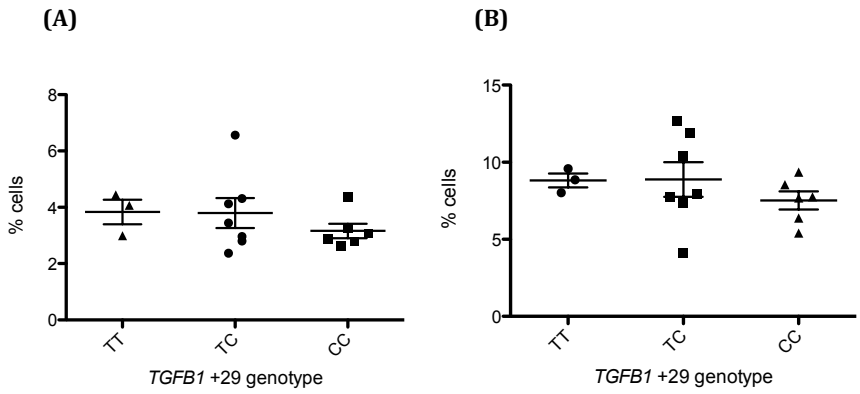


Figure 4.11 The percentage of regulatory T cells (Treg) does not differ according to *TGFB1* codon 10 +29T>C genotype. The frequency of Treg was recorded in the majority of the activation experiments at the time of cell procurement. Total peripheral blood mononuclear cells (PBMC) were stained with fluorescently-labeled antibodies against CD4, CD25, and CD127, and the frequency of CD4+CD25+CD127lo cells among the total PBMC (A) and in the CD4+ subset (B) was assessed by flow cytometry.

4.3 The kinetics of TGF-β1 mRNA expression in regulatory T cells (Treg)

The kinetics observed for the expression of LAP on the surface of Treg upon TCR stimulation, with a peak of expression at approximately 24 of culture elicited interest since it could mean that the production of TGF-β1 is regulated in these cells, and this could be an important biological observation with potential *in vivo* relevance. To further study this phenomenon at the mRNA level, I used the same culture conditions as those used for the surface LAP experiments, but used total RNA extraction and RT-PCR for the assessment of the levels of *TGFB1* mRNA in the isolated cell subsets. The RNA procedure was done as described in Chapter 2, and the RT-PCR assay used was the one whose development is presented in Chapter 3.

In a first set of cultures, RNA was extracted from Treg and effector CD4+ cells at the time of isolation and at 8, 16 and 24 h of culture in activating conditions. The activation of the cells was confirmed by additional 24 h cultures that were analysed by flow cytometry as done previously. Also, non-stimulated cell cultures were also set up for the 16 h time point. The data for this experiment is presented in **Figure 4.12**. As shown in this graph, the levels of *TGFB1* mRNA in isolated Treg cultures subject to activation decreased significantly as early as 8 h of incubation, and continued to do so until the 24 h time point. mRNA levels also decreased for non-stimulated cells, whereas the levels in the effector cell cultures remained at approximately 75% of those seen at the time of isolation, albeit slightly lower in non-stimulated cells. Moreover, as shown in **Figure 4.13**, when the mRNA levels were compared between subsets, the levels in Treg were 1.48 times higher than those in effector cells, but the ratio was inversed from 8 h onwards. These unexpected results seemed to suggest that, if present, the induction of *TGFB1* expression could be happening before the first 8 h of culture. Consequently, I decided to repeat the experiments using earlier assessment time points.

In the next experiments, I used cells from a different healthy donor, and set up the activation cultures as done for the previous ones. However, the harvesting and total RNA extraction of the cells was done at 2, 4 and 6 h of culture. The results for these experiments are presented in **Figures 4.14** and **4.15**. As seen in these graphs, once again, there was a differential regulation of the amount of *TGFB1* mRNA between Treg and effector cell cultures. Isolated effector CD4+ cells showed levels of message that were 2-3 x those found at the time of isolation, before any stimulus was added.

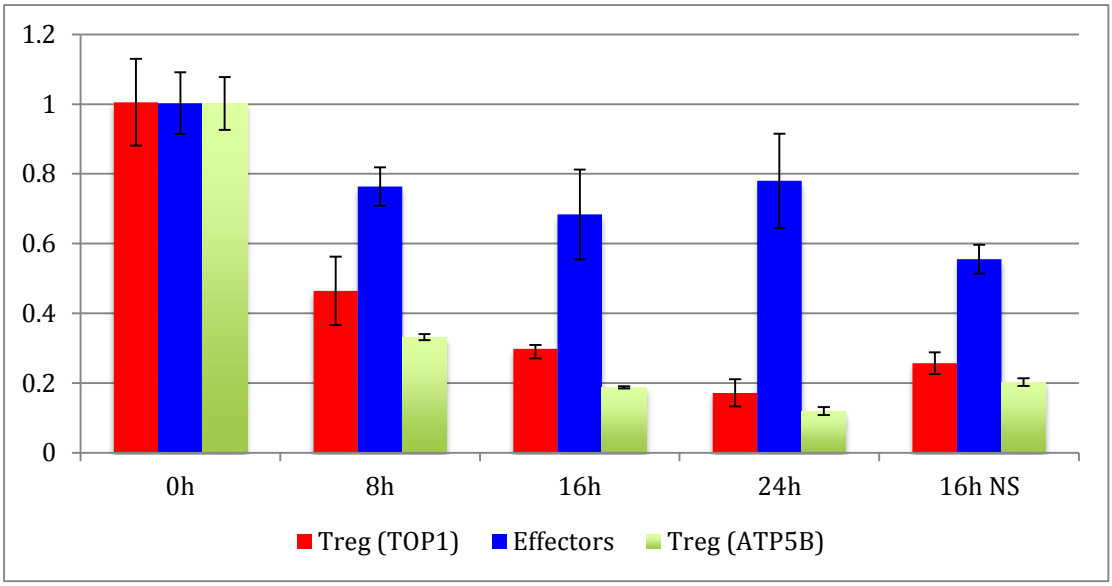


Figure 4.12 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) and effector CD4+ cells subject to stimulation. Isolated Treg and effector cells were cultured for 8 to 24 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. An additional 16 h non-stimulated (NS) culture was also analysed. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using one or two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

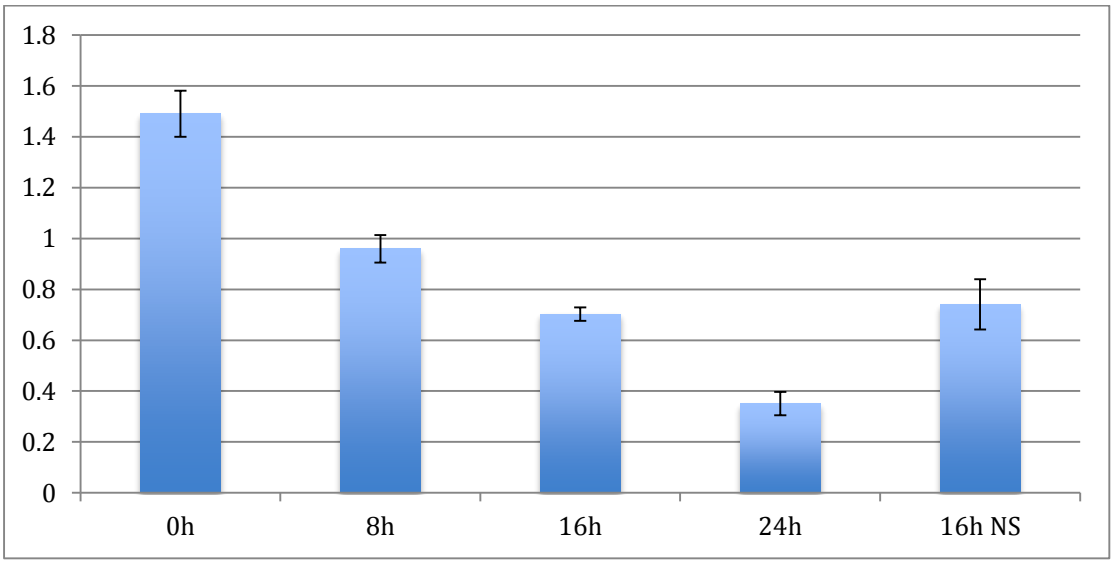


Figure 4.13 Levels of expression of *TGFB1* mRNA in regulatory t cells (Treg) and effector CD4+ cells subject to stimulation. Isolated Treg and effector cells were cultured for 8 to 24 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. An additional 16 h non-stimulated (NS) culture was also analysed. The levels of target mRNA are presented as normalized ratios of Treg levels to effector cell levels at each time point using an average of two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

These levels were approximately constant from 2 to 8 h of culture. On the contrary, Treg showed once again a severe reduction in the amount of *TGFB1* message upon culture in activating conditions, even as early as 2 h of incubation. The levels of *TGFB1* message in stimulated Treg dropped to 40 to 50% of those seen at the time of isolation. When the levels of *TGFB1* mRNA were compared between Treg and effector cells, those at the time of isolation were several times higher in Treg, but this ratio was once more reverted after stimulation, hovering just about equal relative amounts in both cell subsets. A second experiment partly confirmed those of the previous experiment: a progressive decrease in the amount of *TGFB1* message in Treg from as early as 2 h of incubation, and reaching levels that were approximately 30% of those seen at the time of isolation by 8 h of culture. In this experiment, effector cells showed an increase in *TGFB1* mRNA levels, although it was more subtle than that seen in the previous experiment (about a 45% increase), and more transient (quantities returned to isolation levels by 6 h, and decreased to 40% by 8 h of culture). When the levels of target mRNA were compared between subsets, Treg were found to have 20-60% more than effector cells, and the ratio was inverted at 2 (0.8), 4 (0.3) and 6 (0.3) h of incubation, reaching comparable levels once more at 8 h.

These experiments were repeated using plate-bound stimulus and also no stimulus. When the activation experiment was repeated using plate-bound stimulus, the amount of *TGFB1* mRNA was progressively reduced in Treg from 2 to 8 h of activation (**Figure 4.16**). By 2 h, target mRNA levels dropped to approximately 70% of those found at the time of isolation, and reached a fourth of the original levels by 8 h. On the contrary, target mRNA levels in stimulated effector CD4 cells had a discrete increase between 2 and 4 h of culture, which receded by 6 h (**Figure 4.17**).

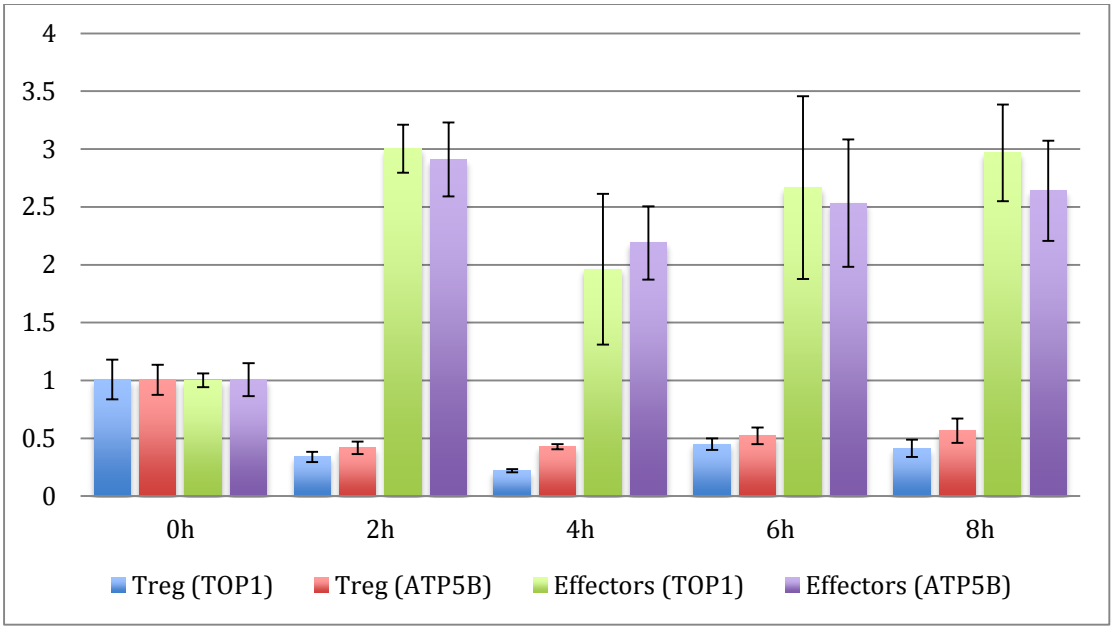


Figure 4.14 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) and effector CD4+ cells subject to stimulation. Isolated Treg and effector cells were cultured for 2 to 8 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using one or two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

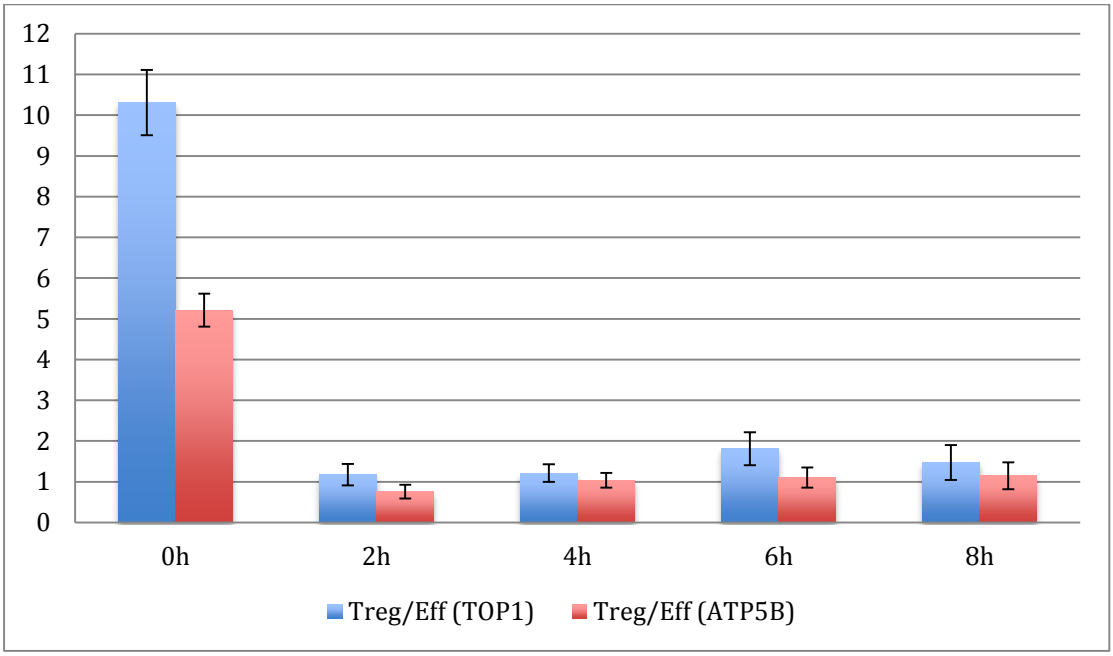


Figure 4.15 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) and effector CD4+ cells subject to stimulation. Isolated Treg and effector cells were cultured for 2 to 8 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios of Treg levels to effector cell levels at each time point using an average of two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

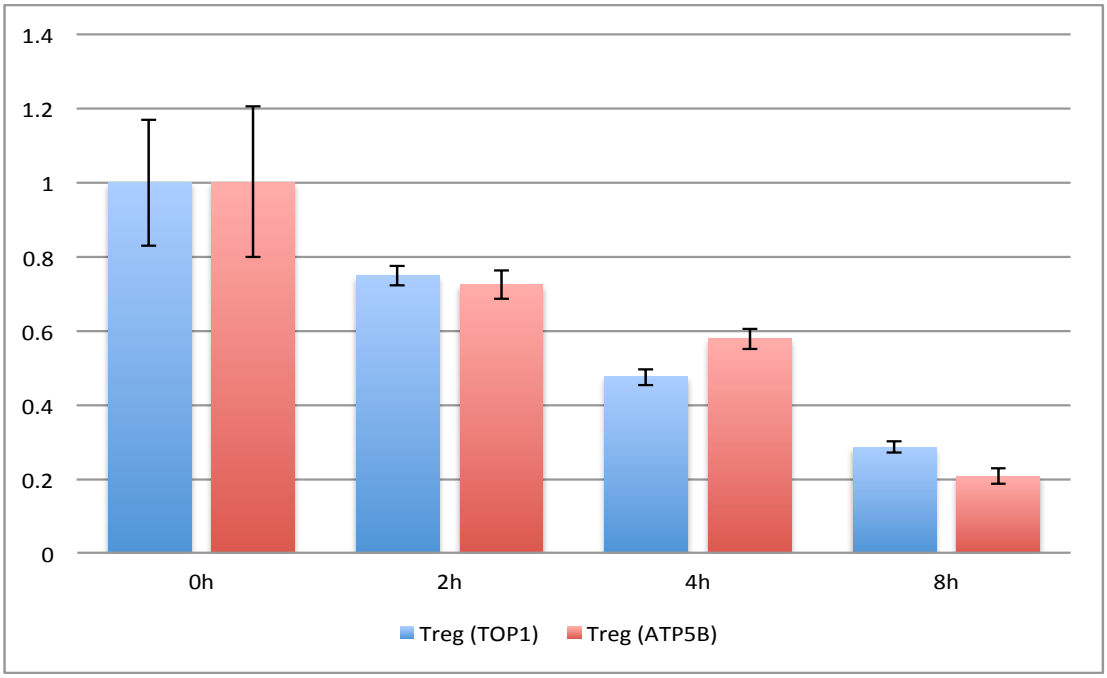


Figure 4.16 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) subject to plate-bound stimulation. Isolated Treg were cultured for 2 to 8 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

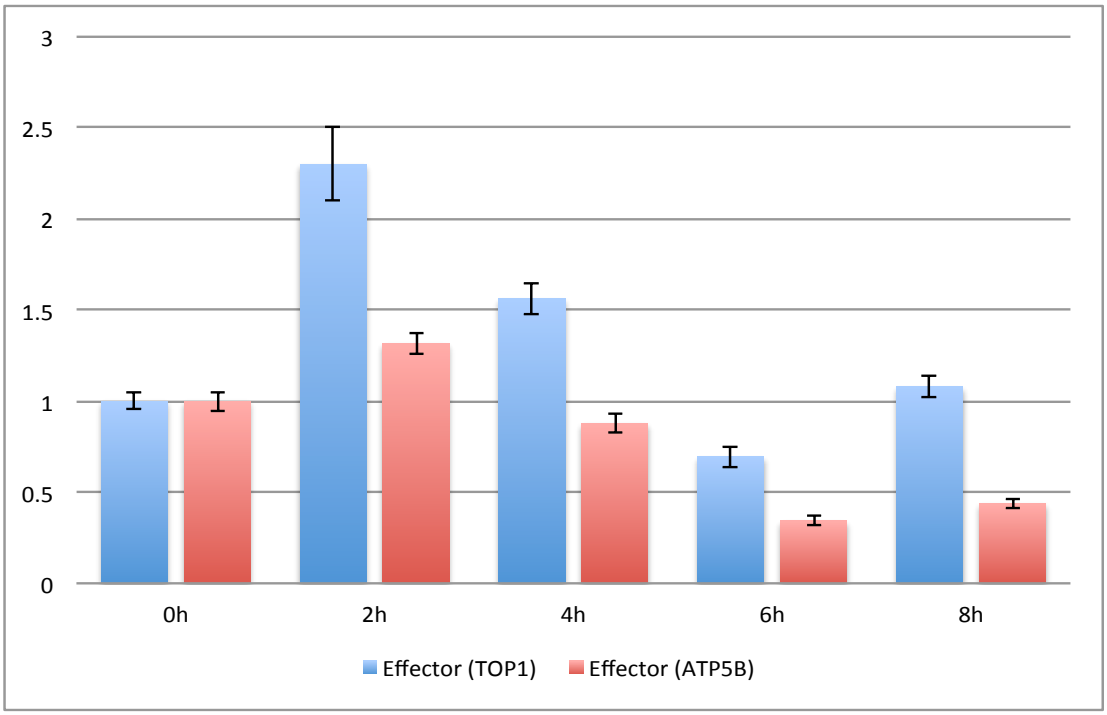


Figure 4.17 Levels of expression of *TGFB1* mRNA in effector CD4+ cells subject to plate-bound stimulation. Isolated effector cells were cultured for 2 to 8 h in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

As shown in the charts, when isolated Treg were cultured without stimulus, these cells kept *TGFB1* mRNA levels similar to those found at the time of isolation until 3 h of culture (**Figure 4.18**). However, these levels decreased by 6 h to approximately 50%. On the other hand, effector cells behaved differently, once more exhibiting a slow decrease in target mRNA levels from 2 h and up to 8 h, when these reached about 50% of the original ones (**Figure 4.19**). Consequently, the absence of stimulus seemed to confer a different picture in both cell subsets, suggesting a role for anti-CD3 and anti-CD28 antibodies in the previous observations.

Because it was still possible that the peak could be occurring before 2 h of incubation, I decided to repeat these experiments using, once again, earlier time points. Once again, Treg and effector CD4 subsets were isolated from PBMC of a healthy donor and cultures in activating conditions with soluble antibodies. In this occasion the cells were harvested for RNA extraction at 40, 80 and 120 minutes of culture. In the first experiment using this setup, Treg exhibited a moderate increase (1.5 to 2.4 times) in the levels of *TGFB1* mRNA at 40 minutes of culture. This increase was transient, since by 80 minutes the levels returned to slightly below isolation levels. On the other hand, effector cells did not show any significant increase in target mRNA levels. mRNA levels were comparable between Treg and effector fractions at three time points except at 40 minutes.This experiment was repeated using cells from another healthy donor. **Figures 4.20 and 4.21** show the results for these experiments.

As seen in **Figure 4.20**, the levels of *TGFB1* mRNA in Treg decreased as early as 20 minutes of culture, while those in effector CD4 cells increased 2-3 times between 40 and 70 minutes of activation. Interestingly, the levels of *FOXP3* mRNA followed a similar trend (**Figure 4.21**): at 40 minutes of culture, the levels were dramatically reduced in Treg, while in effectors they increased to approximately twice those found at the time of isolation. As has been observed previously, the levels of *TGFB1* mRNA were higher in Treg than in effectors at the time of isolation. However, the ratio was inverted from 20 to 70 minutes of incubation. Similarly, *FOXP3* mRNA levels were 12-20 x higher in Treg at the time of isolation, and continued to be higher than in effectors at 40 and 70 minutes but at only about 4x.

In summary, a clear induction of the amount of *TGFB1* message in Treg caused by TCR stimulation that would explain the kinetics of surface LAP expression on these cells was not consistently confirmed. Rather, a reduction of the cytokine's message was seen consistently in Treg upon stimulation. A differential behavior between isolated Treg and effector CD4 cells was constant, and effector cells were the only subset seen to upregulate *TGFB1* mRNA upon stimulation.

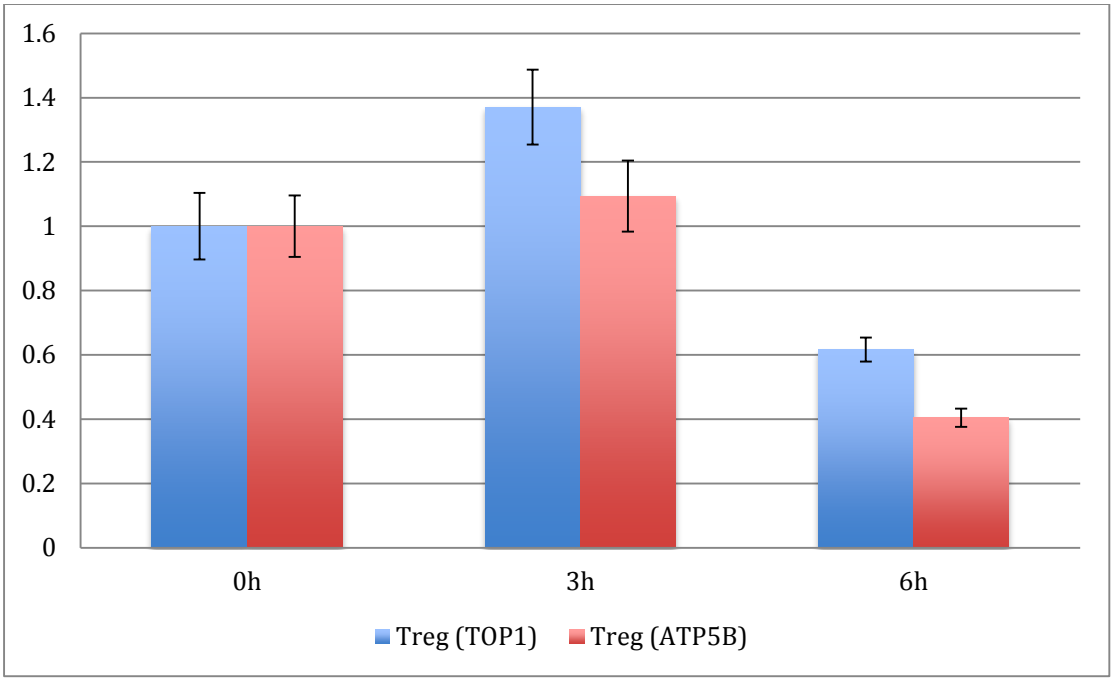


Figure 4.18 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) subject to culture in the absence of stimulation. Isolated Treg were cultured for 3 to 6 h in non-activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Because of the isolation's yield, only 3 time points could be set up for Treg. Bars indicate standard error from triplicate measurements.

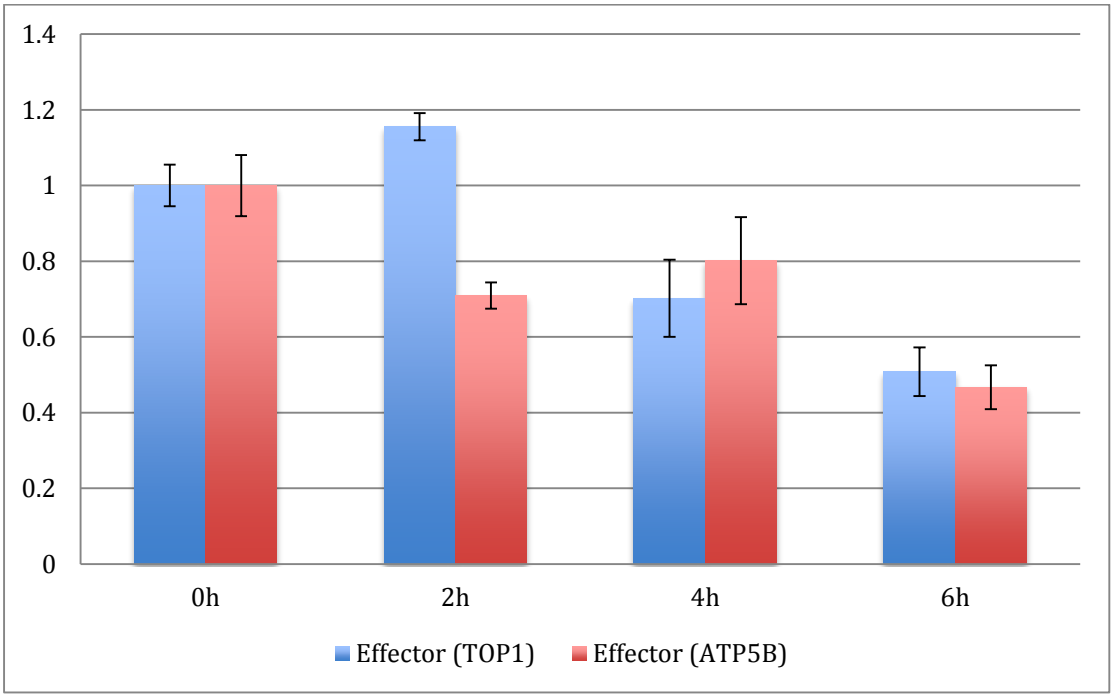


Figure 4.19 Levels of expression of *TGFB1* mRNA in effector CD4+ cells subject to culture in the absence of stimulation. Isolated effector cells were cultured for 2 to 6 h in non-activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

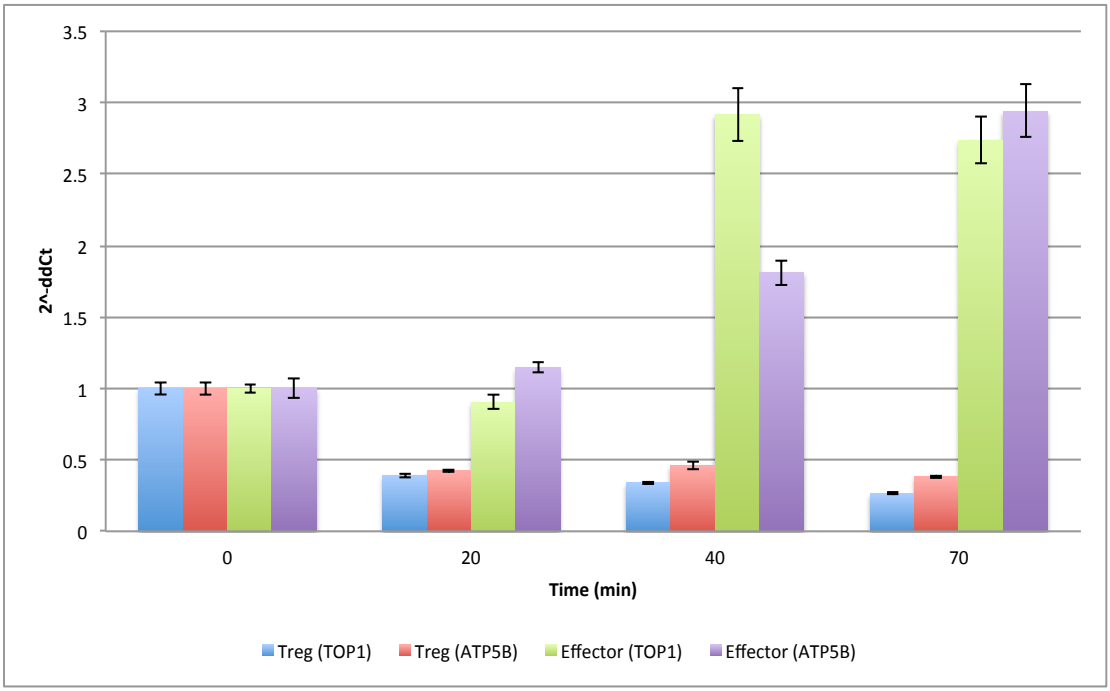


Figure 4.20 Levels of expression of *TGFB1* mRNA in regulatory T cells (Treg) and effector CD4 cells subject to stimulation. Isolated subsets were cultured for 20 to 70 minutes in activating conditions and the levels of *TGFB1* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

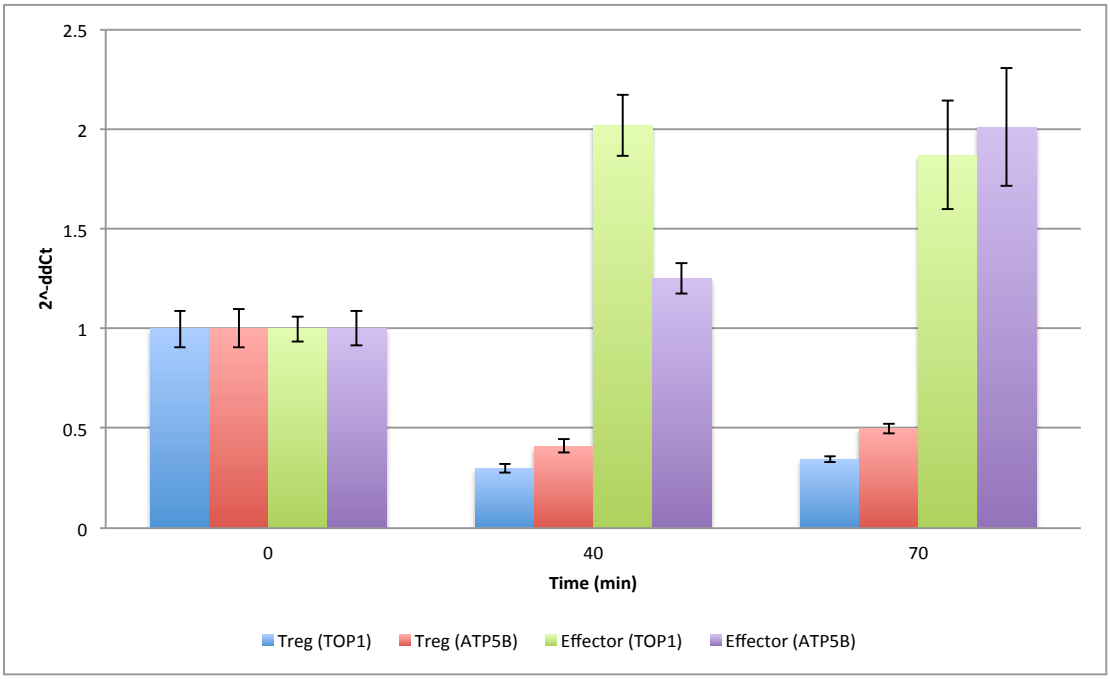


Figure 4.21 Levels of expression of *FOXP3* mRNA in regulatory T cells (Treg) and effector CD4 cells subject to stimulation. Isolated subsets were cultured for 20 to 70 minutes in activating conditions and the levels of *FOXP3* mRNA were measured by RT-PCR. The levels of target mRNA are presented as normalized ratios to levels at the time of isolation using two reference genes (*TOP1*, *ATP5B*). Bars indicate standard error from triplicate measurements.

4.4 Intracellular TGF-β1 in regulatory and effector CD4+ T cells

The lack of correlation between the results of the surface LAP expression experiments and the *TGFB1* mRNA experiments prompted my interest in studying the production of transforming growth factor-β1 (TGF-β1) by regulatory T cells (Treg) and also by their effector counterparts further. Another strategy to study the way TGF-β1 is used by Treg was to look for it in the cells rather than on their surface. Surface expression of TGF-β1 was well known and was also confirmed by my experiments. However, little work had been done on the presence of this cytokine in any of its forms in the intracellular compartment.

For these experiments, I decided to follow the activation experimental settings used for surface TGF-β1 expression. PBMC from a healthy donor, both fresh and activated in the presence of IL-2, were stained for intracellular as well as intracellular LAP and mature TGF-β1, using a matched isotype control in parallel.

The results of this pilot staining of PBMC revealed that LAP was seen extracellularly only on monocytes, as was previously described (**Figure 4.22**). However, intracellular LAP was found in both CD4+ and CD4- lymphocytes, as well as in monocytes. The levels of intracellular LAP were slightly increased after activation only in CD4- lymphocytes. Intracellular LAP levels in CD4+ lymphocytes were similar in freshly isolated and 16h-stimulated cells. Interestingly, in monocytes the levels of intracellular LAP were higher than those found for extracellular LAP. **Figures 4.22** and **4.23** show the results for these experiments. When CD4+ lymphocyte subsets are examined, it became clear that only CD127loCD25+ cells express significant amounts of surface LAP upon stimulation, as was previously observed. However, both CD127loCD25+ and CD127hiCD25- cells seem to have intracellular LAP.

In contrast, surface expression of mature TGF-β1 was not seen for any PBMC subset except for monocytes (**Figure 4.22**). However, intracellular mature TGF-β1 could be identified in all PBMC subsets, albeit with much lower intensity that that seen for LAP, suggesting that its levels are relatively low in comparison with its SLC. Upon activation of the cells, the levels of intracellular mature TGF-β1 seem to increase in lymphocytes, although this increment is only slight. Interestingly, the levels of surface mature TGF-β1 on monocytes seem to be higher than the intracellular levels. Overall, this preliminary experiment showed that LAP is indeed found intracellularly at a resting stage in both lymphocytes and monocytes.

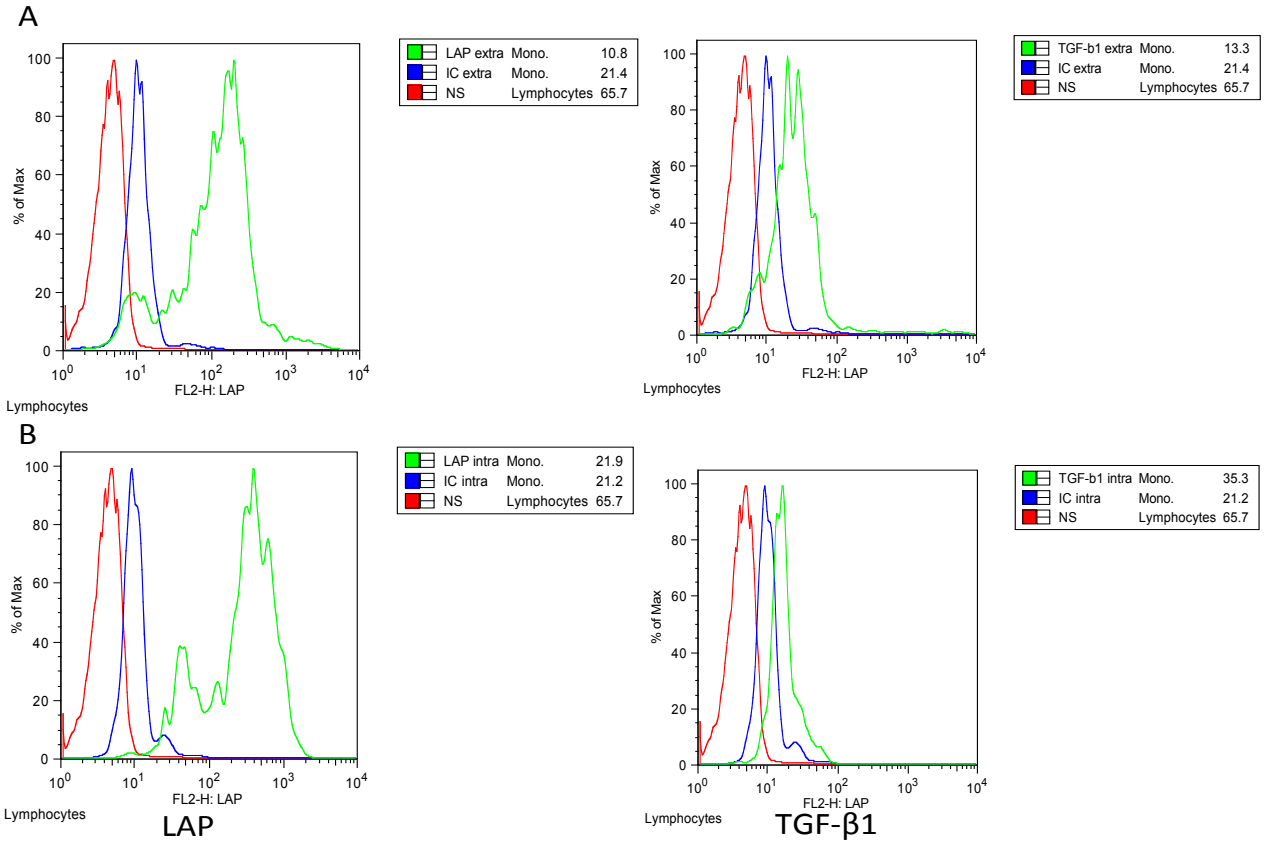


Figure 4.22 Extra and intracellular latency-associated peptide (LAP) and mature transforming growth factor-β1 (TGF-β1) are present in freshly isolated monocytes. Peripheral blood mononuclear cells (PBMC) from a healthy donor were stained for extra (A) and intracellular (B) forms of TGF-β1 at the time of isolation. Monocytes were analysed by gating on live cells and forward and side scatters. Data are shown as histograms. Isotype control (IC) fluorescence intensity for monocytes is also shown with the same gating. Histograms for non-stained (NS) lymphocytes are shown for comparison. Left panels represent LAP staining while those on the right show mature TGF-β1 staining. Top panels: extracellular stainings; bottom panels intracellular stainings.

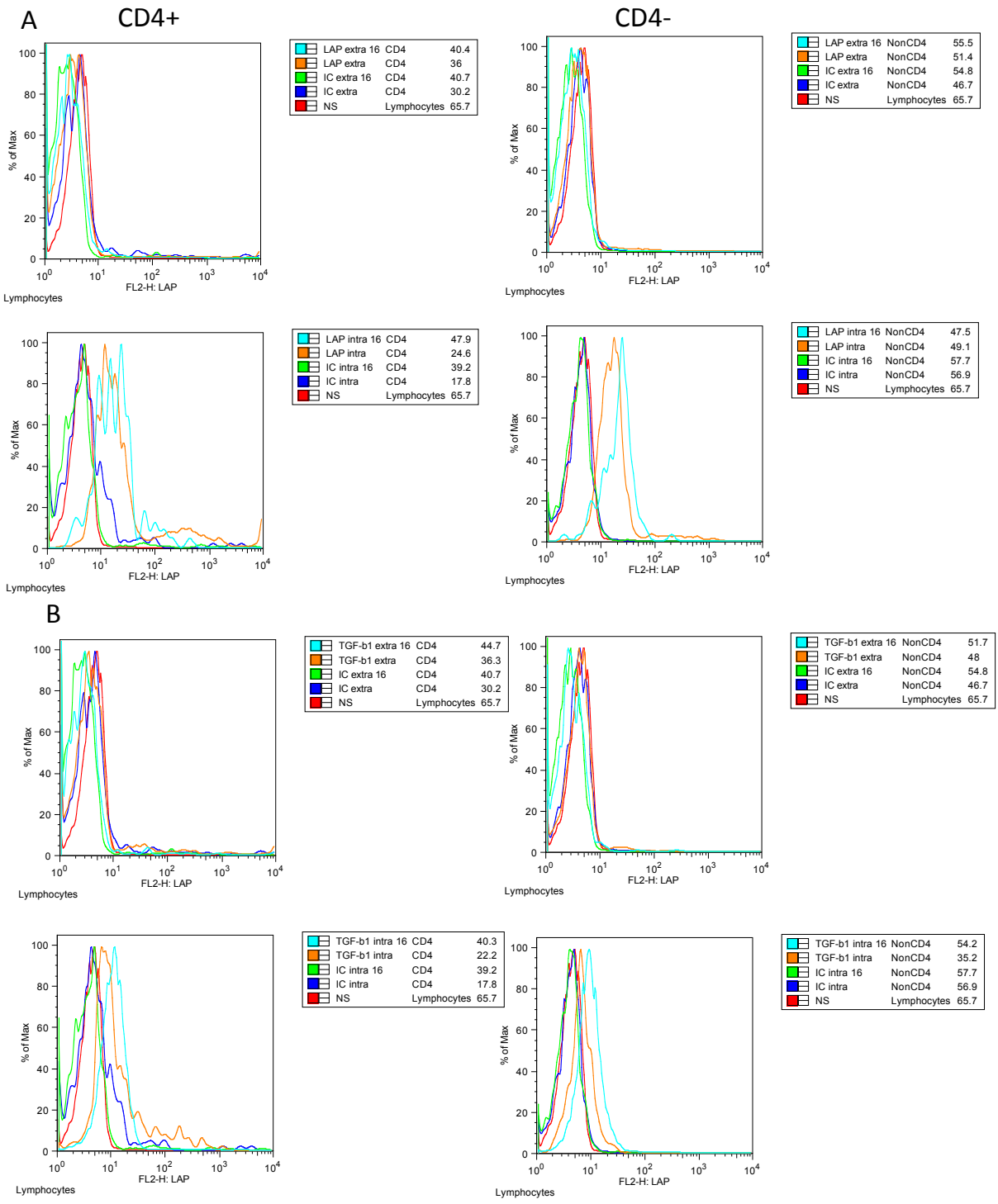


Figure 4.23 Intracellular latency-associated peptide (LAP) and mature transforming growth factor-β1 (TGF-β1) are present in resting and activated lymphocytes. Peripheral blood mononuclear cells (PBMC) from a healthy donor were stained for intracellular forms of TGF-β1 at the time of isolation or after 16 h (indicated on the legend) of activation with soluble antibodies against CD3 and CD28. Lymphocytes were analysed by forward and side scatters, and subsets were gated according to expression of CD4. Data are shown as histograms. Left panels represent CD4+ cells while those on the right show CD4- cells. Top 4 panels (A): extracellular and intracellular LAP; bottom 4 panels (B): extracellular and intracellular TGF-β1. IC, isotype control; NS, non-stained.

In subsequent experiments, Treg and effector CD4+ cells were isolated from PBMC of healthy donors in order to further study the presence of intracellular forms of TGF-β1 in each of the subsets. **Figure 4.24** shows the data for a representative experiment.

The results from this experiment showed that in isolated subsets LAP stores are present at a resting state not only in Treg, but also in CD4+CD25- effector cells. Intracellular stocks of mature TGF-β1 were also present in both cell subsets, but their levels were much lower than those seen for LAP. Nonetheless, both the levels of LAP and TGF-β1 at the time of isolation (0 h) were similar between Treg and effector CD4+ cells. The levels of intracellular mature TGF-β1 increased in effector cells at 16 h of activation, but receded at 30 h. On the contrary, levels of intracellular TGF-β1 in Treg increased at 16 h with respect to 0 h, but kept higher levels at 30 h.

The levels of intracellular LAP in this experiment increased after activation, and continued to be higher at 30 h. MFI increases too. On the contrary, LAP levels in effector cells remain the same. Despite the fact that intracellular LAP levels were higher at 30 h, the levels of surface LAP on Treg decreased with respect to those seen at 16 h of activation. Interestingly, a definitively distinct population with intracellular LAP induction is seen until 30 h post-activation.

This experiment was repeated by activating the cells for 24 and 48 h instead. When this was done, there was evidence for an induction of LAP at 24 h post-activation, which receded at 48 h. On the contrary, mature TGF-β1 levels showed similar levels at the three time points. In the case of effector cells, intracellular LAP induction is also seen at 24 h, but with lower levels than those seen in Treg. However, mature TGF-β1 levels showed a constant increase, and were maximal at 48 h post-activation.

To confirm these results, another experiment using cells from a different healthy donor was set up and included both soluble antibody and plate-bound antibody-mediated activation of the isolated subsets, as well as cells kept in culture with no stimulus. Freshly isolated cells were stained, as were those kept in culture conditions for 16-17 h or 40 h for plate-bound stimulated cells, and for 24 or 48 h for those stimulated with soluble antibodies. **Figure 4.25** and **Figure 4.26**.show the data from this experiment.

As expected, in this experiment surface LAP was strongly induced among the isolated Treg, and not on effector cells. When intracellular LAP was assessed, plate-bound stimulus induced upregulation of LAP stocks in the cells at both 16 and 40 h of stimulation, with the levels forming a plateau. This increase was seen both in terms of percentage of positive cells, as well as in terms of MFI. Effector cells also showed an increase on the levels of intracellular LAP with plate-bound stimulus.

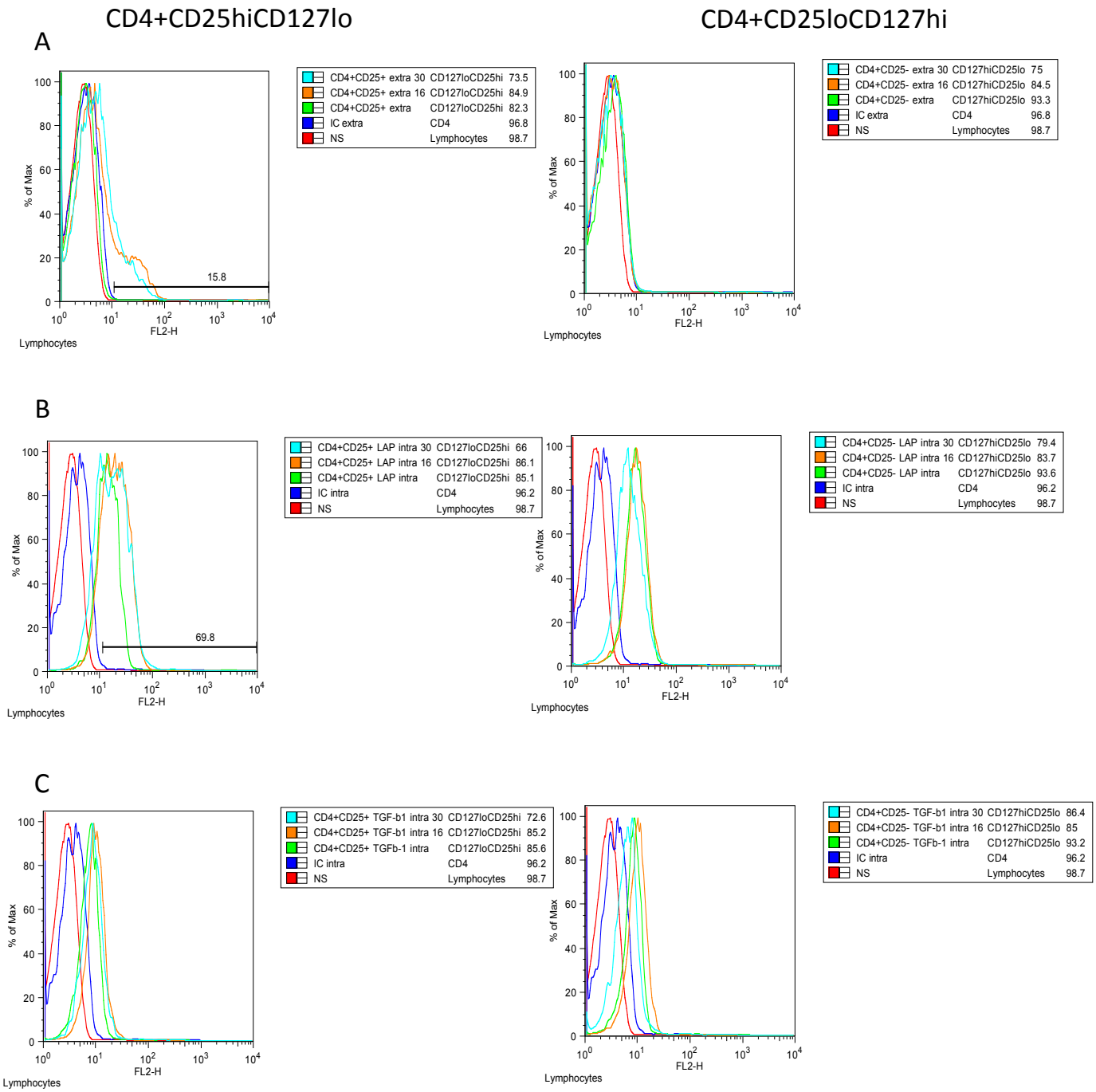


Figure 4.24 Intracellular latency-associated peptide (LAP) and mature transforming growth factor-β1 (TGF-β1) are present in resting and activated regulatory T cells (Treg) and effector CD4+ cells. Treg and effector cells were isolated from peripheral blood mononuclear cells from a healthy donor and were stained for intracellular forms of TGF-β1 at the time of isolation or after 16 or 30 h (indicated on the legend) of activation with soluble antibodies against CD3 and CD28. Lymphocytes were analysed by forward and side scatters, and subsets were gated according to expression of CD4, CD25 and CD127. Data are shown as histograms. Left panels represent CD4+CD25hiCD127lo cells while those on the right show data for CD4+CD25loCD127hi cells. Top 2 panels (A): extracellular LAP; middle 2 panels (B): intracellular LAP; bottom 2 panels (C): intracellular TGF-β1. IC, isotype control; NS, non-stained.

Intracellular LAP levels in freshly isolated Treg were higher than those seen in effector cells, but both subsets had comparable levels at 16 h post-stimulation, especially among cells that showed upregulation of CD25. At 40 h of incubation, similar levels continue to be present, but there seems to appear a clear distinction between 2 populations of Treg: one expressing higher levels of CD25 with higher levels of intracellular LAP, and another showing lower levels for both markers. Moreover, mature TGF-β1 levels inside both cell subsets were again low from the start, and a slight upregulation at 16 h with plate-bound stimulus was seen (**Figure 4.26**), but these levels were lower than autofluorescence at 40 h. Finally, when the cell subsets stimulated with soluble antibodies were assessed, both cell subsets showed a discrete increase in intracellular LAP at 24 h, which was reduced at 48 h, in agreement with previous experiments. Interestingly, a low but noticeable induction of intracellular LAP was also seen in non-stimulated cultured cells, although those levels were much lower than those seen in stimulated cells (**Figure 4.26**).

Overall, the assessment of intracellular forms of TGF-β1 showed that reservoirs of this cytokine are found in all PBMC, especially in CD4+ lymphocytes. Results also showed that most TGF-β1 is in the form of the SLC, since levels of mature cytokine were generally low. TCR stimulation induces only discrete increases in the levels of intracellular LAP.

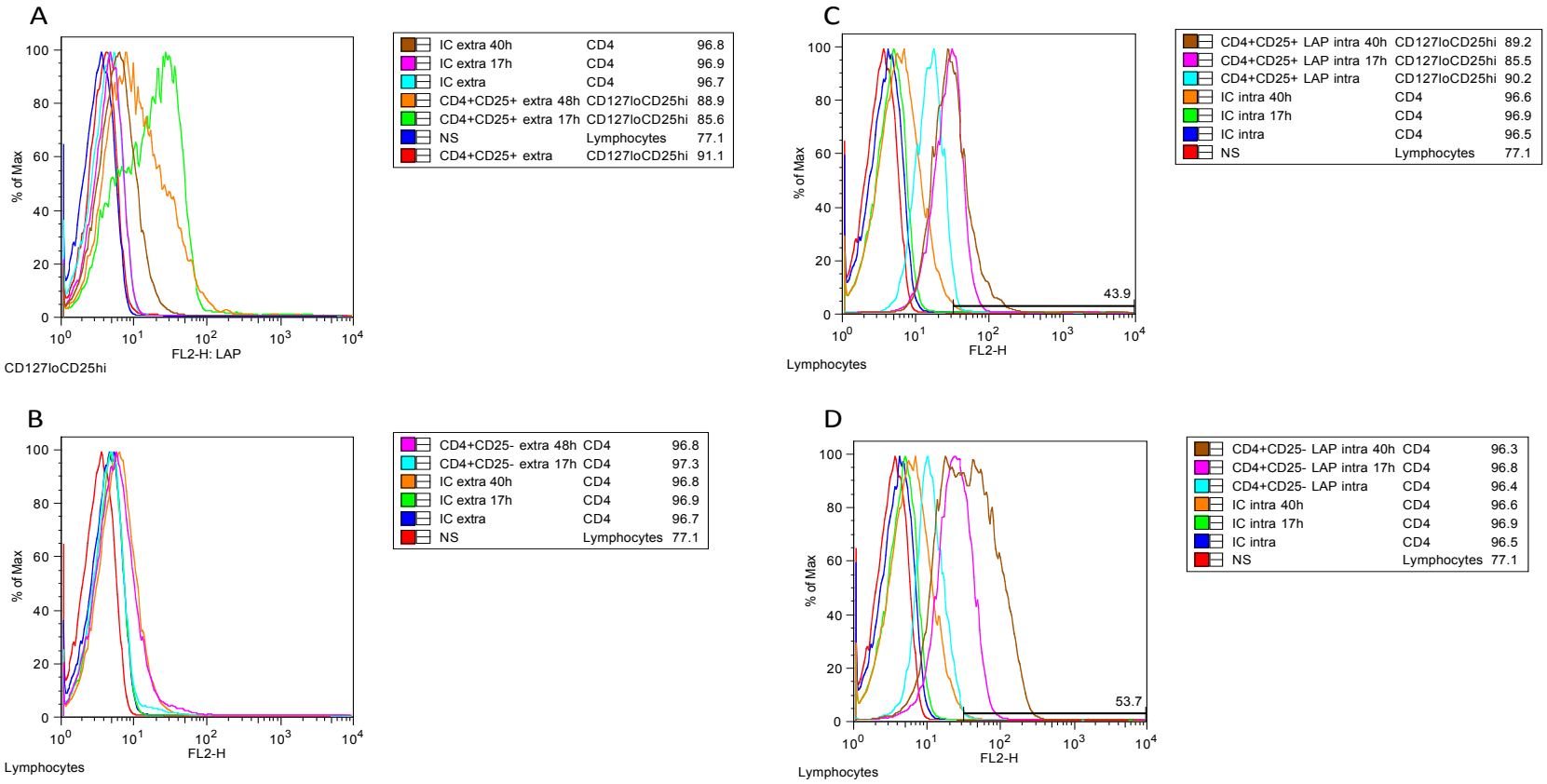


Figure 4.25 Intracellular latency-associated peptide (LAP) continues to be present in resting and plate-bound activated regulatory T cells (Treg) and effector CD4+ cells after 40 h. Treg and effector cells were isolated from peripheral blood mononuclear cells from a healthy donor and were stained for extracellular and intracellular LAP at the time of isolation or after 17 or 40 h of activation with plate-bound antibodies against CD3 and CD28. Lymphocytes were analysed by forward and side scatters, and subsets were gated according to expression of CD4, CD25 and CD127. Data are shown as histograms. Left panels: extracellular LAP on Treg, (A) and effector CD4 cells (B). Right panels: intracellular LAP in Treg (C) and effector CD4 cells (D). IC, isotype control; NS, non-stained; ns, non-stimulated; sol, soluble.

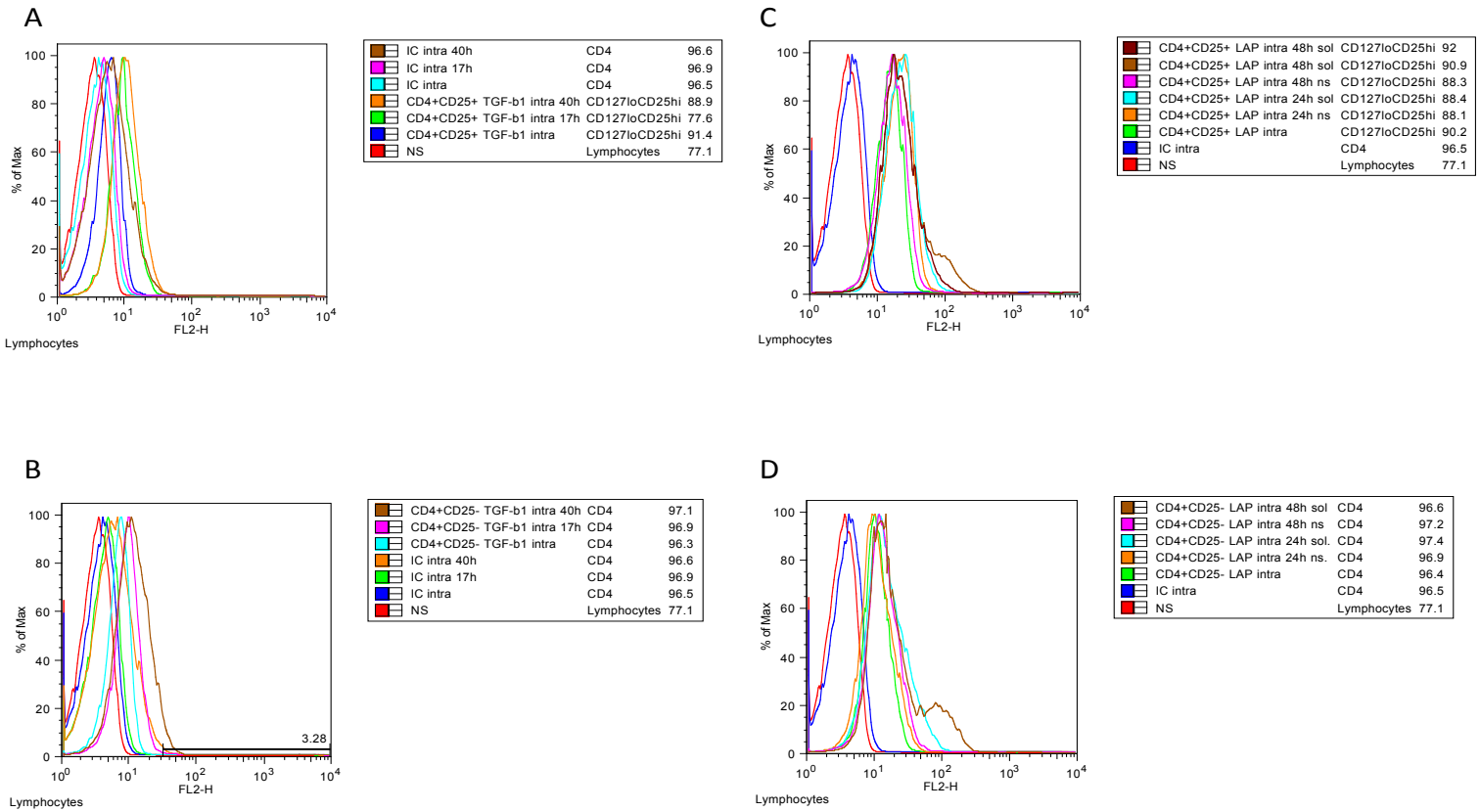


Figure 4.26 Intracellular latency-associated peptide (LAP) and mature transforming growth factor-β1 (TGF-β1) continue to be present in resting and plate-bound activated regulatory T cells (Treg) and effector CD4+ cells after 48 h. Treg and effector cells were isolated from peripheral blood mononuclear cells from a healthy donor and were stained for intracellular forms of TGF-β1 at the time of isolation or after 17 or 40 h (plate-bound) or 24 or 48 h (soluble) of activation with antibodies against CD3 and CD28. Lymphocytes were analysed by forward and side scatters, and subsets were gated according to expression of CD4, CD25 and CD127. Data are shown as histograms. Panels show: intracellular TGF-β1 in plate-bound activated Treg (A) and effector CD4 cells (B), and intracellular LAP in Treg (C) and effector CD4 cells (D) activated with soluble antibodies. IC, isotype control; NS, non-stained; ns, non-stimulated; sol, soluble.

4.5 Discussion

In this chapter, I have presented my findings regarding the surface expression of latent TGF-β1 on Treg upon stimulation and the potential role of the *TGFB1* +29T>C in the amount of LAP that is expressed by these cells. The exclusive expression of LAP on the surface of Treg was confirmed in my experiments, and a trend towards higher expression levels was found for cells bearing a +29 C allele. Also, I presented my findings on the kinetics of *TGFB1* mRNA in isolated Treg and effectors upon stimulation, and the presence of intracellular reservoirs of mostly latent TGF-β1, both in resting Treg and effector CD4+ cells.

4.5.1 Expression of latency-associated peptide (LAP) upon T cell receptor (TCR) stimulation

When the LAP staining levels at the times of isolation were assessed, a significant proportion of CD4+ and CD4- cells within the unmanipulated PBMC showed LAP staining. However, this proportion was reduced progressively in the subsequent derived fractions. In the final isolated CD4+CD25- and CD4+CD25+ subsets, a minority of the cells showed LAP staining, and the percentage was usually higher for the CD4+CD25+ cells than for the CD4+CD25- cells but their levels varied between experiments, with almost no LAP+ cells in some cases. These observations, together with the fact that unmanipulated non-stimulated PBMC gradually lose their LAP staining overtime in culture, suggest the presence of externally rather than internally produced LAP on the fresh PBMC. This staining would be eliminated in the isolated fractions, which, in turn, show the real staining. Since platelets are major carriers of LAP (Assoian *et al.*, 1983) it is possible that freshly isolated PBMC are covered with platelets, which are progressively shed in culture or washed off during the numerous washes performed during the isolation of CD4+CD25- and CD4+CD25+ cells. The specific platelet counts for each donor as well as the amount that persist after the ficoll gradient might influence these levels. This possibility should be taken into account when interpreting LAP staining on PBMC or derived subsets. Nonetheless, monocytes have been reported to also be able to localize latent TGF-β1 on their surface using thrombospondin and CD36 as anchors (Yehualaeshet *et al.*, 1999). These surface complexes are activated by plasmin.

Upon activation through TCR stimulation, surface LAP was upregulated solely on cells that also expressed CD4, CD25, and that had low levels of CD127. This correlates with previous reports showing LAP induction in Treg (CD4+CD25+CD127loFOXP3+) and not in other

lymphocyte subsets (Assoian *et al.*, 1983; Nakamura *et al.*, 2001; Chen *et al.*, 2008; Tran *et al.*, 2009). Thus, a gating strategy for the assessment of LAP by Treg was implemented. By restricting the assessment to cells that are CD4+CD25+CD127lo, the effect of similar but nevertheless varying proportions of contaminating CD4+CD25+CD127hi cells was reduced. In this way, a more accurate measurement of the proportion of LAP+ cells and their staining intensity was sought.

FOXP3+ is still the best-known marker for naturally occurring Treg (Fontenot *et al.*, 2003; Hori *et al.*, 2003). A 90% correlation between low expression of CD127 and FOXP3 in CD4+CD25+ lymphocytes has been clearly stated (Liu *et al.*, 2006; Seddiki *et al.*, 2006). Based on these observations and the fact that the intranuclear staining protocol for FOXP3 is labour-intensive, involves fixation of the cells, and requires higher starting numbers of cells, FOXP3 staining in parallel with LAP staining was done only for a number of experiments. However, despite there being one report describing human CD4+LAP+FOXP3- cells (Gandhi *et al.*, 2010), in my experiments all the cells that showed surface LAP upon TCR stimulation were FOXP3+. With these experiments, the surface staining gating strategy was validated since LAP+ cells generated from activated isolated CD4+CD25+ after 24h of incubation were FOXP3+, and >95% of the CD127lo cells were indeed FOXP3+.

The expression of LAP on Treg upon TCR stimulation with soluble antibodies against CD3 and CD28 showed a peak at 24h followed by a return to levels similar to those before activation and remained constant up to 96h of culture. Nakamura *et al*. reported that LAP appears at 24h after TCR stimulation (Nakamura *et al.*, 2001). However, their activation system differed (it included irradiated non-T cells) and the levels of LAP generated were constant over time. Tran *et al*. reported that freshly isolated murine Treg that are stimulated express increased levels of LAP between 24 and 48 h of stimulation, and that these levels disappear by 72 h (Tran *et al.*, 2009). The fact that soluble antibody stimulation of the TCR on Treg generates a peak in LAP expression suggests that it's well-recognized function in immunomodulation (Nakamura *et al.*, 2004; Chen *et al.*, 2008) occurs mainly in these first hours of activation. However, it is important to note that this observation was made in a simplified *in vitro* system, and thus *in vivo* variables could modify this kinetics. The absence of this induction in effector CD4 cells correlates with the paradigm of immune regulation being restricted to the CD4+FOXP3+ naturally occurring Treg.

The activation of both Treg and effector CD4 cells proved to be very efficient. Once again, LAP induction was observed exclusively on Treg cells and its levels in terms of the percentage of LAP+ cells and fluorescence intensity were much stronger and sustained

than those obtained with soluble antibodies. This strategy was important in order to confirm the viability of the cells, but, because it exceeds any physiological stimulus, it was not selected for comparison between groups.

The level of LAP+ Treg at 24h after TCR stimulation appears to be modified by the presence or absence of a +29C allele in *TGFB1*. Even though it did not reach statistical significance, *TGFB1* +29 T>C genotypes TC and CC showed results which suggested higher percentages of LAP+ cells when compared to TT individuals. This observation prompted the analysis of the proportion of LAP+ cells between individuals with genotypes bearing this allele and TT individuals. This TC-CC vs. TT comparison yielded a trend towards a higher percentage of LAP+ cells in individuals bearing a C allele. The fact that these results did not reach statistical significance might be due to a number of sources of variability between independent experiments and to a limited number of experiments per genotype. However, regardless of the statistical aspect, results suggest variation that seems to be correlated with *TGFB1* +29 T>C polymorphism.

Several sources of variation in these experiments could blur the differences due to polymorphisms. If that is the case, increasing the number of experiments could help reduce this effect and make the differences clearer. Nevertheless, a more likely explanation could be the fact that allelic variation exerts a stronger effect than particular SNPs individually. Some 18 polymorphisms have been identified in the *TGFB1* promoter region and exon 1 (Shah *et al.*, 2006). Many of those have been associated with effects on the production of this cytokine. Moreover, several alleles formed by combinations of these polymorphisms have been recognized and grouped in 3 phyla according to the genotypes present at nucleotides -1347 and +29 (Shah *et al.*, 2006). Taking this into account, there is a possibility that within the *TGFB1* +29T>C genotype and variant groups variation in other *TGFB1* SNPs influences the effect of this particular polymorphism. Consequently, one alternative to further study this question would be to analyse other polymorphic positions in order to unravel the effects that are readily seen in Treg LAP production upon TCR stimulation. However, such an analysis would require much greater numbers of experiments in order to give statistical power in a setting with multiple genotype combinations.

4.5.2 Functional impact of *TGFB1* +29 T>C polymorphism

The presence of a +29C allele causes an amino acid change from Leu to Pro on position 10 of the TGF-β1 pre-pro-peptide. The effect of this change on the secretion of this protein through the endoplasmic reticulum has not been specifically addressed. However, it is possible to infer that the replacement of a hydrophobic residue (Leu) for a small, neutral,

cyclic and rigid one (Pro) will significantly affect the macromolecular properties of that polypeptide chain. In fact, Pro acts as a structural disruptor in the middle of regular secondary structure elements such as alpha helices and beta sheets (Nelson, 2008). A mutation occurring close to this position and involving the insertion of three Leu (LLL12-13ins) has been associated with the Camurati-Engelmann disease (Janssens *et al.*, 2000). This disease is characterised by increased activity of TGF-β1. In this case, a decreased amount of TGF-β1 is secreted, owing to an extension of the central hydrophobic stretch of the signal peptide and making the excision of the signal peptide difficult. This would lead to intracellular accumulation of aberrant cytokine and increased intracellular signaling (Janssens *et al.*, 2003). Thus, the polymorphism at *TGFB1* +29 T>C, within the hydrophobic alpha helix of the signal peptide, could alter the overall hydrophobicity of the core transport sequence and disrupt the alpha-helical structure of the region, thereby altering its ability to direct protein transport across the endoplasmic reticulum (Awad *et al.*, 1998), potentially favoring secretion by eliminating one of the Leu in this region.

4.5.3 Clinical impact of *TGFB1* polymorphisms

Other studies have also reported the importance of the presence or absence of the +29C allele rather than the specific genotypes on the effect of this polymorphism on TGF-β1 production (Suthanthiran *et al.*, 2000; Dunning *et al.*, 2003). For example, Dunning *et al.* (2003) found that carriers of the +29C (Pro10) allele secreted higher quantities of TGF-β1 *in vitro* and had increased incidence of invasive breast cancer. Also, the +29C allele has been associated with increased risk of suffering conditions which may be exacerbated by more TGF-β1 production, such as liver cirrhosis (Gewaltig *et al.*, 2002), hypertension (Niu, 2011), chronic obstructive pulmonary disease (Liu *et al.*, 2010), protection from osteoporosis (Yamada *et al.*, 1998), advanced colorectal carcinoma (Berndt *et al.*, 2007), esophageal squamous cell carcinoma (Wei *et al.*, 2007), diabetic nephropathy (Jia *et al.*, 2011), or skin fibrosis during chronic GVHD following HSCT (Liem *et al.*, 1999). However, some studies have had different results, linking the +29T allele to higher TGF-β1 serum levels (Hinke *et al.*, 2001), higher production and fibrosis (Wang *et al.*, 2008), or finding no association (Hoffmann *et al.*, 2001).

The results presented here are also relevant to studies linking the *TGFB1* +29C allele with increased susceptibility of aGVHD after HSCT (Hattori *et al.*, 2002) (Noori-Daloii *et al.*, 2007) (Berro *et al.*, 2010) (Shah *et al.*, 2009). In general, the presence of a +29C allele has been associated with worst outcome by an increase in aGVHD in HSCT.

There may be multiple ways in which TGF-β1 and the variation of its levels caused by genetic polymorphism could influence HSCT outcome, as it is produced by a variety of cells

and has pleiotropic effects, dependent upon its concentration and the type of target cell. These properties range from being both pro-inflammatory and immunosuppressive to promoting fibrosis (Leffell *et al.*, 2001). Since the main sources of TGF-β1 are epithelial cells and Treg, it is plausible that either the donor (T cell) or the recipient (epithelial cell) genotypes could play a role in and be predictive of aGVHD development (Shah *et al.*, 2009). The association of a polymorphism causing higher production of TGF-β1 in Treg and aGVHD following HSCT could have different interpretations. Even though Treg are expected to counteract the inflammatory events during the pathogenesis of GVHD, the extent of the inflammation and tissue damage prompted by the injury to tissues following the conditioning regimens to which patients are exposed, could overwhelm any control by these cells. Moreover, production of TGF-β1 from these cells in a heavily inflammatory context, where high concentrations of cytokines such as IL-1β and IL-6 are expected, could paradoxically lead to the induction of Th17 cells (Manel *et al.*, 2008; Volpe *et al.*, 2008; Yang *et al.*, 2008) as significant plasticity of FOXP3+ Treg has been identified (Koenen *et al.*, 2008). Th17 cells have indeed been associated with the pathogenesis of GVHD (Chen *et al.*, 2007; Carlson *et al.*, 2009; Kappel *et al.*, 2009). In line with these possibilities, although membrane bound TGF-β1 (LAP) has been characterised in these experiments, the effect of *TGFB1* polymorphism on the amount of secreted TGF-β1 remains to be tested. The fact that the MFI of LAP+ Treg did not differ between *TGFB1* +29 T>C genotypes could be interpreted as a lack of effect of the polymorphism on the amount of LAP on a single-cell basis. However, since LAP on Treg is attached to GARP (Stockis *et al.*, 2009; Tran *et al.*, 2009) it is possible that the latter limits the amount of LAP captured on the cell membrane (and thus its levels), leaving open a possibility that secreted LAP levels differ more overtly than those bound to the Treg membrane. However, GARP has been reported to impair TGF-β1 secretion by Treg, confining the cytokine to their membrane (Wang *et al.*, 2012). Alternatively, normal variation in staining conditions between experiments might have also affected the MFI on these cells.

Alternatively, as previously mentioned, the source of the pathogenic TGF-β1 in an HSCT setting could be attributed to cell types other than Treg, and thus studies in other systems and settings are likely to help elucidate this.

4.5.4 The expression of *TGFB1* mRNA

The RT-PCR experiments analysing at the levels of *TGFB1* mRNA in isolated Treg and effector cells subject to TCR activation showed puzzling results. Based on the observations of the LAP expression experiments, I expected to be able to find an induction of these mRNA levels in response to TCR stimulation, at least in Treg. However, the results showed

the opposite situation. *TGFB1* mRNA levels in Treg subject to activation showed a decrease in their levels very early after the start of the culture. At first, it appeared that the induction might be taking place at much earlier time points that those used for assessment in the early experiments. Taking into account that changes in cytokine mRNA levels have been reported to be very transient and quick in some cases (Abbas, 2012), there was a chance that this was the case. However, as shown before, a decrease in mRNA levels was seen even at less than one hour of incubation in activating conditions. In one experiment, it was seen as early as 20 minutes of activation. There remains the possibility that the upregulation of *TGFB1* mRNA occurs within 20 minutes, however, this seems unlikely. Of note, a report by Koenen and collaborators showed that stimulation of human Treg, characterised as CD4+CD25hiFOXP3+CD127-CD27+, did not enhance mRNA levels of *TGFB1* (Koenen et al., 2008).

Disparities between mRNA and protein levels have been reported for several genes. Specifically for *TGFB1*, discrepancies between the levels of message and of protein are well described for other cell types. For example, resting B cells have been reported to express TGF-β1 mRNA, but secrete very little protein (Kehrl et al., 1986). Conversely, when these cells are stimulated with *Staphylococcus aureus* components, they produce high amounts of TGF-β1 with little change in mRNA levels (Kehrl et al., 1986). An early report by Assoian *et al*. showed that *TGFB1* mRNA levels in resting and activated monocytes were similar, but TGF-β1 was only secreted by activated monocytes (Assoian *et al.*, 1987). Human PC-3 prostate adenocarcinoma cells were reported to secrete predominantly TGF-β2 and little TGF-β1 despite the levels of mRNA of the former being lower than those of the latter (Ikeda *et al.*, 1987). Moreover, rat pheochromocytoma PC12 cells are known to secrete large amounts of TGF-β1, but possess mRNA levels that are much lower than those found in other cells lines such as PC-3 cells (Kim *et al.*, 1992). In keratinocytes, stimulation with retinoic acid produces an increase in *TGFB1* mRNA transcription, but no increase in the secretion of TGF-β1 (Glick *et al.*, 1989). The opposite is true of fibroblasts treated with progestin gestodene, in which, secretion occurs without changes in mRNA levels (Colletta *et al.*, 1990). Moreover, in a study looking at the induction of TGF-β1 by treatment of human renal proximal tubule cells with pro-inflammatory cytokines IL-1 and TNF-α, the authors found that despite the induction of TGF-β1 mRNA following stimulation with either of these cytokines, neither stimulus increased TGF-β1 protein synthesis or release (Phillips *et al.*, 1996). In another report in which a murine model of chemical carcinogenesis of the skin was used, papillomas and carcinomas induced *in vivo* had elevated levels of TGF-β1 mRNA within basal keratinocytes, but did not contain significant levels of TGF-β1 protein within the tumor. The authors postulated that the tumor evaded

TGF-β1-controlled negative growth regulation by altered translational and/or post-translational processing mechanisms of this growth factor (Fowlis *et al.*, 1992).

The *TGFB1* promoter is known for post-transcriptional regulation of the production of its cytokine (Kim *et al.*, 1992). *TGFB1* mRNA is poorly translated, and contains a long, GC-rich 5'UTR (Derynck *et al.*, 1985; Kim *et al.*, 1989). This 5'UTR is known to have different length variants, and to be able to negatively control translation by stem-loop formation (Kim *et al.*, 1992). In fact, there is evidence that a 137-nucleotide (nt) region of the *TGFB1* 5'-UTR potently inhibits the expression of heterologous reporter genes by inhibiting mRNA translation and that it involves the specific binding of a cytosolic factor (Romeo *et al.*, 1993). Deletion or mutation of the potential stem-loop-forming region abolished binding of this factor and partially restored reporter gene production (Romeo *et al.*, 1993; Jenkins *et al.*, 2010). Moreover, polysome analysis of synthetic TGF-β1 mRNA transfected into MCF-7 cells suggested that the cell contains a limited pool of trans-acting factors that interact with its 5'-UTR and confer it with inhibitory capacity *in vivo* (Allison *et al.*, 1998). More specifically, Y box binding protein-1 has been found to be physically associated with *TGFB1* mRNA, and this association is reduced when translation of the mRNA is activated (Fraser *et al.*, 2008). Furthermore, the presence of microRNA-mediated regulation of TGF-β1 production has been identified within the 3'-UTR of *TGFB1* mRNA (Martin *et al.*, 2011). Two different 3'-UTRs have been described, both with translational inhibitory properties. The microRNA miR-744 has been shown to be able to bind these regions and repress the production of TGF-β1 (Martin *et al.*, 2011). Thus, it is evident that the regulation of *TGFB1* expression occurs at various stages, especially post-transcriptionally. This complexity in its regulation, which is compatible with TGF-β1's central role in many crucial biological processes, must consequently be taken into account when attempting to interpret the various experiment presented in this chapter.

One aspect that was constant in these RT-PCR experiments was that the effector CD4+CD25- cells showed results that were different from those of Treg. In most cases, an induction of *TGFB1* mRNA was confirmed in these cells as opposed to their regulatory counterparts. This might seem at odds with the fact that Treg were the sole subset to express surface TGF-β1 upon stimulation. However, it is important to remember that this cytokine can indeed be produced by most leukocytes, especially by T lymphocytes. Induction of *TGFB1* mRNA in human T cells as early as 2 hours after stimulation and reaching a plateau that continued up to 48 hours has been reported for a long time (Kehrl *et al.*, 1986). In this report, the secretion of TGF-β1 was nonetheless delayed and gradual, only reaching its maximal levels after 72-96 h of culture. However, these cells were total T cells, and hence could be reflecting the behavior of effectors, which would be the vast

majority of the cells. Moreover, in this report, PHA was used as the stimulus, which strongly activates T cells. Furthermore, the expression of *FOXP3* mRNA in activated effectors has been observed previously (Morgan *et al.*, 2005).

It remains unclear though why these cell subsets show this differential regulation of *TGFB1* mRNA. One possibility is that, due to the close relationship between Treg and TGF-β1 in terms of their development, maintenance, induction and function, Treg possess a specific kinetics of TGF-β1. Treg possess the capacity of expressing this cytokine on their surface, but they also express the receptor for TGF-β1 on their membranes. This co-expression of signal and receptor might mean that an autologous signaling by TGF-β1 is fundamental for Treg homeostasis. Autologous TGF-β1 signaling has indeed been described in other contexts such as the regulation of haematopoiesis (Ruscetti *et al.*, 2005). Consequently, it would be conceivable that the regulation of the expression of this cytokine is specific to this cell type. In view of the results, it is possible that TGF-β1 plays a different role in effector and Treg states: it would be constantly active and maintaining, thus requiring no upregulation of mRNA levels upon activation, and would be a de novo control mechanism for effector CD4 cells, which would increase their levels of mRNA in order to eventually control the expansion elicited upon them by the activating stimulus. In fact, in a study examining the role of TGF-β1 in *in vitro* suppression of human effector T cell function and proliferation, Oberle and collaborators found that the addition of anti-TGF-β1 monoclonal Ab to cultures containing effector T cells and APC led to an increased proliferation, suggesting that TGF-β1 produced by the effectors themselves inhibits their proliferation (Oberle *et al.*, 2007).

4.5.5 Intracellular TGF-β1 in regulatory T cells (Treg) and effector CD4 cells

The intracellular staining of latency-associated peptide (LAP) and TGF-β1 in peripheral blood mononuclear cells (PBMC) revealed the presence of widespread deposits of mostly latent TGF-β1 among different cell subsets. LAP staining was found to be positive in both monocytes and lymphocytes, and both in CD4+ and CD4- fractions of the latter. Mature TGF-β1 was also found in most subsets, but its levels were comparatively low. This agrees with the fact that the formation of the SLC occurs quickly inside the cell shortly after synthesis of the pre-pro-TGF-β1, and the translocation of the pro-TGF-β1 to the lumen of the endoplasmic reticulum. The low amounts of mature TGF-β1 would be those molecules that were in process of forming the SLC with LAP, or potentially misfolded complexes. In any case, the fact that the intracellular levels of LAP are many times higher than those of mature TGF-β1 agrees with the necessity of precise regulation of the activation of the cytokine, and the quick formation of the SLC upon the action of furin enzymes on the

cytokine's precursor. The Treg specific expression of GARP (Wang *et al.*, 2008; Probst-Kepper *et al.*, 2009) would then confer them with the unique characteristic of surface LAP expression upon TCR stimulation. Indeed, *GARP* is a gene exclusively induced in CD4+CD25hi cells upon stimulation (Probst-Kepper *et al.*, 2009). However, GARP mRNA was also found in Th clones that did not express the protein, suggesting post-transcriptional regulation mechanisms also play a role in its expression (Stockis *et al.*, 2009).

Of note, the extracellular staining for mature TGF-β1 on Treg was always negative, despite the presence of LAP (or the SLC) on the same cells. Nakamura *et al*. reported the presence of TGF-β1 (and of LAP as well) on stimulated murine CD4+CD25+ cells, but used a different antibody for TGF-β1, and this factor could be the reason for this discrepancy (Nakamura *et al.*, 2001). In any case, it has been shown that the surface-bound TGF-β1 is essentially folded in the SLC after processing by furin (Oida & Weiner, 2010), thus raising the possibility that the epitope on the mature TGF-β1 that the antibody used by myself recognizes is hidden by LAP.

Intracellular latent TGF-β1 reservoirs in murine Treg have only recently been described (Edwards *et al.*, 2013). However, the presence of similar stores of SLC in human Treg and, more importantly, in human effector CD4+ cells have not been reported yet. The fact that intracellular LAP was found both in Treg and in effector CD4+ cells but only Treg express it on their surface upon stimulation raises the question of the purpose of those deposits in effector cells. One possibility is that intracellular TGF-β1 in effector cells is ready for secretion. However, since the levels of TGF-β1 in culture supernatants of stimulated CD4 lymphocytes are reported to appear until after 72-96 h after stimulation (Kehrl *et al.*, 1986; Oida & Weiner, 2010), it would appear that most of this secretion occurs by means of de novo synthesis of the cytokine. On the contrary, the preformed intracellular TGF-β1 could be used by effectors for steady state secretion and the maintenance of tolerance. Indeed, TGF-β1 has been shown to play a fundamental role in the maintenance of constitutive inhibition of resting CD4+ T cells (Classen *et al.*, 2007).

TCR stimulation resulted in only discrete increases in the amount of intracellular mature TGF-β1 and LAP. This could mean that, since the cells have preformed cytokine inside, these intracellular deposits would be mobilized, reducing the need for the novo synthesis. It is important to remember that these experiments were performed in short term cultures, and that, as it has been mentioned before, CD4 effector cells start noticeable secretion of TGF-β1 not before 72-96 h of stimulation (Kehrl *et al.*, 1986; Oida & Weiner, 2010). Consequently, a later increase of intracellular levels cannot be ruled out. Alternatively, since both Treg and effector CD4 cells either externalize or secrete the SLC

molecules, a slight but constant increase in the intracellular stocks of TGF-β1 could reflect a steady state of expulsion/activation of the cytokine, preventing larger accumulations of molecules inside the cells.

The fact that Treg have preformed SLC at a resting state would also be reconcilable with the fact that no increase in *TGFB1* mRNA levels was seen upon TCR stimulation. In the context of a normal activation level, the cell would not depend largely on the synthesis of new SLC molecules, and perhaps the mRNA molecules already present can provide the templates for the slight increase in the levels of TGF-β1 production. Hence, no more mRNA would be needed, and it would be in fact reduced as a negative control loop caused by the activating stimulus.

Under this model, in the context of an immune response, Treg would be activated, mobilize preformed and newly synthesized latent TGF-β1 for surface expression that would be activated and signal to target cells (i.e. effectors or APC) and to themselves. Autologous TGF-β1 signaling would continue to be the main force driving the maintenance of their anergy and regulatory phenotype. In fact, ectopic expression of GARP in Th cells caused the development of anergy and the expression of FOXP3+ (Probst-Kepper *et al.*, 2009). This TCR stimulus would cause the reduction of the expression of TGF-β1 mRNA, and this would contribute to limit the expression of surface LAP, down-regulating its levels after 24 h of stimulation with soluble antibodies. Moreover, autocrine TGF-β1 signaling by Treg has been shown to reduce the expression of GARP (Zhou *et al.*, 2013), thus enhancing the control of the surface TGF-β1 response. In contrast, effector cells would initiate their activation aiming at expansion, and would overcome the steady-state inhibition by means of the TCR stimulus. TGF-β1 production by effector cells would be a late byproduct of the expansion process, possibly aimed at curbing its extent in the longer term. In summary, the regulatory programme, know to be established since the early developmental stages in the thymus, as well as the intricate relationship between Treg and TGF-β1, appear to modify the way in which these cells manage their production of this cytokine both in resting and in activating conditions.

In conclusion, the results from these experiments suggest that *TGFB1* +29T>C seems to confer a higher capacity of expression of surface TGF-β1 upon TCR stimulation for Treg that bear a C allele. However, TCR stimulation does not seem to cause upregulation of *TGFB1* mRNA in Treg. Finally, intracellular deposits of TGF-β1, mainly as the SLC, have been confirmed to exist in human Treg, and have also been discovered to be present in human effector CD4+ T cells. Moreover, the effect of other polymorphisms in *TGFB1*'s regulatory region and exon 1 should be explored in more detail.

Chapter 5. The effect of *TGFB1* +29T>C polymorphism on the suppression of immune responses by regulatory T cells

5.1 Introduction and aim

One of the main objectives of this project was to analyse the functional effect of the *TGFB1* +29T>C polymorphism in the way that CD4 Treg exert their regulatory functions. Apart from the potential effect observed for *TGFB1* +29T>C on the amount of surface latent TGF-β1 expressed by Treg, I decided to determine if this polymorphism would have an effect on the way that cells with one genotype or the other control the immune response.

As has been discussed before, TGF-β1 production is one of the mechanisms proposed to be central to Treg function in the control of immune responses (Nakamura *et al.*, 2001; Chen & Wahl, 2003; Nakamura *et al.*, 2004). TGF-β1 acts on different immune cells and does so in different ways. Consequently, there are a number of potential assays in which the effector function of immune cells, and the effect of Treg on this, could be measured both *in vitro* and *in vivo*. *In vitro* assays include: inflammatory cytokine production, immunoglobulin production, NK cell destruction of target cells, inhibition of activation surface marker expression, and inhibition of the proliferative response, among others. The most common assay used for the assessment of the suppressive capacity of Treg is the *in vitro* proliferation (or suppression) assay (Collison & Vignali, 2011; McMurchy & Levings, 2012; Tran, 2013). In this system, the ability of Treg to impair the activation and expansion of a population of responder (effector) cells upon stimulation is tested. The end-point of these assays is always proliferation, measured by different techniques such as incorporation of radio-labeled nucleotides to DNA synthesized toward cell division, or the dilution of an intracellular dye by cell division.

In this chapter, I present the data produced during the optimisation of an *in vitro* autologous suppression assay, as well as the data from the testing of the effect of the *TGFB1* +29T>C on Treg suppressive capacity. In addition, I present the development of a variation of the suppression assay in which the kinetics of LAP expression by Treg are shown in the context of the suppression of an immune response.

5.2 The effect of *TGFB1* +29T>C on the suppressive capacity of regulatory T cells (Treg)

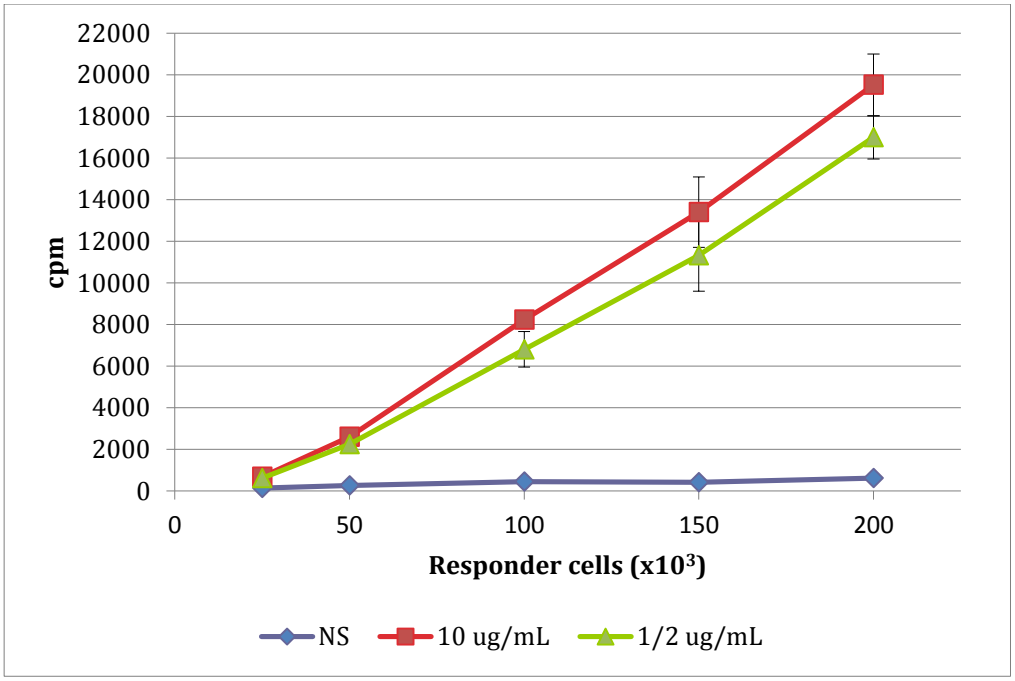
The autologous suppression assay model was adopted for the testing of the hypothesis that the *TGFB1* +29T>C SNP affects the capacity of Treg to suppress the expansion of effector CD4 cells through TCR stimulation. The incorporation of tritiated thymidine by responder cells during the synthesis of DNA was chosen as the endpoint for the assessment of the proliferation of these cells.

The first step for the implementation of this technique in subsequent experiments was to optimise the assay by finding the best conditions for both proliferation and suppression. The variables tested for this optimisation were the number of responder (i.e. effector CD4) cells per well, the ratio of Treg to effectors, the concentration of stimulating antibodies, the presence of accessory cells, and the presence or absence of additional IL-2. In a series of experiments, these variables were tested in autologous co-cultures run for 72-96 h and with the addition of tritiated thymidine for the last 16 h of culture as described in Chapter 2.

In the first series of experiments, the concentration of antibodies against CD3 and CD28 were tested on different numbers of responder cells per well. In these experiments, responder PBMC ranging from $25x10^3$ to $200x10^3$ were incubated in 200 µL cultures in round-bottom 96-well plates. These cells were stimulated with antibody concentrations of either 1 and 2 µg/mL or both 10 µg/mL for anti-CD3 and anti-CD28, respectively. Representative results for these experiments are shown in **Figure 5.1**. As shown in this figure, the proliferation of responder cells increased proportionally to the number of cells included in the wells. Also, the minimum cell number that provided a clear difference between the stimulated and non-stimulated cells was $100x10^3$. Lower responder cell numbers would not allow for proper differentiation of the proliferative response, while after $150x10^3$ there appeared to be a plateau of proliferation capacity. Moreover, there was not a significant difference between the two antibody concentration combinations, despite the increase of 10 or 5 times in the amount of soluble antibody.

These experiments were then repeated using isolated CD4 cells as responders in order to determine if their proliferation would develop similarly to total T cells in PBMC and also to determine the effect of the presence of other cell types such as monocytes or B cells. The results for the isolated CD4 responding cells are shown in **Figure 5.2**.

(A)



(B)

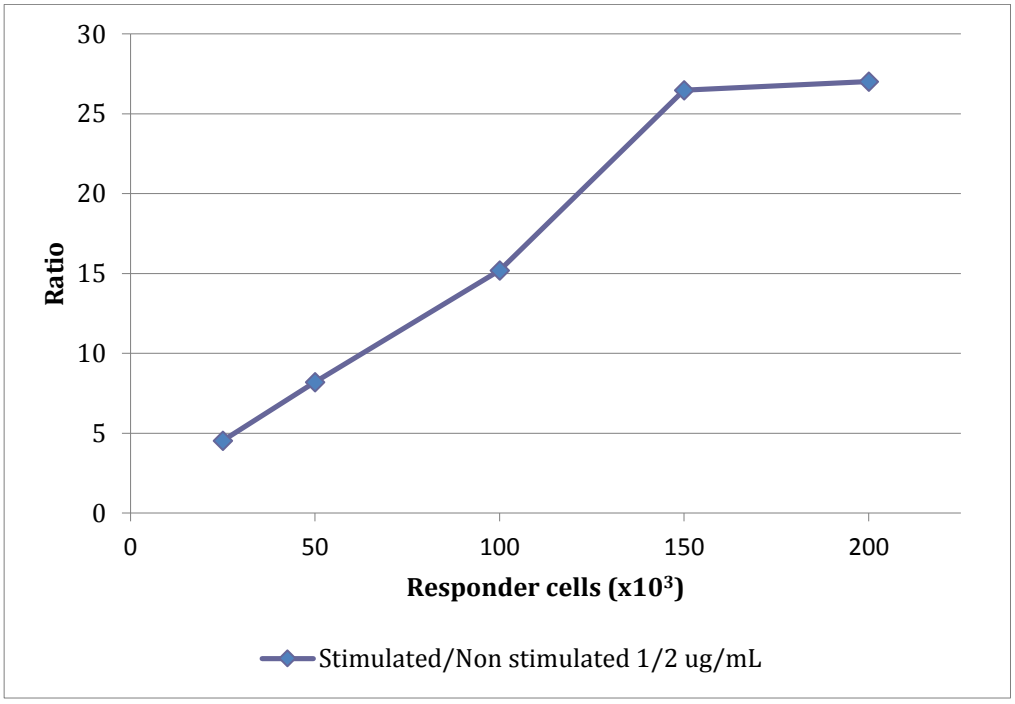
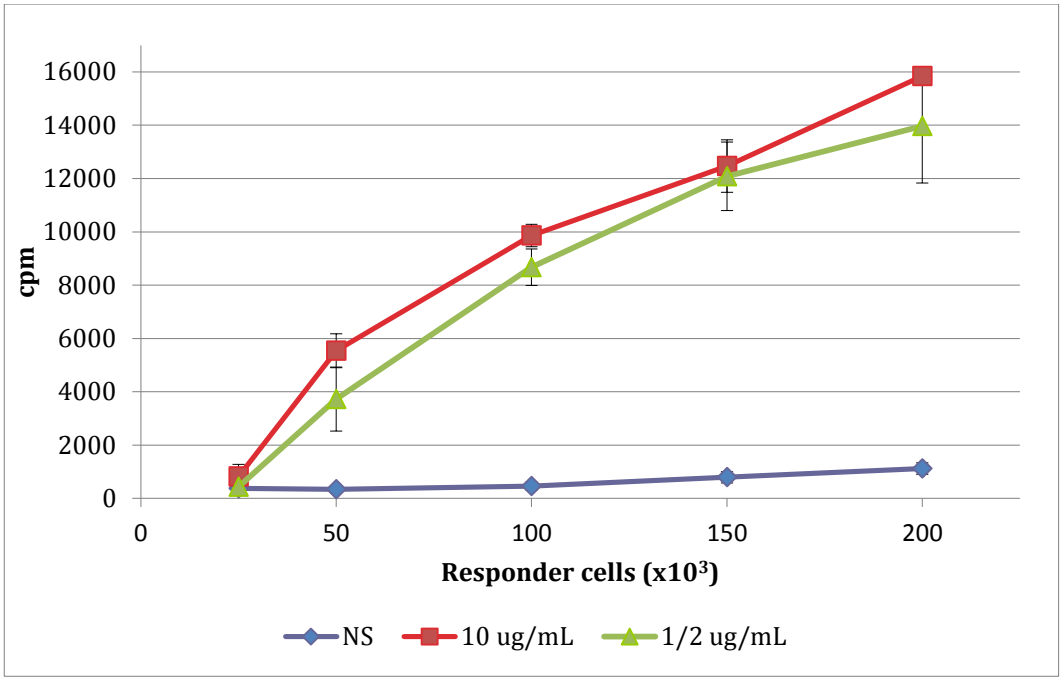


Figure 5.1 Optimisation of the proliferation of peripheral blood mononuclear cells (PBMC) in terms of number of responder cells and the concentration of stimulating antibodies. (A) Different amounts of PBMC from healthy volunteer donors were cultured in 96-well round bottom plates and stimulated with two concentrations of antibodies against CD3 and CD28 for 72-96 h. The cells were pulsed with tritiated thymidine for the last 16 h of culture. Tritium incorporation was then measured by liquid scintillation. Non-stimulated (NS) cells were also included as controls. (B) The proliferative response ratio at each responder cell concentration was compared between stimulated and non-stimulated cells. All cultures were set up in triplicate and bars represent the standard deviation. cpm, counts per minute.

(A)



(B)

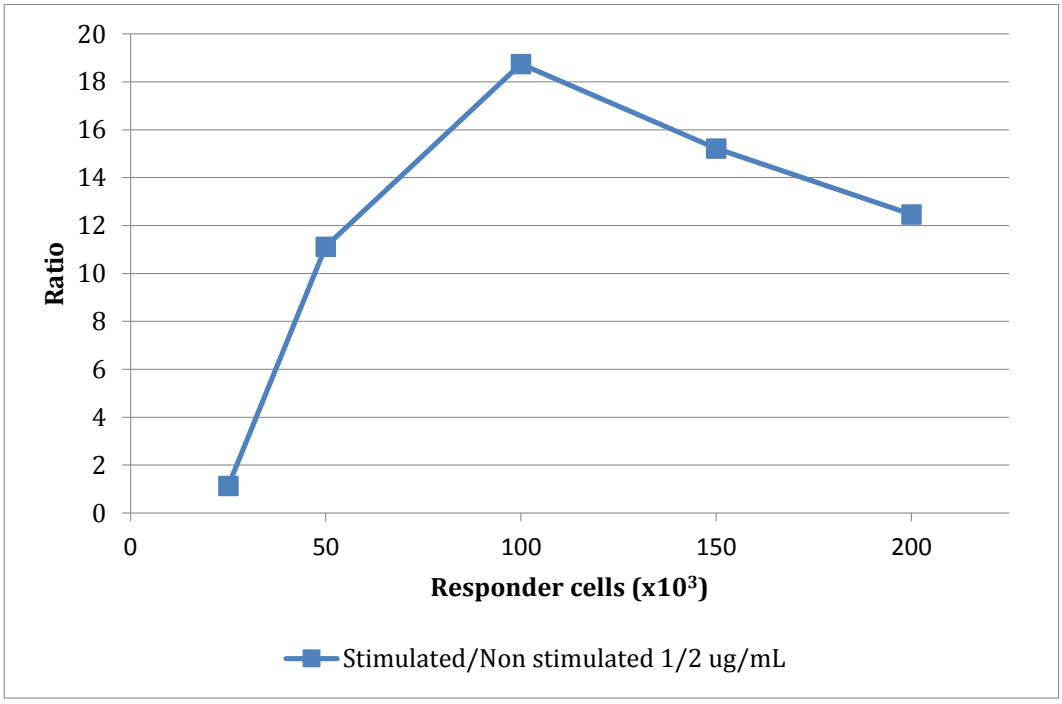


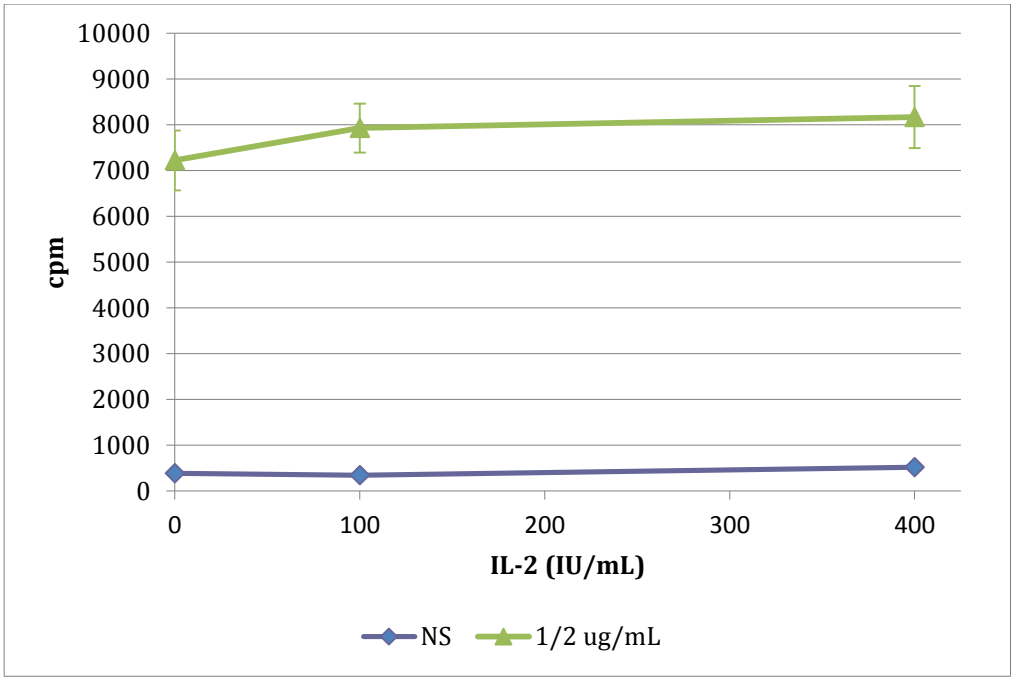
Figure 5.2 Optimisation of the proliferation of CD4+ cells in terms of number of responder cells and the concentration of stimulating antibodies. (A) Different amounts of isolated CD4+ cells from healthy volunteer donors were cultured in 96-well round bottom plates and stimulated with two concentrations of antibodies against CD3 and CD28 for 72-96 h. The cells were pulsed with tritiated thymidine for the last 16 h of culture. Tritium incorporation was then measured by liquid scintillation. Non-stimulated (NS) cells were also included as controls. (B) The proliferative response ratio at each responder cell concentration was compared between stimulated and non-stimulated cells. All cultures were set up in triplicate and bars represent the standard deviation. cpm, counts per minute.

The results show that the response increased with an increased cell number, albeit with a lower slope than that seen with the total PBMC. Once again, there appeared to be little added value in increasing concentrations of stimulating antibodies 10 or 5-fold. Interestingly, the difference in radiation signal between the stimulated cells and the resting cells was maximal at $100x10^3$ responder cells per well. Higher and lower cell numbers decreased this difference. The addition of more than $100x10^3$ cells per well did not improve but rather reduced the difference with non-stimulated cell background levels. This might be explained by a saturation effect in a 200-µL culture, which can be counter-productive. Consequently, $100x10^3$ responder cells per well and the 1/2 µg/mL antibody concentration combination were chosen for further experiments.

In another set of experiments, different concentrations of exogenous IL-2 were tested for their effect on the proliferation of the responder cells. Two concentrations of IL-2, as well as no added IL-2, were tested in similar conditions as those used for the previous experiments. The results for these tests are shown in Figures **5.3 and 5.4**. As shown in these figures, the presence of added IL-2 increased proliferation only marginally in both PBMC and isolated effector CD4 cells. In fact, increasing the concentration of IL-2 four times from 100 IU/mL to 400 IU/mL did not significantly change the amount of proliferation that was obtained. Moreover, the difference between stimulated and resting cells was not improved in PBMC in the presence of 400 IU/mL IL-2 with respect to the other concentration. In the case of CD4 effectors it only increased this difference modestly. Because of the low effect of IL-2 on the proliferation of both PBMC and isolated effector CD4 cells, likely to be due to their own production of IL-2 upon stimulation, and its potential effects on Treg suppression, I decided not to include any exogenous IL-2 to the suppression assays. This was also to ensure that the activation and potential suppression of this activation occurred in a system as close to an *in vivo* setting as possible.

In order to further visualise the activation of isolated CD4 cells with the aforementioned conditions more clearly, another set of experiments was carried out. On this occasion, the assessment of proliferation was done by another method using carboxyfluorescein succinimidyl ester (CFSE) staining. CFSE is a compound that is able to enter the responder cells and become trapped by metabolic modification. It fluoresces when excited by a laser, and it is thus used in order to visualise cellular division by means of its dilution and the reduction of its fluorescence.

(A)



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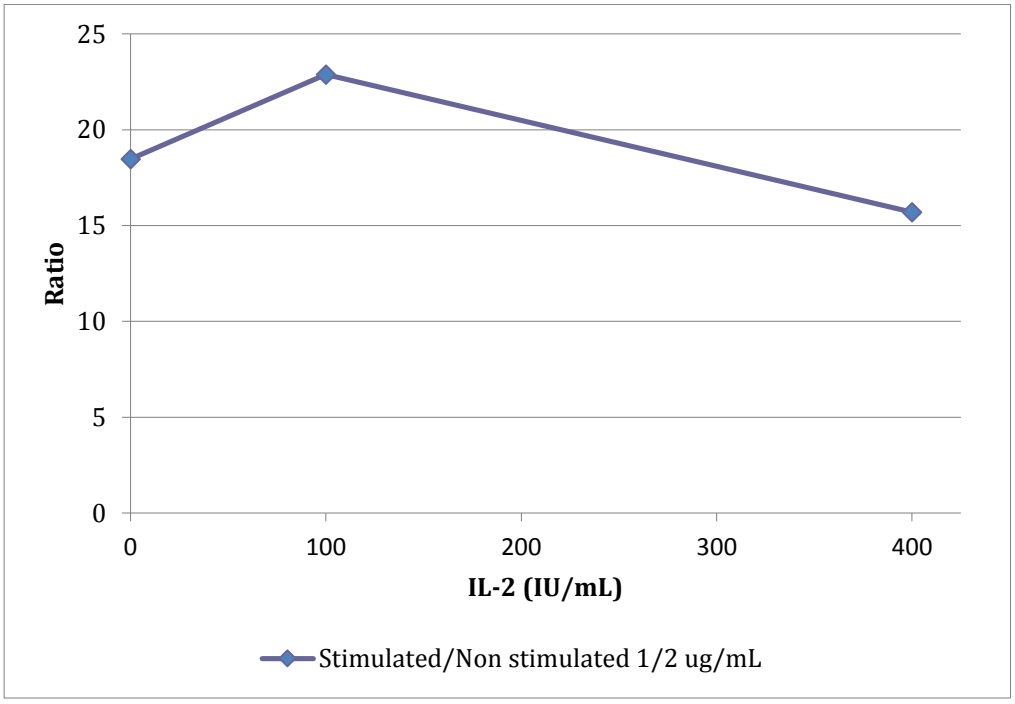
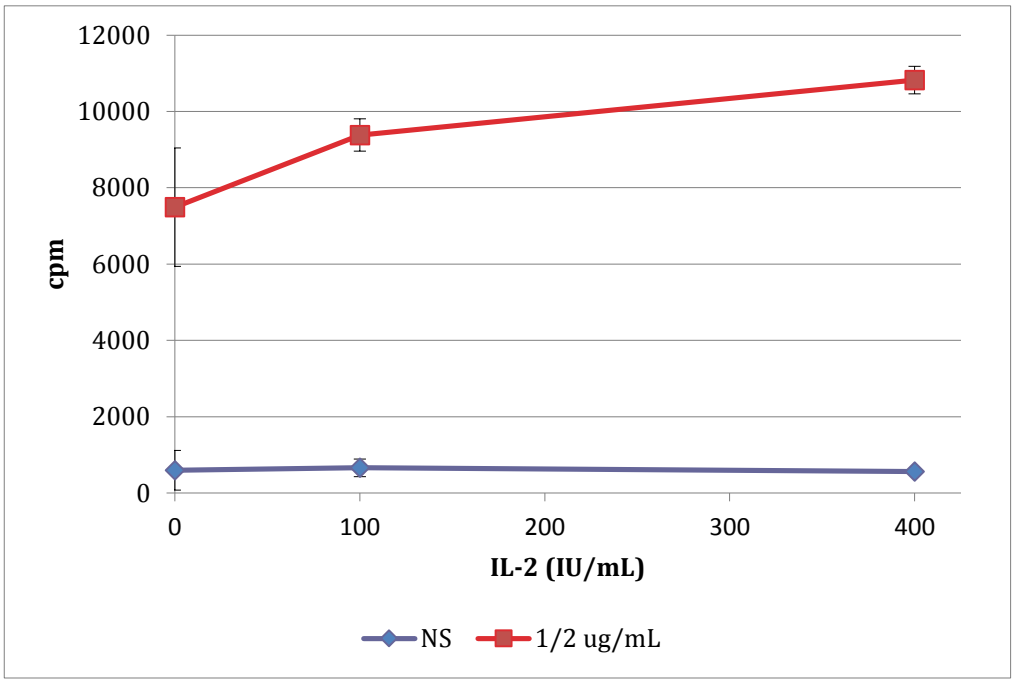


Figure 5.3 Optimisation of the proliferation of peripheral blood mononuclear cells (PBMC) in terms of the concentration of exogenous IL-2. (A) 100x10^3 PBMC from healthy volunteer donors were cultured in 96-well round bottom plates and stimulated with 1/2 ug/mL of antibodies against CD3 and CD28 for 72-96 h. The cells were pulsed with tritiated thymidine for the last 16 h of culture. Tritium incorporation was then measured by liquid scintillation. Non-stimulated (NS) cells were also included as controls. (B) The proliferative response ratio at each concentration of exogenous IL-2 was compared between stimulated and non-stimulated cells. All cultures were set up in triplicate and bars represent the standard deviation. cpm, counts per minute.

(A)



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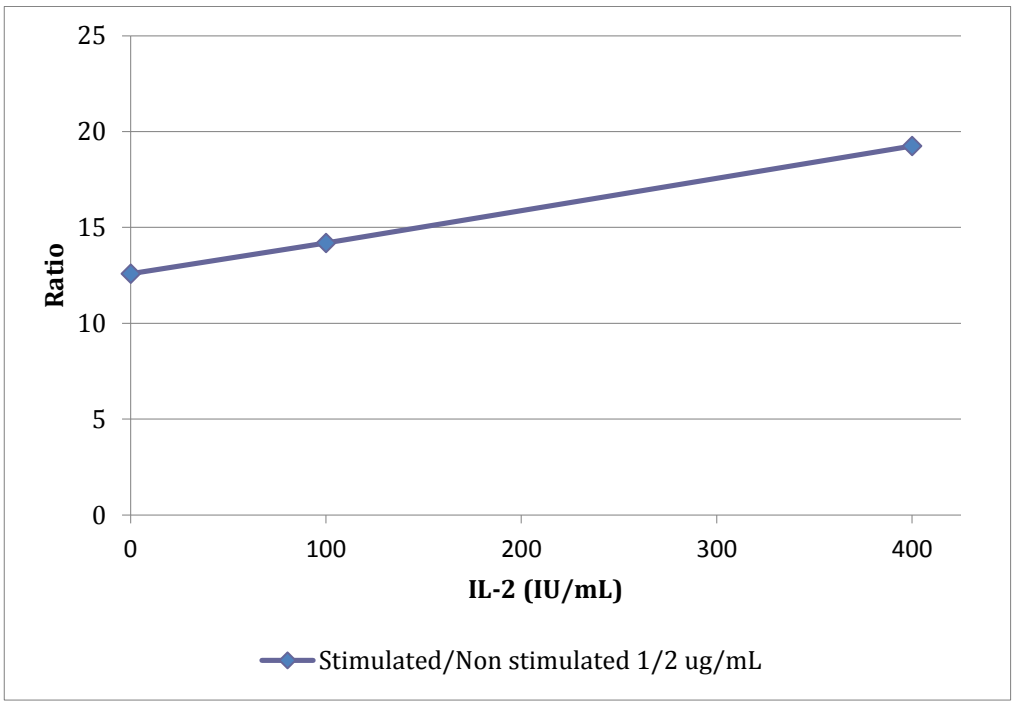

Figure 5.4 Optimisation of the proliferation of CD4+ cells in terms of the concentration of exogenous IL-2. (A) 100x10^3 isolated CD4+ cells from healthy volunteer donors were cultured in 96-well round bottom plates and stimulated with 1/2 ug/mL of antibodies against CD3 and CD28 for 72-96 h. The cells were pulsed with tritiated thymidine for the last 16 h of culture. Tritium incorporation was then measured by liquid scintillation. Non-stimulated (NS) cells were also included as controls. (B) The proliferative response ratio at each concentration of exogenous IL-2 was compared between stimulated and non-stimulated cells. All cultures were set up in triplicate and bars represent the standard deviation. cpm, counts per minute.

In these experiments, the responder cells were labeled with CFSE as explained in Chapter 2. 100x10^3 CD4 cells were seeded in 200-µL cultures in the presence of 1/2 µg/mL of anti-CD3/CD28. After 72 h of incubation at 37˚C in a 5% CO2 atmosphere, the cells were harvested, washed and stained with fluorescently-labeled antibodies against extracellular antigens. The cells were then analysed by flow cytometry as explained in Chapter 2. As a control, MACS activation beads were also used as stimulation in some cultures. The results of this set of experiments are shown in **Figure 5.5**. As shown in this figure, the CFSE staining was efficient, and it allowed the visualization of the activation and division of stimulated CD4 cells.

Co-stimulation is known to play an important role in the T lymphocyte activation and proliferation process. These second signals are known to come mainly from other cell types, such as APC. Moreover, the production of soluble factors by accessory cells can also enhance T cell activation. Finally, the Treg suppression model has been proposed to involve a three-cell structure between the effector cell, the Treg and an APC. Consequently, the use of irradiated autologous PBMC was implemented for the final conformation of the suppression assay. 100x10^3 irradiated PBMC were added per well in order to provide sufficient numbers of APC (approximately 5,000-10,000 monocytes) for co-stimulation for both effectors and Treg. As controls for background tritium incorporation and anergy, wells with irradiated PBMC only and irradiated PBMC with Treg in the presence of activating stimulus, as well as non-stimulated effectors in the presence of irradiated PBMC were included in the subsequent experiments. Furthermore, in order to assess differences in suppressive capacity, three different Treg:effector ratios were included in most subsequent experiments: 1:2, 1:4, and 1:8. Finally, 10 µg/mL PHA was added to a set of wells as a positive control for proliferation. All of the conditions were set up in triplicate wells.

Once the suppression assay was defined a series of experiments were performed in which effector CD4 cells and Treg were isolated from healthy donors of known *TGFB1* +29T>C genotypes. Autologous suppression assays were then set up and the normalized suppression seen for every Treg:effector ratio was recorded. The normalization was done with the radiation counts for each well using the stimulated effectors (with no Treg) as the reference value (i.e. 1.0 or 100% proliferation). **Figure 5.6** shows a representative example of the results for these experiments. The experiment was performed for 3 different subjects per genotype. The normalized and complete results for this series of experiments are shown in **Figure 5.7**.

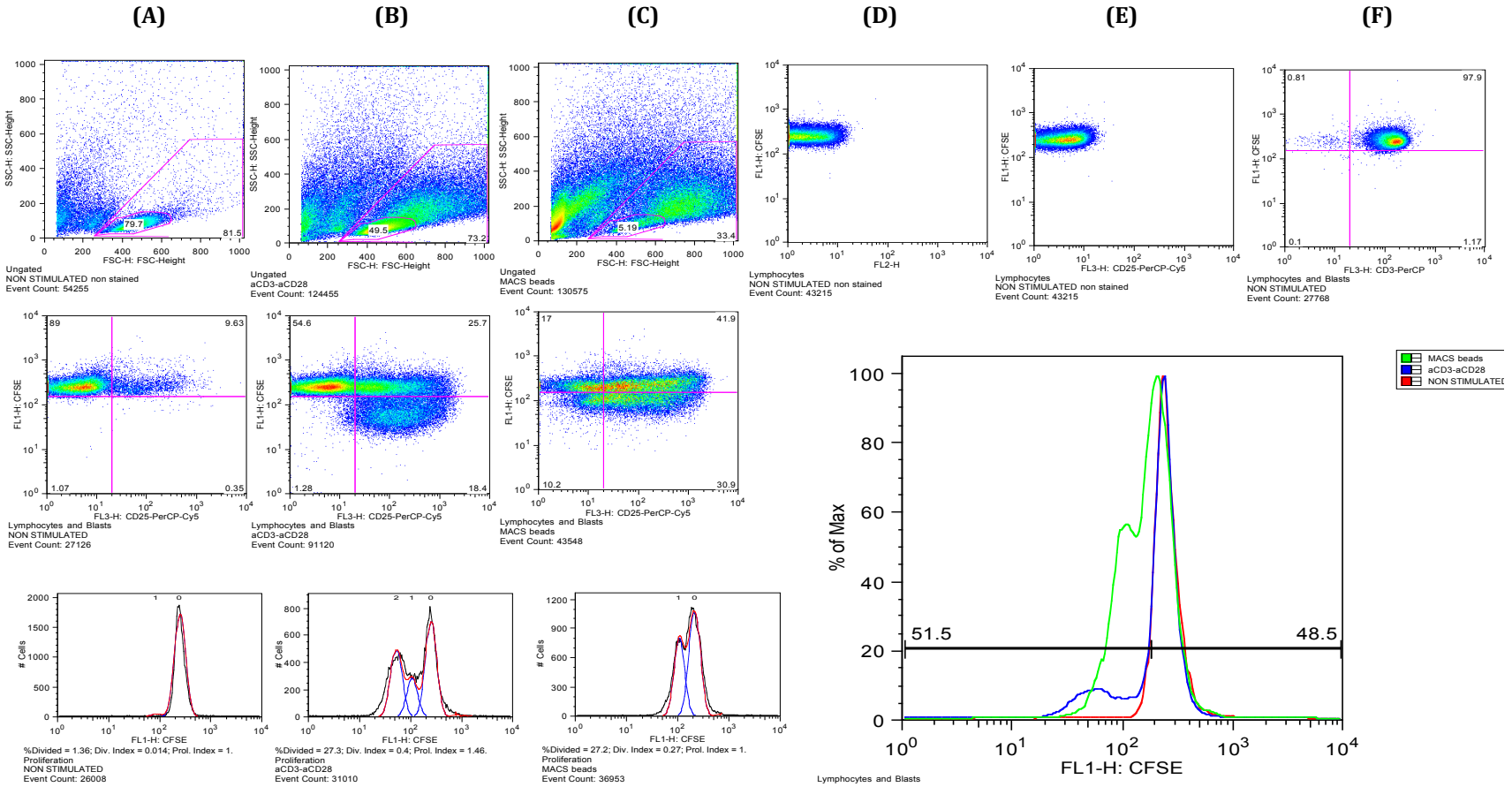

Figure 5.5 Visualization of the optimisation of the proliferation of isolated CD4+ cells by carboxyfluorescein succinimidyl ester **(CFSE) dilution.** (A) 100x10^3 isolated CD4+ cells from healthy volunteer donors were cultured in 96-well round bottom plates and stimulated with 1/2 ug/mL of antibodies against CD3 and CD28 or with MACS T cell stimulation beads for 72 h. After culture, the cells were harvested and stained with fluorescently labeled antibodies. CFSE dilution was assessed by flow cytometry. Non-stimulated (NS) cells were also included as controls. Columns A-C: proliferation assessment for non-stimulated, antibody-stimulated and bead-stimulated cells. (D) and (E): Compensation of CFSE staining in non-stimulated CD4 cells. (F) CFSE staining vs CD3 staining in non-stimulated CD4 cells. The histogram shows CFSE dilution in non-stimulated and stimulated cells.

As shown in the figure, when normalised proliferation in the different experiments was assessed, no clear tendencies towards a difference in the efficiency of suppression between *TGFB1* +29 T>C genotypes could be observed in any of the effector:Treg ratios used.

The analysis of these experiments suggested that the standard autologous *in vitro* suppression assay might not be a system that would allow for the easy assessment of the effect of the *TGFB1* genotype. Since these 9 series of experiments did not show any clear differences in suppression levels, I decided to explore other alternatives of studying the effect of this polymorphism on the suppressive capacity of Treg.

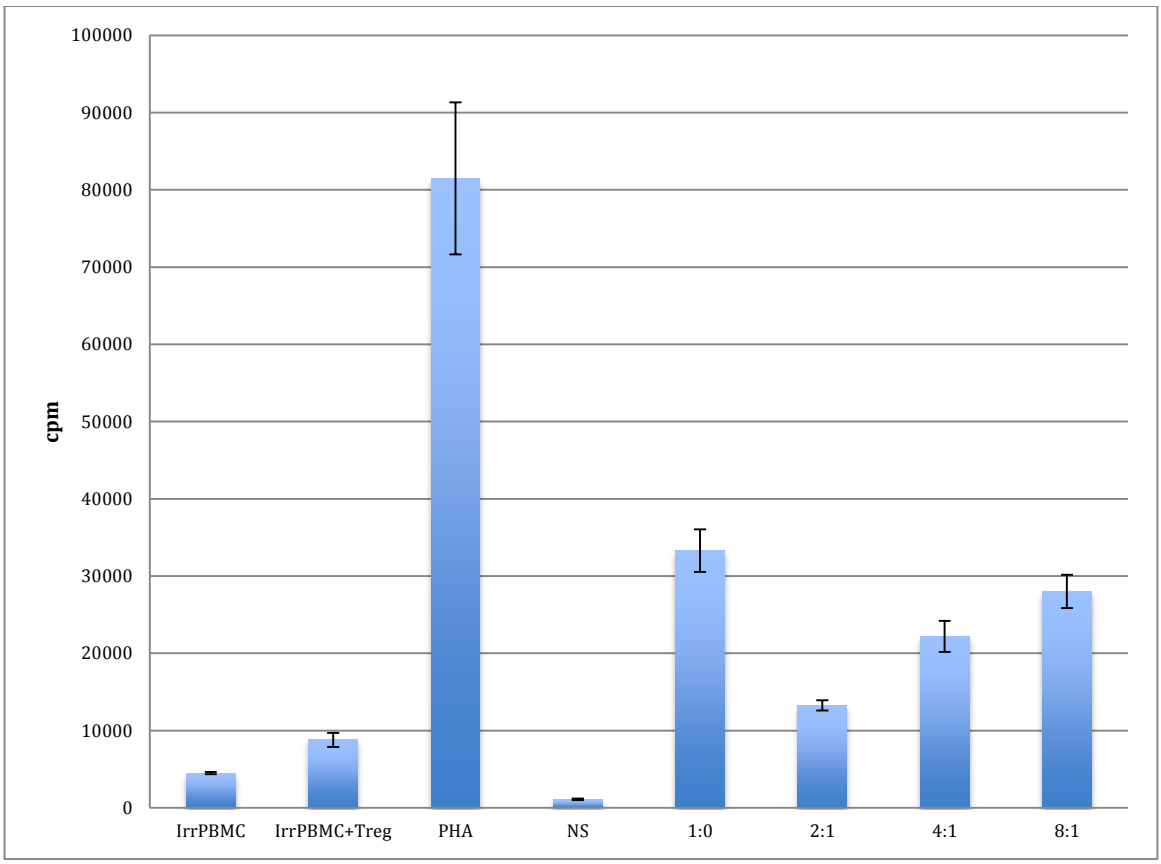

Figure 5.6 Suppression of the proliferation of effector CD4 cells by autologous regulatory T cells from a *TGFB1* +29CC individual. 100x10^3 effector cells per well were cultured in the presence of an equal number of irradiated peripheral blood mononuclear cells (IrrPBMC) with or without various amounts of autologous Treg. The cells were either stimulated with soluble anti-CD3 and anti-CD28, or non-stimulated (NS). Activated IrrPBMC on their own or with Treg, plus PHA-activated effectors without Treg were used as background and positive controls, respectively. Levels of proliferation shown are mean and standard deviation of triplicate wells. cpm, counts per minute.

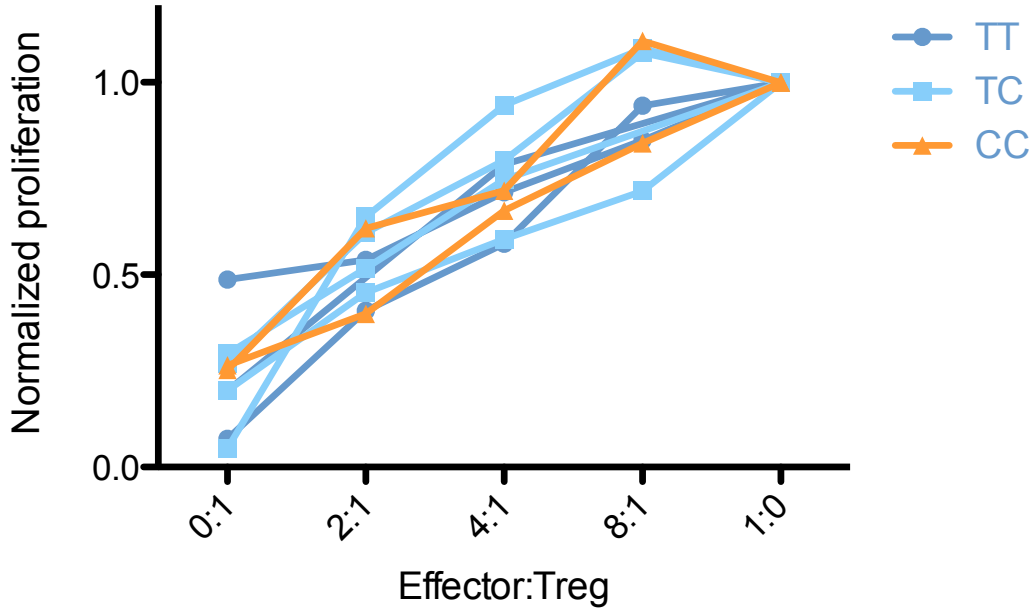

Figure 5.7 Proliferation of effector CD4 cells in the presence or absence of autologous regulatory T cells (Treg) from individuals of different *TGFB1* +29 T>C genotypes. Autologous suppression assays were performed as detailed previously and proliferation was normalized to the counts 1:0 ratio.

Thorough reflection on the fact that the data obtained for the standard suppression assay did not show any clear trends in terms of differential suppressive capacities raised the possibility that various factors could potentially blur the effect of *TGFB1* +29T>C genotype on Treg function. These factors include the fact that TGF-β1 would be only one of various mechanisms by which Treg suppress effector proliferation in this assay, the presence of additive effects of variations between these complex experiments such as the activation efficiency, the quality and quantity of accessory cells (i.e the irradiated PBMC), or the presence of contaminating cells in the isolated fractions, and the fact that the endpoint variable was blunt proliferation as opposed to other possible effects of TGF-β1 like cytokine production. Consequently, I decided that the functional study of the effect of *TGFB1* +29T>C genotype on the suppressive capacity of Treg would require an assay that would be more specific for TGF-β1 and that reduced the effect of confounding factors between experiments. For this purpose, I decided to develop a different suppression assay in which the induction of a marker in CD4+ effectors being suppressed by TGF-β1 would be the endpoint to assess Treg suppressive capacity.

5.3 "Real-time" suppression assays and the assessment of the role of *TGFB1* +29T>C in regulatory T cell (Treg) function

Following the results obtained for the standard autologous suppression assays, I decided to test a different strategy to assess the effect of the codon 10 polymorphism on the suppressive capacity of Treg. For this purpose, I looked for a way to measure the action of TGF-β1 on effector CD4 cells. Nakamura *et al*. had reported that murine CD4+CD25+ T cells induce CD103 expression on CD4+CD25- T cells in co-cultures with soluble TCR activation by anti-CD3 and in the presence of irradiated autologous PBMC and IL-2 (Nakamura *et al.*, 2004). The fact that this observation appeared to be a direct effect of TGF-β1 produced by Treg on effector cells, suggested that CD103 induction could potentially be used as a direct marker of the action of TGF-β1 in the suppression of T cell activation. Consequently, I designed a modified suppression assay based on flow-cytometric assessment of CD103 expression on effector cells as an end-point. The rationale behind this was that if Treg produced more surface TGF-β1, then they could potentially induce more CD103 in effectors, and this could allow for the visualization of functional differences arisen from different genotypes for *TGFB1* +29T>C.

Because the induction of TGF-β1 (LAP) on the surface of Treg was shown to occur in a transient manner, with a peak around 24 h of activation (see Chapter 4), I decided to follow the expression of LAP on Treg in the context of the suppression assay and to also follow the potential expression of CD103 on effector cells. In this way, I could determine if CD103 was induced at early or later stages in the assay, and what the kinetics of expression would be. Because of the necessity of being able to tell the effector CD4 cells apart from the Treg in order to assess the levels of the markers on each subset, I decided to label the effector cells with CFSE. In this way, it would be possible to isolate a population by flow-cytometry for analysis, plus it would allow for the assessment of proliferative capacity within the suppression assay. The CFSE-negative cells would then include the Treg, as well as any irradiated PBMC added to the assay. The labeled and non-labeled cells would then be put into co-cultures, activated and their behavior would be followed daily for 3-4 days.

In a first round of experiments, PBMC from healthy donors were obtained and subject to isolation of both Treg and effector CD4+ cells. A fraction of the PBMC would be irradiated and used as a source of accessory cells. Once the fractions were isolated, effector cells were stained with CFSE as explained in Chapter 2. The cultures were set up using the same combinations as the standard assays. All wells contained $100x10^3$ irradiated PBMC. Cultures with only irradiated PBMC in the presence of stimulation, and with non-

stimulated effectors were used as background controls. Wells with Treg and irradiated PBMC in stimulating conditions were used to monitor the expression of LAP by Treg in the context of soluble activation in the presence of accessory cells. These wells would allow for the study of LAP expression kinetics and for the comparison of this kinetics with that seen in the Treg activation assays explained in Chapter 4. Of note, anti-CD28 antibodies were not included in these new experiments because the analysis of the standard suppression assays pointed out the fact that CD28 co-stimulation could be blunting TGF-β1-mediated suppression of effector cells (Sung *et al.*, 2003; Gunnlaugsdottir *et al.*, 2005). Moreover, since Nakamura *et al*. did not use anti-CD28 in the assay in which they observed the induction of CD103 on suppressed effectors, I decided to rely on co-stimulation by irradiated PBMC. All the cultures were set up in triplicate, which were then pooled and split again for staining with 2 different fluorescently labeled antibody cocktails: one for LAP and one for CD103. All the triplicates were set up at the same time, and each time-point was assessed on a daily basis. For the suppression wells, a 2:1 effector:Treg ratio was used in order to have sufficient amounts of cells for the assessment and to favor the induction of CD103 on effectors. $100x10^3$ isolated effectors were added per well.

The representative results for these experiments are shown in **Figures 5.8-5.12**. **Figures 5.8** shows the PHA-stimulated effector cells with irradiated PBMC were used as positive controls for activation. As shown in **Figures 5.9 and 5.10**, the presence of CFSE-negative Treg effectively quenches the full activation and division of the CFSE-positive effector CD4 cells. CFSE-diluting cell division starts from 72 h of culture and increases by 96 h. However, not a single division of effector cells takes place if Treg are present despite CD25 being upregulated by the responder cells. It became clear that irradiated PBMC show progressive death, which is expected because of the damage they suffer by the radiation, except in a minority of cells that do become activated and potentially even turn blastic. These cells represent the background tritium incorporation seen for this control in the standard suppression assays. Despite their quick death, irradiated PBMC perform their main role in the activation of both effector cells and Treg in the first stages of the process. On the contrary, the other cell combinations showed a survival of the majority of the cells until 72 h of culture. It is also evident that Treg are anergic when exposed to activating stimulus, and that their addition to wells with CD4+ effectors effectively block the blastic transformation and proliferation of the latter. Consequently, the suppression assay showed efficient suppression by the activated Treg.

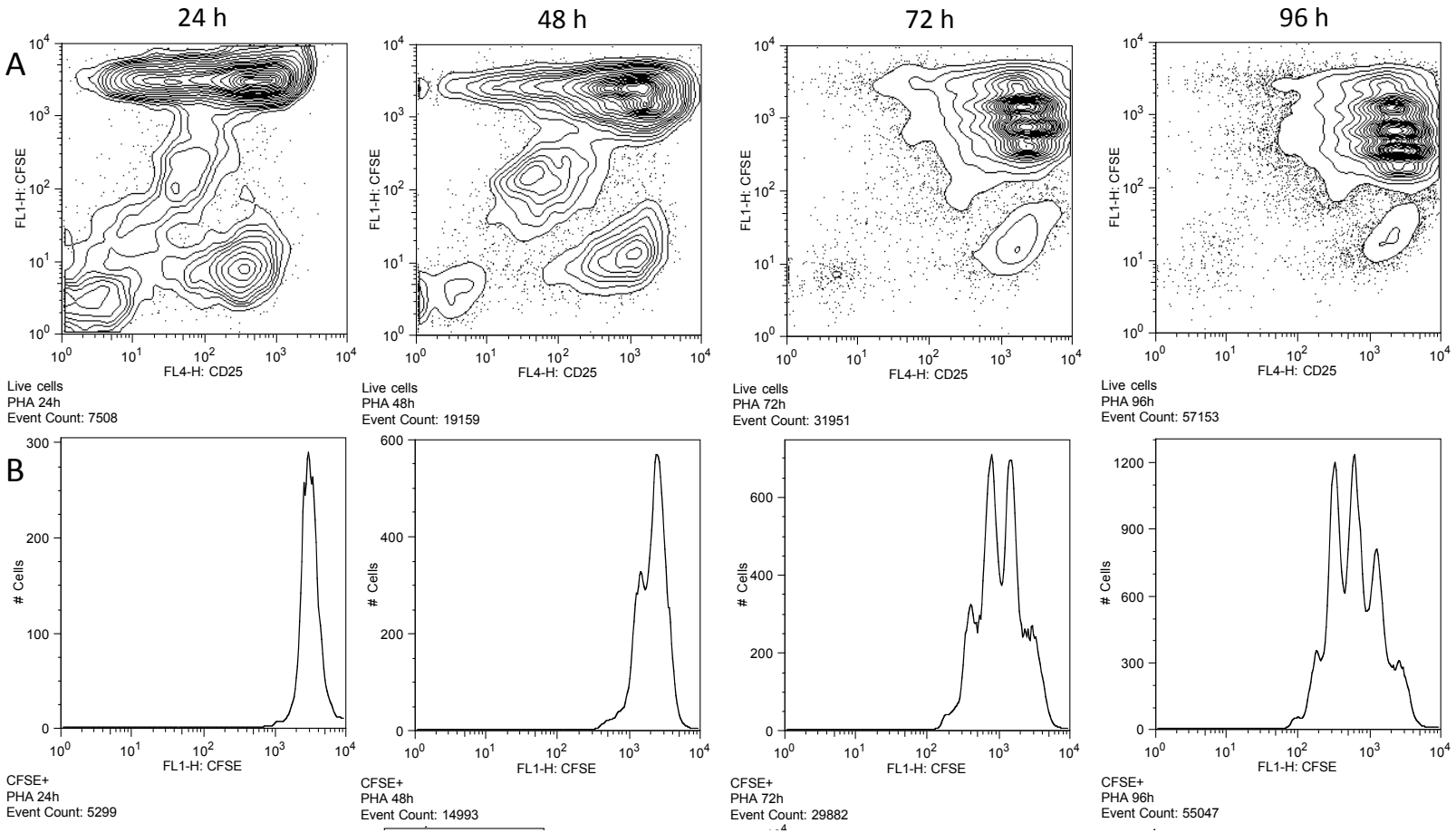

Figure 5.8 Proliferation control in 'real-time' suppression assay. The proliferation of isolated effector CD4+ T cells was assessed daily by CFSE dilution using flow cytometry from culture set up to 96 h of incubation. 100x10^3 irradiated PBMC (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The cells were stimulated with 10 ug/mL of PHA. The cells were harvested daily and stained with fluorescently-labeled antibodies against CD4 and CD25. (A) CD25 expression vs CFSE levels among live cells. (B) Histograms show the intensity of CFSE levels in effector cells. Columns correspond to the different time points.

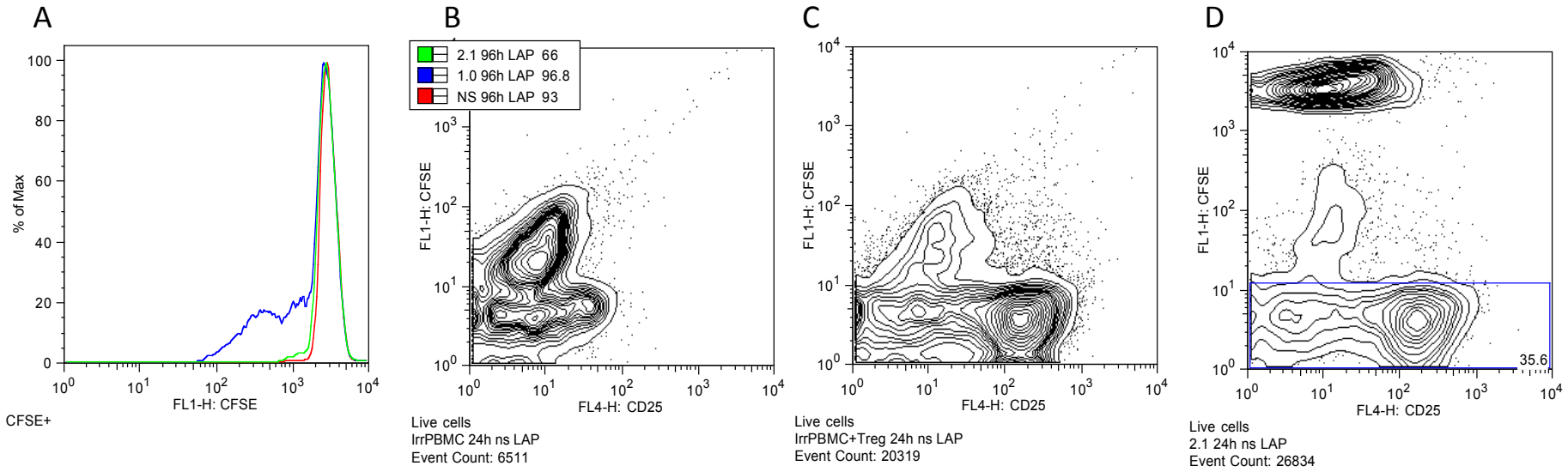

Figure 5.9 'Real-time' suppression assay shows effective suppression of effector CD4+ T cell activation and proliferation by Treg. Representative results of a series of experiments in which an autologous suppression assay was followed from the start of incubation until 96 h of culture. $100x10^3$ irradiated PBMC (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The suppression wells included a 2:1 effector:Treg ratio. The cells were stimulated with 1 ug/mL of soluble anti-CD3. Non-stimulated (NS) 1:0 wells as well as wells with stimulated irradiated PBMC (IrrPBMC) and irradiated PBMC with $50x10^3$ Treg (IrrPBMC+Treg) were included as controls. The cells were harvested stained with fluorescently labeled antibodies against CD4 and CD25. Panels show a histogram of CFSE fluorescent intensity at 96 h in non-stimulated, stimulated (1:0) and suppressed (2:1) (A). Plots show 24 h CD25 vs CFSE levels in non-stimulated controls for IrrPBMC (B), IrrPBMC+Treg (C), and the 2:1 suppression wells (D).

The assessment of the expression of LAP on Treg in the context of the suppression assay showed a peak at the 24 h time point. As shown in **Figure 5.11**, as had been reported for the activation experiments, LAP expression arose only on activated Treg cells, found among the CFSE-negative cells. The upregulation of LAP occurred in both the Treg+irradiated PBMC wells and the 2:1 suppression wells. LAP expression is strongest at 24 h in the 2:1 suppression cultures, while high levels are maintained between 24-48 h in the Treg activated in the presence of irradiated PBMC only. The isotype control stainings confirm the validity of the LAP stainings.

When the presence of CD103 was assessed both on effector (CFSE+) cells and Treg, no clear induction of this marker was observed in either subset. As shown in **Figure 5.12**, very few cells were CD103 positive at 24 h of incubation, and autofluorecence of blastic cells could be responsible for any positivity seen at later time points for CFSE-negative cells. Finally, to determine if CD103 induction could occur at a later stage of the suppression assay, the 2:1 cells cultured for 96 h were labeled with anti-CD103 instead of isotype control. However, once again, there was no upregulation of this marker on suppressed effectors.

The lack of CD103 induction was unexpected, taking into account that the activation conditions were similar to those reported by Nakamura and collaborators. The only difference was that their cells were activated for a longer period (5 days). It appeared thus that unlike murine cells, human CD4+ effectors co-cultured with their Treg counterparts in activating conditions do not show CD103 upregulation and nor do activated Treg.

Despite the fact that CD103 induction was not observed, the real time assessment of an autologous suppression assay allowed for the observation of the kinetics if LAP expression by Treg in the presence of accessory cells (including APC) and in the context of a suppression assay. The fact that these data suggested that the kinetics observed when the Treg were stimulated in the activation assays presented in Chapter 4 were also present in a suppression assay was interesting since this phenomenon could then be a feature of Treg biology and suggest that it could happen *in vivo*. For this reason, I repeated these experiments in a new series with a few modifications that would allow for a more definite characterization of their surface TGF-β1 expression.

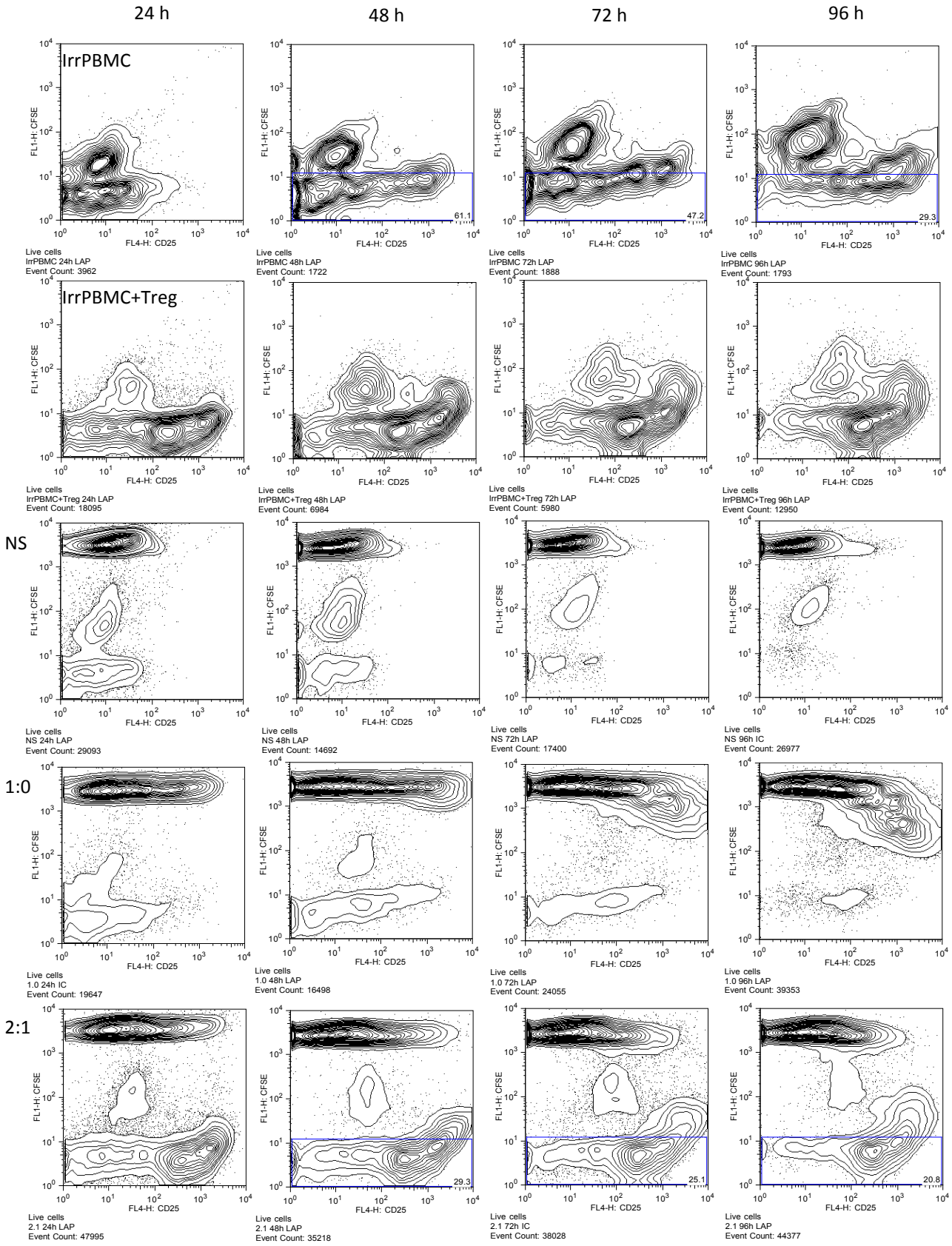

Figure 5.10 'Real-time' suppression assay showing the development of the suppression of effector CD4+ T cell activation and proliferation by regulatory T cells (Treg) (see below).

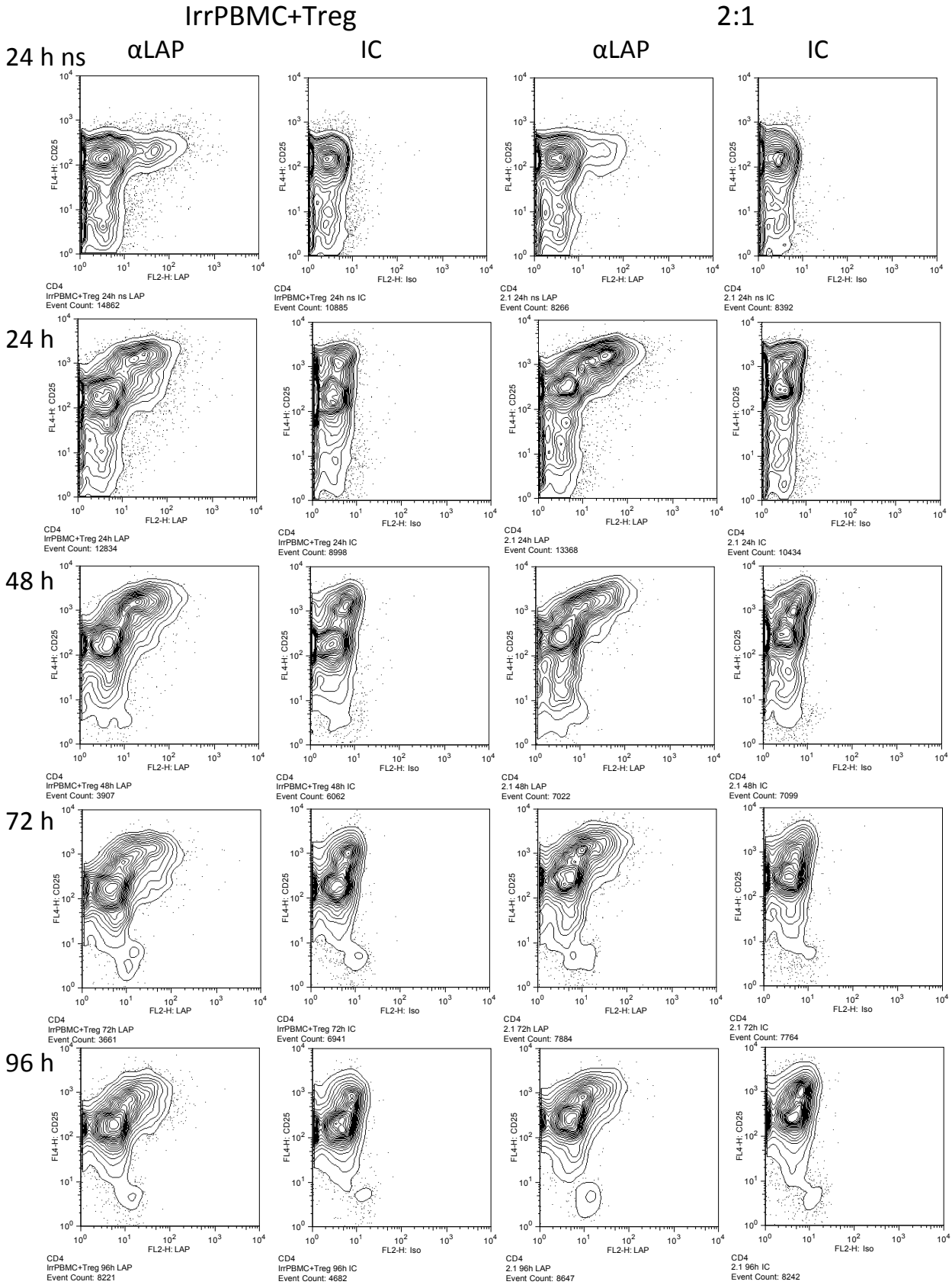

Figure 5.11 Regulatory T cell (Treg) expression of surface latent TGF-β1 in 'real-time' suppression assay shows a peak at 24 h of culture (see below).

Figure 5.10 'Real-time' suppression assay showing the development of the suppression of effector CD4+ T cell activation and proliferation by regulatory T cells (Treg). Representative results of a series of experiments in which an autologous suppression assay was followed from the start of incubation until 96 h of culture. $100x10^3$ irradiated peripheral blood mononuclear cells (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The suppression wells included a 2:1 effector:Treg ratio. The cells were stimulated with 1 ug/mL of soluble anti-CD3. Non-stimulated (NS) 1:0 wells as well as wells with stimulated irradiated PBMC (IrrPBMC) and irradiated PBMC with $50x10^3$ Treg (IrrPBMC+Treg) were included as controls. The cells were harvested daily and stained with fluorescently labeled antibodies against CD4 and CD25. Panels show CD25 vs CFSE staining among live cells. Columns represent each time point, and rows correspond to each of the cell combinations.

Figure 5.11 Regulatory T cell (Treg) expression of surface latent TGF-β1 in 'real-time' suppression assay shows a peak at 24 h of culture. Representative results of a series of experiments in which the expression of surface latency-associated peptide (LAP) on non-CFSE labeled cells in an autologous suppression assay was followed from the start of incubation until 96 h of culture. $100x10^3$ irradiated peripheral blood mononuclear cells (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The suppression wells included a 2:1 effector:Treg ratio. The cells were stimulated with 1 ug/mL of soluble anti-CD3. Irradiated PBMC with $50x10^3$ Treg (IrrPBMC+Treg) were included as controls for the observation of LAP expression in the absence of responder cells. Moreover, non-stimulated (ns) cultures were also set for the 24 h time point. The cells were harvested daily and stained with fluorescently-labeled antibodies against CD4, CD25 and LAP or its isotype control (IC), and these were analysed by flow cytometry. Plots show LAP (or isotype control) vs CD25 staining on live, CFSE-negative, CD4+ cells. Rows represent each time point, and columns correspond to LAP staining or isotype control staining for IrrPBMC+Treg (left panels) and for 2:1 suppression wells (right panels).

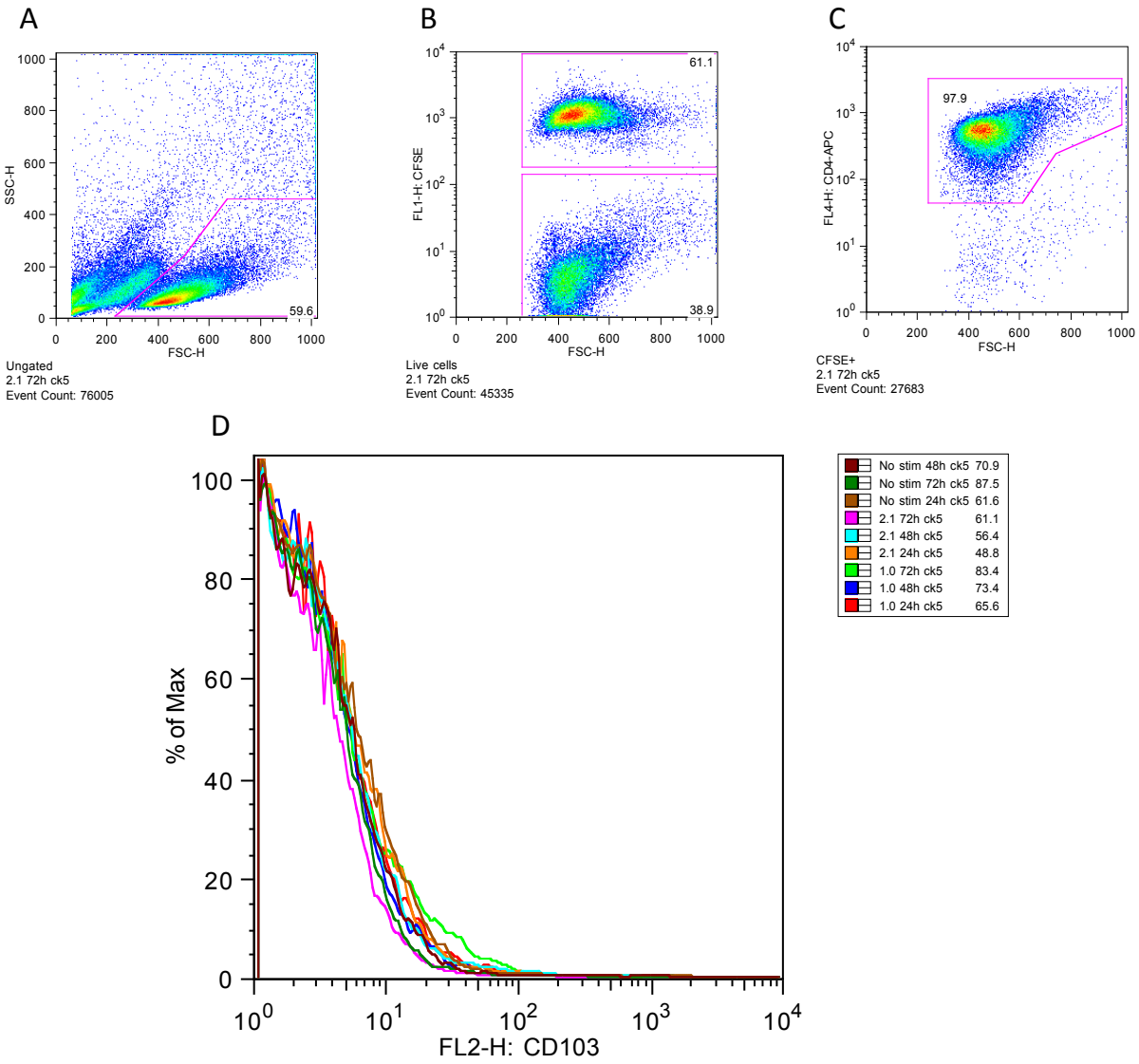

Figure 5.12 Effector CD4+ cells appear not to express surface CD103 in 'real-time' suppression assay. Representative results of a series of experiments in which the expression of surface CD103 on CFSE-labeled responder cells in an autologous suppression assay was followed from the start of incubation until 72 h of culture. 100x10^3 irradiated PBMC (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The suppression wells included a 2:1 effector:Treg ratio. The cells were stimulated with 1 ug/mL of soluble anti-CD3. Non-stimulated (No stim) 1:0 wells were included as controls. The cells were harvested daily and stained with fluorescently-labeled antibodies against CD4, CD127 and CD103, and these were analysed by flow cytometry. Top plots show the gating strategy on live (A), CFSE-labeled (B), CD4+ cells (C). Histogram (D) shows the intensity of CD103 staining in effector cells in different culture combinations and at the different time points.

In the new series of experiments, the suppression assays included once again activated irradiated PBMC on their own and with Treg, as well as 1:0 and 2:1 activated CFSE-labeled effector CD4 with or without Treg, and non-stimulated labeled effectors. The cells were activated with 1 ug/mL anti-CD3. Also, PHA-stimulated effector cells with irradiated PBMC were used as positive controls for activation. Additionally, I included 24 h resting cultures for the irradiated PBMC, irradiated PBMC+Treg and the 2:1 suppression in order to define more clearly the levels of LAP induction in stimulated Treg. The cultures were once more incubated for 24-96 h and the cells were harvested, washed and stained with fluorescently labeled antibodies for assessment. Anti-LAP antibodies and their isotype-matched control were used at each time-point.

Figure 5.13 shows the combined histograms for LAP (and isotype control) staining for both of the cultures having Treg at the different time points, confirming the transient expression of surface latent TGF-β1.

In summary, these experiments did not show evidence of the induction of CD103 expression on either human activated Treg or on human effector CD4+ cells on being suppressed by Treg. However, the real time assessment of the suppression assay confirmed that LAP expression on the surface of activated Treg does occur in the context of the suppression of effector T cell proliferation, and that this induction occurs with similar kinetics to that seen in the Treg activation experiments. These data suggest that the transient expression of surface TGF-β1 by Treg upon TCR stimulation also occurs in the context of the control of effector cell function.

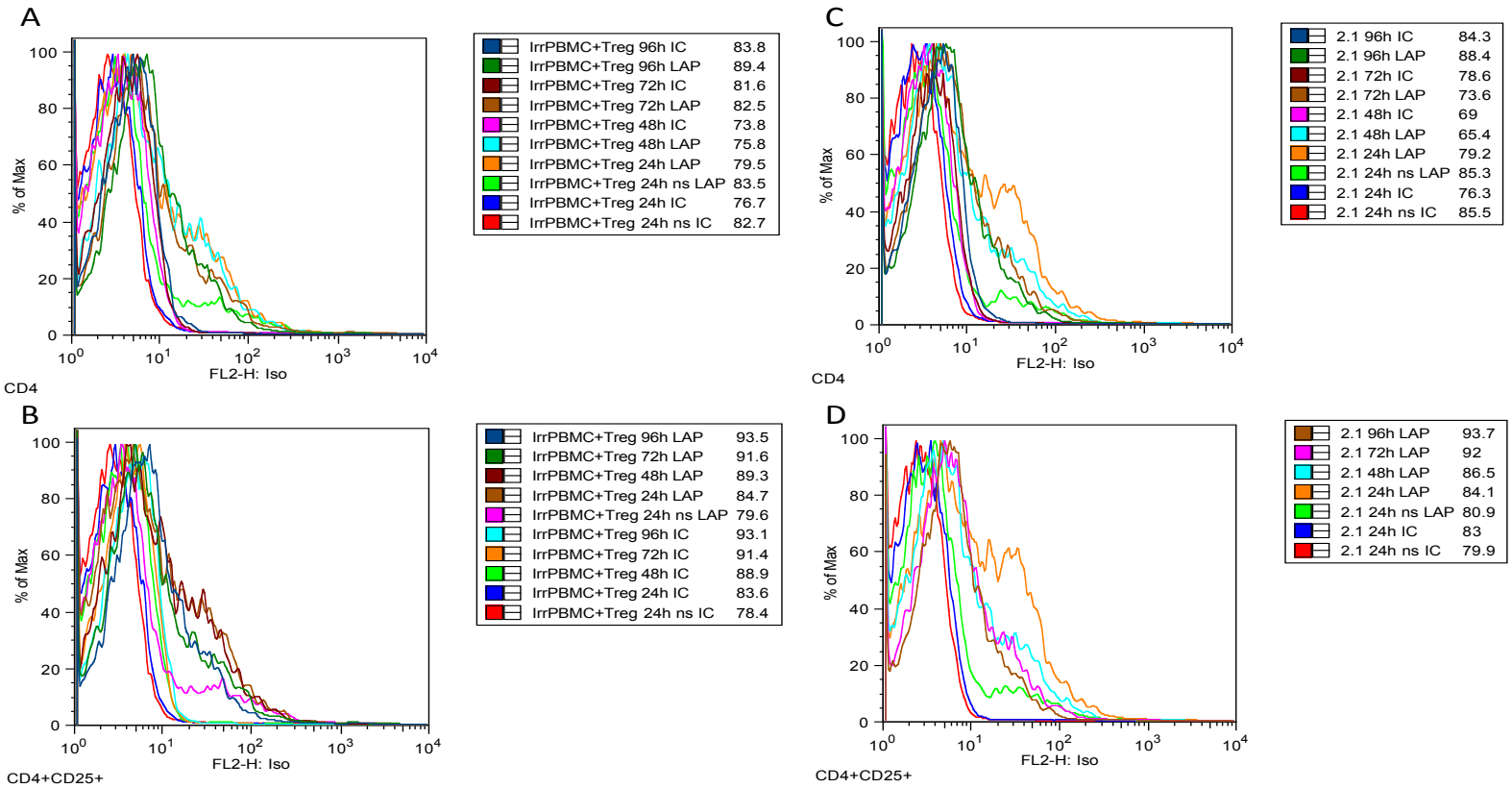

Figure 5.13 Regulatory T cell (Treg) expression of surface latent TGF-β1 in 'real-time' suppression assay shows a peak at 24 h of culture. Representative results of a series of experiments in which the expression of surface LAP on non-CFSE labeled cells in an autologous suppression assay was followed from the start of incubation until 96 h of culture. $100x10^3$ irradiated PBMC (IrrPBMC) were used as a source of co-stimulation, and the same number of isolated, CFSE-labeled CD4+CD25- effector cells were used as responders. The suppression wells included a 2:1 effector:Treg ratio. The cells were stimulated with 1 ug/mL of soluble anti-CD3. Irradiated PBMC with $50x10^3$ Treg (IrrPBMC+Treg) were included as controls for the observation of LAP expression in the absence of responder cells. Moreover, non-stimulated cultures were also set for the 24 h time point. The cells were harvested daily and stained with fluorescently-labeled antibodies against CD4, CD25 and LAP or its isotype control (IC), and these were analysed by flow cytometry. Histograms show LAP (or isotype control) staining levels on live, CFSE-negative, CD4+ (A, C) or CD4+CD25+ (B, D) cells. Results shown for IrrPBMC+Treg wells (A , B) and for the 2:1 suppression panels (C, D).

5.4 Discussion

In this chapter, I have presented my findings regarding the effect of *TGFB1* +29T>C on the suppressive capacity of Treg in the context of a standard *in vitro* autologous suppression assay. Also, I presented the development of a novel strategy for the visualization of the standard suppression assay, which I called 'real-time' suppression assay. The results of the standard suppression assays were inconclusive in terms of any impact of a differential genetic effect. Moreover, the use of the induction of CD103 as an end-point of TGF-β1-mediated suppression was not successful. However, the strategy allowed for the discovery that the transient expression of surface latent TGF-β1 by Treg upon activation also occurs in the context of a suppression assay.

5.4.1 *TGFB1* +29T>C in the standard suppression assays

Autologous suppression of effector CD4 proliferation by Treg is a well-known feature of this cell subset. Suppression assays have been a way for identification and functional characterisation of Treg. Moreover, the production of TGF-β1, and specifically membrane-bound LAP, by these cells is one of their distinctive characteristics and is believed to participate in their modulation of immune responses (Nakamura *et al.*, 2001; Chen & Wahl, 2003; Nakamura *et al.*, 2004; Wan & Flavell, 2007; Andersson *et al.*, 2008; Chen *et al.*, 2008; Chen *et al.*, 2009). Although a polymorphism in *TGFB1*, which is capable of modifying this cytokine's production, is likely to have an effect on these cells' suppressive capabilities, there are a number of factors that could affect the capacity of the assay to show consistent differences between genotypes.

Suppression assays are subject to considerable variation that must be taken into account and can exert influence on their results. During the optimisation of the autologous suppression system used a considerable number of optimisation steps had to be performed. Namely, the presence of sufficient numbers of cells for adequate signal, the presence and number of APC, the ratio between effector, Treg and irradiated PBMC, the nature and strength of the stimulus, the incubation period, and the way by which proliferation was assessed had to be addressed.

In the initial attempts, without the presence of APC, there was not sufficient proliferation of effectors, and, paradoxically, absence of suppression. Recognition that sufficient activation of both effector CD4 cells and Treg is needed in order to obtain suppression (Sakaguchi *et al.*, 2010) prompted the inclusion of APC to provide the system with costimulatory signals. The increase of the number of seeded cells and the inclusion of

irradiated PBMC as a source of APC drastically improved both the proliferation and the suppression within the assay. These modifications allowed the observation of dose (ratio)-dependent suppression of ^{3}H incorporation. In agreement with what has been reported, Treg showed anergy upon stimulation.

The lack of a clear tendency of differential suppressive capability between *TGFB1* +29 T>C genotypes may be due to confounding effects of the numerous variables that must be controlled for in this system. Also, the fact that the ultimate measurement in this assay is DNA synthesis and, thus, division of effector cells, restricts the interpretation to just one of the various immunomodulatory effects that Treg exert over effectors. For example, this assay does not measure inhibition of cytokine production by effectors, a phenomenon that is not necessarily codependent on suppression of cellular division.

Furthermore, many mechanisms other than production of TGF-β1 have been recognised to be important in Treg function (Sakaguchi *et al.*, 2010), and consequently these can participate in the suppression of proliferation and obscure the differences in TGF-β1-mediated immune modulation between *TGFB1* +29T>C genotypes.

One aspect that should be taken into account is the fact that the role of TGF-β1 in the *in vitro* suppression assay remains controversial, and the evidence for the importance of TGF-β1 in the control of immune responses seems to be more solid *in vivo* than *in vitro* (Nakamura *et al.*, 2004). Nakamura and collaborators have reported that suppression of the proliferation of effector cells and of the production of immunoglobulin by B cells by murine Treg were abolished in their assays by the addition of anti-TGF-β1 antibodies (Nakamura *et al.*, 2001). Similar results have been reported by other groups (Zhang *et al.*, 2001; Annunziato *et al.*, 2002). Moreover, the addition of recombinant LAP to co-cultures inhibited the suppression of both human and murine effector CD4 cells by their Treg counterparts (Nakamura *et al.*, 2004). Tran and collaborators showed that human Treg transfected with siRNA for *TGFB1*, as well as for *GARP*, were significantly less suppressive than Treg treated with control siRNA in an *in vitro* suppression assay (Tran *et al.*, 2009). Moreover, in an suppression assay using allogeneic B cells as stimulus, human T_h (effector) cells transduced to overexpress GARP were able to efficiently suppress the proliferation of their untransduced Th counterparts to levels comparable to those caused by natural Treg (Probst-Kepper *et al.*, 2009).

However, despite being considered as one of the main mechanisms that Treg use in order to control immune responses, the role of TGF-β1 has been questioned as a relevant actor in *in vitro* assays. A number of early studies showed a failure in blocking suppression of effector cells with TGF-β1-neutralizing antibodies (Takahashi *et al.*, 1998; Thornton & Shevach, 1998). Results presented by Oberle and collaborators showed that immediate

Th1 cytokine mRNA suppression in human effector T cells was not dependent on TGF-β1. In this report, the authors show that blocking TGF-β1 signaling with compound SB431542 resulted in only 35% reduction of the suppressive activity of Treg. Direct addition of recombinant TGF-β1 inhibited effector T cell proliferation alone by 50%, whereas Treg suppression reached over 75% of effector proliferation independently of further addition of recombinant TGF-β1. Addition of anti-TGF-β1 monoclonal Ab to purified effector CD4 cells resulted in increased IL-2 transcription, but the suppression of IL-2 induction by Treg was not altered. Blocking TGF-β1 signaling with inhibitor SB431542 did not significantly reduce Treg-mediated suppression of IL-2 mRNA production by the effector T cells (Oberle *et al.*, 2007). Gandhi and collaborators saw a reduction of only 24% in the suppression levels elicited by human Treg in an *in vitro* assay in the presence of recombinant LAP (Gandhi *et al.*, 2010). Another report showed that murine effector CD4+ cells with a deletion of the gene encoding the TGF-β1 receptor or with SMAD3 deficiency could be suppressed by normal Treg. Moreover, Treg cells from TGF-β1-deficient mice could also suppress the proliferation of normal effector cells (Piccirillo *et al.*, 2002). More recently, Edwards and collaborators tested the suppressive capacity of both murine GARP$^{-/-}$ and *TGFB1*$^{-/-}$ Treg in a standard *in vitro* suppression assay and found that they were equally effective in suppressing the proliferation of CD4+CD25- responder cells as their wild-type counterparts (Edwards *et al.*, 2013).

In another report, knockdown of *TGFB1* expression by RNA interference with silencing RNA significantly reversed the *in vitro* suppressive function of isolated murine CD4+CD25+LAP+ cells, but had no effect on the suppressive function of their CD4+CD25+LAP- counterparts (Chen *et al.*, 2008). The same TGF-β1 dependency was seen in an *in vivo* model of suppression by CD4+CD25+LAP+ using neutralizing antibodies against TGF-β1. Moreover, Ito and collaborators showed that *in vitro* suppression by human ICOS- Treg, which express higher levels of membrane-bound TGF-β1, was ablated by the use of an antibody that inhibits the type I TGF-β1 receptor (Ito *et al.*, 2008). On the contrary, the function of ICOS+ Treg, which also use IL-10 for suppression, was not as thoroughly affected by the use of this receptor inhibitor. Another report suggested that TGF-β1-mediated suppression of the activation of CD4+ cells would act differently on CD4+CD25-FOXP3- and CD4+CD25+FOXP3- cells, it being dispensable for the control of the former (Andersson *et al.*, 2008). These results suggest that, both in mice and humans, the dependency of suppression on TGF-β1 might be associated with a specific subset of Treg. If this subset is not predominant numerically or functionally in the system used to test Treg function, then the effect of TGF-β1-mediated suppression might be obscured.

In view of this, the role of TGF-β1 in the standard suppression assay might not be sufficient for the differences that would arise from genetic polymorphisms in its gene to be adequately assessed. Alternatively, despite a relevant role in the suppression assay, compensation from other suppression mechanisms employed by Treg might quench the effect of differential production of the cytokine caused by genetic polymorphisms.

5.4.2 The 'Real-time' suppression assays

Based on the arguments presented above, the need for a different system for the functional characterisation of the effect of *TGFB1* polymorphisms on Treg control of immune responses became evident. Since the upregulation of CD103 (α_ϵ integrin) on CD4+CD25- cells which are being suppressed following exposure to TGF-β1 from Treg had been described (Nakamura *et al.*, 2004), a suppression assay where this induction, rather than a coarser ^{3}H incorporation, is assessed could potentially provide a more direct answer to the possibility of an effect of *TGFB1* polymorphism on suppression. CD103, or α_ϵ β7 integrin, is known to mediate lymphocyte retention in epithelial tissues. In mice, it is detected mainly on T cell populations within the mucosal epithelium of the gut and on intestinal lamina propria leukocytes. Its receptor is E-cadherin expressed by epithelial cells, thus reinforcing its role in the retention of lymphocytes in epithelial tissues. However, around 20% of murine Treg in the spleen express this marker (Anz *et al.*, 2011). In fact, only 0.67% of murine spleen CD4+ T cells express CD103, but this proportion increases to 30% in the CD25+LAP+ subset (Chen *et al.*, 2008).

CD103 has been reported to be expressed on CD8+ T cells found in bronchoalveolar lavage of patients with chronic obstructive pulmonary disease and also as intraepithelial lymphocytes in the lungs of healthy individuals (Glader *et al.*, 2005). Moreover, these CD103+ CD8+ cells could be generated *in vitro* by co-culture of peripheral T cells and APC (monocytes) in the presence of TGF-β1. Interestingly, this group also reported that these differentiated T cells down regulated their production of IFN-γ and TNF-α. In addition to exogenous TGF-β1 and monocytes, their activation protocol included soluble anti-CD28, plate-bound anti-CD3, and IL-2, and 2 rounds of stimulation (Glader *et al.*, 2005). CD103 expression on murine Treg has been observed to be present in many murine tumors and Treg expressing CD103 were found to have stronger suppressive capability than their CD103-negative counterparts (Lehmann *et al.*, 2002; Anz *et al.*, 2011). The authors also report that the levels of CD103 expression correlated positively with intra-tumoral TGF-β1 levels, making this a hallmark of tumor-infiltrating Treg.

The fact that no induction of CD103 on suppressed effector CD4+ cells was seen in these experiments could be due to various reasons. First, although the activation used in the

'real-time' suppression assays was similar to that used by Nakamura *et al*. (2004), there remains the possibility that the stimulation was not sufficient to elicit the expression of the integrin on effectors. Also, as mentioned before, the cells in their experiments were murine, while I worked with human cells, and there could be a difference between species. In fact, Gandhi and collaborators found that among freshly isolated human CD4+LAP-, CD4+LAP+ and CD4+LAP-CD25hi cells, only 0.74%, 1.7%, and 1.8% were positive for CD103, respectively (Gandhi *et al.*, 2010). Moreover, the generation of CD103+ CD8+ cells reported by Glader and collaborators (2005) involved the addition of exogenous TGF-β1, whose levels could have been considerably higher than those present in my experiments. Alternatively, despite TGF-β1 being expressed on the surface of Treg present in the suppression assay, the suppressive mechanisms in use in this system could be others (e.g. IL-2 consumption, IL-10 production, cAMP transfer, APC modulation, blocking of Ca^{++} influx, induced effector cell death, etc.) potentially excluding the participation of TGF-β1 and impairing the visualization of CD103 induction. Finally, even if TGF-β1 is participating in the suppression process, its effective quantity might not be high enough for the induction of CD103.

It is important to remark that the differences between the activation and the suppression assays, such as the presence of APC in the system, might affect the levels of activation and of LAP production. The inclusion of APC, in the form of T cell-depleted PBMC has indeed been used by some authors in their characterisation of LAP expression (Nakamura *et al.*, 2001). Consequently, studying LAP production by Treg bearing different *TGFB1* +29 T>C genotypes in the presence of APC was something that required further study. In this respect, the 'real-time' suppression assay proved very useful. As explained before, this approach was initially conceived to determine the point at which CD103 would be induced on effectors being suppressed by TGF-β1. However, since I wanted to correlate this with the appearance of LAP on Treg, surface latent TGF-β1 was also monitored. The results of these observations showed evidence that the kinetics of LAP expression on Treg in the context of the suppression assay (i.e. in the presence of APC and other accessory cells, as well as of effector CD4+ cells) were very similar to those found in the activation experiments depicted in Chapter 4, where the isolated Treg were on their own. The data suggested that the presence of APC and other accessory cells did not modify this phenomenon significantly, which reinforces the validity of the observations made in the activation experiments. However, these experiments showed that there is a strong possibility that the transient nature of the expression of surface LAP could be a mechanism ingrained in the Treg's physiologic response to the TCR stimulus. This leads to the hypothesis that these observations could indeed happen *in vivo*. However, this must be

confirmed. The importance of this observation lies in the potential coordinated control of both the effector cell response and the Treg response. If this mechanism actually exists *in vivo*, then how well it is controlled in normal and in pathogenic or inflammatory conditions could be relevant to the outcome of the immune process.

In conclusion, since the objective of determining the effect of the *TGFB1* +29T>C was not attained, it is clear that other systems could perform better toward this aim and should be considered in future work. Possible options are to study the specific effect of this variation on the inhibition of cytokine production by effector cells. Alternatively, since the control of NK cell function by Treg appears to be mediated directly by TGF-β1 (Barao *et al.*, 2006; Ralainirina *et al.*, 2007; Frimpong-Boateng *et al.*, 2010), a way to test *TGFB1* polymorphism's effect could be the use of an NK-directed suppression assay.

Chapter 6. The effect of *TGFB1* promoter and exon 1 alleles on the outcome of unrelated donor haematopoietic stem cell transplantation

6.1 Introduction and aim

The presence of a significant amount of genetic variation in the regulatory regions 5' of the *TGFB1* gene as well as within its first exon has attracted researchers due to this cytokine's central role in many physiological and pathological processes. As was presented in Chapter 1, the role of *TGFB1* polymorphisms has also been a subject of research in immunogenetics of HSCT. However, the results of many of the clinical studies have not been consistent. The main reasons for these inconsistencies are most likely to be related to the wide variations between the studies' methodologies, target populations and the clinical end-points that are studied. Moreover, most studies have been small, and have focused on a single polymorphism. Different studies have tested the effect of different SNPs, and thus comparison is not always possible. In addition, many studies include patient-donor pairs of a very diverse origin, with wide variations in the cohort in terms of disease type and status, HLA matching, type of transplant, ethnicity, etc. For these reasons, there is need of larger, more homogenous and more comprehensive studies that could shed a light on the question of the role of *TGFB1* polymorphisms in the outcome of UD-HSCT.

Because of this, I decided to carry out a study that would address the limitations of the previous ones, and that would allow us to reach more solid conclusions on this matter. Consequently, I decided to develop a study in which a large and more homogeneous cohort of patient-donor pairs would be typed for all known of *TGFB1* regulatory region polymorphisms. Moreover, I decided not to base my analysis solely on the role of individual SNPs or other forms of genetic polymorphism, but to include the combined effect of all variations in this region.

TGFB1's regulatory region extends from position -2,665 to position +423 taking the major translation start site as +1. As has been stated before, the work of Shah and collaborators allowed for the definition of *TGFB1* regulatory region alleles, which are formed by the

combination of 18 SNPs and other kinds of variation (Shah *et al.*, 2006; Shah *et al.*, 2009). These studies defined the presence of 17 alleles in this region. The data presented in Chapter 3 show that I have increased this number with the discovery of a novel allele during the optimisation of the typing strategy for this genetic region. Consequently, I used this knowledge to define *TGFB1* regulatory region and exon 1 alleles in a cohort of unrelated-donor HSCT patient-donor pairs and tested their association with the outcomes of the transplant. In this chapter, I present the use of the technique I developed in Chapter 3 for the typing of clinical samples, and the results of the statistical analyses of genetic-outcome association.

6.2 Cohort

As explained in Chapter 2, the cohort used for the typing of *TGFB1* regulatory region and exon 1 polymorphisms is part of the Anthony Nolan Research Institute's patient-donor sample repository. This is a well-characterised biobank that stores PBMC, DNA and plasma. Clinical data from every transplant that takes place in the United Kingdom for which an Anthony Nolan-supplied donor was used was obtained from the British Society of Blood and Marrow Transplantation. All of the samples had high-resolution HLA typing data available.

The cohort selected for typing on this study was composed of myeloablative transplants performed between 1996 and 2008. A total of 542 pairs (i.e. 1,084 DNA samples) were selected for this study out of the complete repository. Out of these, 1,024 samples were successfully typed for all regions and known polymorphisms, which corresponded to 502 complete pairs. From the original cohort, 40 samples (i.e. 20 pairs) were excluded because their DNA was no longer available and/or it could not be tested successfully. These samples failed repeatedly during the amplification process, most likely because of the degradation of the DNA. The vast majority of these samples were old and thus probably suffered fragmentation or contamination. For another 20 pairs from the original cohort, typing was possible only for either the donor (11 cases) or the patient (9 cases) because their paired sample was unavailable. Consequently, the pairs with donor typing were 513 while those with patient typing were 512. Of note, out of the 522 patient-donor pairs included for typing of their *TGFB1* regulatory region and exon 1 alleles, clinical data for 18 pairs were not available for analysis. This means that, for analyses in which clinical data were included, the number of eligible pairs was 504.

Although for most samples typed successfully DNA was available from the start, a proportion had to be re-extracted from stored material. As a control for their identity,

these re-extracted samples were also typed for HLA class I as described in Chapter 2. Their HLA genotype was then compared to the data stored for them in the sample repository. All but one of the samples tested had results that agreed with those on the database. The incongruent patient sample was excluded from analysis. The characteristics of the patients, their donors and the transplants are presented in **Table 6.1**.

Table 6.1 Patient, donor and transplant characteristics in the HSCT cohort (n=504).

		N	%
Patient age (years)	Range 0.4-63.8		
	0-20	176	34.9
	20-40	201	39.9
	40-60	126	25.0
	>60	1	0.2
Donor age (years)	Range 19.2-60.4		
	0-20	2	0.4
	20-40	369	73.2
	40-60	132	26.2
	>60	1	0.2
Sex (male)	Patients	322	63.9
	Donors	382	75.8
Sex matching	Patient-donor		
	Male-male	256	50.8
	Male-female	66	13.1
	Female-female	56	11.1
	Female-male	126	25.0
HLA-matching	10/10 matched	358	71.0
	1 mismatch	100	19.8
	>1 mismatch	46	9.1
Disease	AML	136	27.0
	ALL	155	30.8
	CML	111	22.0
	MDS	45	8.9
	Other[1]	57	11.3
Disease status	Complete remission/chronic phase	417	82.7
	Other	77	15.3
	Unknown	10	2.0
CMV status	Patient(+)-donor(+)	64	12.7
	Patient(+)-donor(-)	88	17.4
	Patient(-)-donor(+)	51	10.1
	Patient(-)-donor(-)	282	56.0
	Unknown	19	3.8
TBI	Present	434	86.1
	Absent	55	10.9
	Unknown	15	3.0
SC source	BM	340	67.5
	PB	159	31.5
	Both	2	0.4
	Unknown	3	0.6
T cell depletion	Yes	427	84.7
	No	22	4.4
	Unknown	55	10.9
GVHD prophylaxis	None	4	0.8
	Cyclosporin	131	26.0
	Cyclosporin+MTX	329	65.3
	Other[2]	28	5.6
	Unknown	12	2.4

1 Includes secondary acute leukaemia, Non-Hodgkin lymphoma, primary immune deficiency, bone marrow failure, multiple myeloma, metabolic disease, myeloproliferative neoplasia, biphenotypic acute leukaemia, Hodgkin's disease, undifferentiated acute leukaemia.
AML, acute myeloid leukaemia; ALL, acute lymphoid leukaemia; CML, chronic myeloid leukaemia; BM, bone marrow; CMV, cytomegalovirus; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; MDS, myelodysplastic syndrome; MTX, methotrexate; PB, peripheral blood; SC, stem cell; TBI, total body irradiation.

6.3 Descriptive results for the typing of *TGFB1* regulatory region and exon 1 alleles in the patient-donor cohort

The samples for which DNA was available were subject to typing using the strategy described in Chapter 3. In brief, the genetic material was used to separately amplify 4 sub-regions of *TGFB1*'s regulatory region and exon 1. These 4 amplicons were then purified in order to be used as templates for bidirectional Sanger sequencing using the M13 tags attached to the amplification primers. The sequences were then analysed and the genotypes for each of the 18 basic polymorphic positions were recorded and input in a database. Additionally, the rest of the sequence was scanned for the presence of potentially novel polymorphisms outside the basic 18 sites. Based on the genotype at these basic polymorphic positions and the alleles described by Shah and collaborators, the allele combination for each sample was inferred, and an allelic genotype was assigned to each patient or donor.

In cases where theoretical ambiguities existed, the phase of the relevant polymorphic positions was defined by allele-specific amplification strategies using different primer combinations and following either the standard amplification protocol or the long-range amplification protocol used for the cloning of the rare allele described in Chapter 3. For example, when the genotype included a heterozygous result for the -2389dupAGG, then the -2389dupAGG-specific primers were used instead of the RI-F primer in combination with any of the reverse primers. The choice of reverse primer depended on the location of the relevant polymorphisms in order to create a -2389AGG-specific amplicon that would set their phase. Likewise, the +29 T>C-specific primers were also used to amplify either the +29T-bearing or the +29C-bearing allele and set the phase of other relevant polymorphisms that would define the allelic combination.

Ten percent of the samples were selected to confirm their typing results. For this, a new amplification from the stock DNA was carried out and sequencing of Region IV was repeated. All of these samples had results that were consistent with the original ones.

Overall, the 1,024 samples typed showed polymorphism for 9 of the 18 previously known variable positions. These were -2410A>G, -2389dupAGG, -1638G>A, -1347C>T, -1169delTT, -778G>A, -768insC, +29T>C and +74G>C. The results for the variant and genotype frequencies observed for each of these positions are shown in **Table 6.2**. Two of these positions (-1169delTT and -778G>A) showed extremely rare variation, leaving the other 7 positions as the most frequently polymorphic. It is important to remember that 5

out of the 18 basic positions are specific for allele p005. Consequently, of the remaining 13 positions, only 4 positions did not show polymorphism in this cohort.

Deviation from Hardy-Weinberg equilibrium (HWE) was tested for 5 of the SNPs (**Table 6.3**). The two most infrequent polymorphisms were excluded because of their rarity. From polymorphisms that show the same observed genotypic frequencies (i.e. -2389dupAGG and -1347C>T, and -768insC and+74G>C) only one was selected. *TGFB1* +29T>C showed a significant deviation from HWE (p=0.03) caused by an excess of heterozygotes. Deviation from HWE was also tested for the 4 major *TGFB1* regulatory region and exon 1 alleles (**Table 6.4**). The genotypic distributions were found not to be significantly deviated from expected frequencies (p>0.25).

These polymorphic positions allowed for the definition of the allelic genotypes for each patient-donor pair. Among the 17 previously defined regulatory region and exon 1 alleles, only 6 were seen in the cohort: p001, p003, p006, p009, p013, and p014. Moreover, only 4 were the predominant ones: p003 (53.71%), p001 (29.35%), p014 (8.25%), and p006 (8.11%). p019 and p013 were only seen in 1 and 2 samples, respectively. **Table 6.5** shows the frequencies of each of these alleles in patients, donors, and the whole cohort. As shown in this table, 80% of the subjects carried a p003 allele and 50% carried a p001 allele, making these two alleles by far the predominant ones both in patients and in donors.

The genotype frequencies observed in the cohort are presented in **Table 6.6**. Twelve genotypes formed by the known *TGFB1* regulatory region and exons 1 were observed. Both the allele and the genotype frequencies did not differ significantly between patients and donors (Z test; p>0.05).

Some of the variants at the known polymorphic positions were very much allele-specific. For example, the -768insC and the +74C variants were always found together, accounting for the presence of the p014 allele. This tight linkage was also reported by other groups (Awad *et al.*, 1998; Shah *et al.*, 2006). Other polymorphic sites such as -1347C>T and the 29T>C show variants that are more promiscuous owing to their presence in more than one allele.

It was not possible to solve the genotype of 9 samples based on the previously known 17 *TGFB1* regulatory region and exon 1 alleles. These samples were typed again in order to confirm the preliminary results. The results were confirmed, and this consequently suggested the presence of a new allele formed by a previously unknown combination of the basic 18 polymorphic positions. As will be explained in the next section, this was confirmed to be the case. Consequently, 9 samples carried a combination of a known allele and a novel one. The known allele was p001 in 4 cases, p003 in 3 cases and p014 in 2 cases. These rare alleles were found in 5 donor samples and 4 patient samples.

Table 6.2 Variant and genotype frequencies for known *TGFB1* regulatory region and exon 1 polymorphisms found in the UD-HSCT patient-donor cohort.

Polymorphic position	Variants and genotypes	Whole cohort		Patients		Donors		Z	p value
		count	frequency	count	frequency	count	frequency		
-2410G>A	G	1269	**0.6196**	632	**0.6184**	637	**0.6209**	0.1	0.91
	A	779	**0.3804**	390	**0.3816**	389	**0.3791**	0.1	0.91
	GG	378	**0.3691**	186	**0.3640**	192	**0.3743**	0.3	0.73
	AG	513	**0.5010**	260	**0.5088**	253	**0.4932**	0.5	0.62
	AA	133	**0.1299**	65	**0.1272**	68	**0.1326**	0.3	0.80
-2389dupAGG	AGG	1439	**0.7026**	712	**0.6967**	727	**0.7086**	0.6	0.56
	…	609	**0.2974**	310	**0.3033**	299	**0.2914**	0.6	0.56
	AGG/AGG	499	**0.4873**	246	**0.4814**	253	**0.4932**	0.4	0.71
	AGG/…	441	**0.4307**	220	**0.4305**	221	**0.4308**	0.0	0.99
	…/…	84	**0.0820**	45	**0.0881**	39	**0.0760**	0.7	0.49
-1638G>A	G	1882	**0.9189**	945	**0.9247**	937	**0.9133**	0.9	0.34
	A	166	**0.0811**	77	**0.0753**	89	**0.0867**	0.9	0.34
	GG	863	**0.8428**	438	**0.8571**	425	**0.8285**	1.3	0.21
	GA	156	**0.1523**	69	**0.1350**	87	**0.1696**	1.5	0.12
	AA	5	**0.0049**	4	**0.0078**	1	**0.0019**	N/A	N/A
-1347C>T	C	1439	**0.7026**	712	**0.6967**	727	**0.7086**	0.6	0.56
	T	609	**0.2974**	310	**0.3033**	299	**0.2914**	0.6	0.56
	CC	499	**0.4873**	246	**0.4814**	253	**0.4932**	0.4	0.71
	CT	441	**0.4307**	220	**0.4305**	221	**0.4308**	0.0	0.99
	TT	84	**0.0820**	45	**0.0881**	39	**0.0760**	0.7	0.49
-1169delTT	TT	2046	**0.9990**	1020	**0.9980**	1026	**1.0000**	N/A	N/A
	..	2	**0.0010**	2	**0.0020**	0	**0.0000**	N/A	N/A
	TT/TT	1022	**0.9980**	509	**0.9961**	513	**1.0000**	N/A	N/A
	TT/..	2	**0.0020**	2	**0.0039**	0	**0.0000**	N/A	N/A
	../..	0	**0.0000**	0	**0.0000**	0	**0.0000**	N/A	N/A
-778G>A	G	2047	**0.9995**	1022	**1.0000**	1025	**0.9990**	N/A	N/A
	A	1	**0.0005**	0	**0.0000**	1	**0.0010**	N/A	N/A
	GG	1023	**0.9990**	511	**1.0000**	512	**0.9981**	N/A	N/A
	GA	1	**0.0010**	0	**0.0000**	1	**0.0019**	N/A	N/A
	AA	0	**0.0000**	0	**0.0000**	0	**0.0000**	N/A	N/A
-768insC	.	1879	**0.9175**	943	**0.9227**	936	**0.9123**	0.9	0.39
	C	169	**0.0825**	79	**0.0773**	90	**0.0877**	0.9	0.39
	./.	862	**0.8418**	436	**0.8532**	426	**0.8304**	1.0	0.32
	./C	155	**0.1514**	71	**0.1389**	84	**0.1637**	1.1	0.27
	C/C	7	**0.0068**	4	**0.0078**	3	**0.0058**	N/A	N/A
+29T>C	T	1275	**0.6226**	635	**0.6213**	640	**0.6238**	0.1	0.91
	C	773	**0.3774**	387	**0.3787**	386	**0.3762**	0.1	0.91
	TT	380	**0.3711**	187	**0.3659**	193	**0.3762**	0.3	0.73
	TC	515	**0.5029**	261	**0.5108**	254	**0.4951**	0.5	0.62
	CC	129	**0.1260**	63	**0.1233**	66	**0.1287**	0.3	0.79
+74G>C	G	1879	**0.9175**	943	**0.9227**	936	**0.9123**	0.9	0.39
	C	169	**0.0825**	79	**0.0773**	90	**0.0877**	0.9	0.39
	GG	862	**0.8418**	436	**0.8532**	426	**0.8304**	1.0	0.32
	GC	155	**0.1514**	71	**0.1389**	84	**0.1637**	1.1	0.27
	CC	7	**0.0068**	4	**0.0078**	3	**0.0058**	N/A	N/A

Only polymorphic positions are shown. del, deletion; dup, duplication; ins, insertion. A dot (.) indicates a deletion or the absence of insertion. Z test and associated probability (p) for the comparison of frequencies between patient and donor subsets are shown. N/A, non-applicable.

Table 6.3 **Analysis of Hardy-Weinberg equilibrium for selected *TGFB1* regulatory region and exon 1 SNPs in the patient-donor cohort.**

Polymorphic position	Genotype	Observed numbers	Expected numbers	p value (Fisher's exact test)
-2410G>A	GG	378	393.15	0.05
	AG	513	482.69	
	AA	133	148.15	
-1347C>T	CC	499	505.55	0.37
	CT	441	427.91	
	TT	84	90.55	
+29T>C	TT	380	396.88	0.03
	TC	515	481.24	
	CC	129	145.88	
+74G>C	GG	862	861.97	1.00
	GC	155	155.05	
	CC	7	6.97	

Table 6.4 **Analysis of Hardy-Weinberg equilibrium for *TGFB1* regulatory region and exon 1 alleles in the patient-donor cohort.**

Genotype	Observed numbers	Expected numbers	p value (Chi^2)
p001/p001	81	88.05	>0.25
p001/p003	346	322.69	
p001/p006	49	48.96	
p001/p014	40	49.26	
p003/p003	280	295.66	
p003/p006	91	89.72	
p003/p014	97	90.27	
p006/p006	5	6.81	
p006/p014	16	13.70	
p014/p014	7	6.89	

Table 6.5 ***TGFB1*** **regulatory region and exon 1 allele frequencies found in the UD-HSCT patient-donor cohort.**

Allele	Whole cohort				Patients				Donors				Z	p value
	Frequency	Copies	Carriers	Carrier frequency	Frequency	Copies	Carriers	Carrier frequency	Frequency	Copies	Carriers	Carrier frequency		
p001	**0.2935**	601	520	0.5078	**0.3004**	307	264	0.5166	**0.2865**	294	256	0.4990	0.7	0.49
p003	**0.5371**	1100	820	0.8008	**0.5411**	553	415	0.8121	**0.5331**	547	405	0.7895	0.4	0.72
p006	**0.0811**	166	161	0.1572	**0.0753**	77	73	0.1429	**0.0867**	89	88	0.1715	0.9	0.34
p009	**0.0005**	1	1	0.0010	**0.0000**	0	0	0.0000	**0.0010**	1	1	0.0019	N/A	N/A
p013	**0.0010**	2	2	0.0020	**0.0020**	2	2	0.0039	**0.0000**	0	0	0.0000	N/A	N/A
p014	**0.0825**	169	162	0.1582	**0.0773**	79	75	0.1468	**0.0877**	90	87	0.1696	0.9	0.39
Total	**0.9956**	**2039**			**0.9961**	1018			**0.9951**	1021				

Z test and associated probability (p) for the comparison of frequencies between patient and donor subsets are shown. N/A, non-applicable.

Table 6.6 *TGFB1* regulatory region and exon 1 genotype frequencies found in the UD-HSCT patient-donor cohort.

Genotype	Whole cohort		Patients		Donors		Z	p value
	count	frequency	count	frequency	count	frequency		
p001/p001	81	**0.0791**	43	**0.0841**	38	**0.0741**	0.6	0.55
p001/p003	346	**0.3379**	182	**0.3562**	164	**0.3197**	1.2	0.22
p001/p006	49	**0.0479**	21	**0.0411**	28	**0.0546**	1.0	0.31
p001/p014	40	**0.0391**	16	**0.0313**	24	**0.0468**	1.3	0.20
p003/p003	280	**0.2734**	138	**0.2701**	142	**0.2768**	0.2	0.81
p003/p006	91	**0.0889**	43	**0.0841**	48	**0.0936**	0.5	0.59
p003/p014	97	**0.0947**	49	**0.0959**	48	**0.0936**	0.1	0.90
p006/p006	5	**0.0049**	4	**0.0078**	1	**0.0019**	N/A	N/A
p006/p014	16	**0.0156**	5	**0.0098**	11	**0.0214**	1.5	0.13
p014/p014	7	**0.0068**	4	**0.0078**	3	**0.0058**	N/A	N/A
p003/p009	1	**0.0010**	0	**0.0000**	1	**0.0019**	N/A	N/A
p003/p013	2	**0.0020**	2	**0.0039**	0	**0.0000**	N/A	N/A
Other[1]	9	**0.0088**	4	**0.0078**	5	**0.0097**	N/A	N/A
Total	1024	**1.0000**	511	**1.0000**	513	**1.0000**		

[1] Other genotypes formed by one previously described allele and one of three novel alleles found in the cohort. Z test and associated probability (p) for the comparison of frequencies between patient and donor subsets are shown. N/A, non-applicable.

6.4 Novel variants and alleles within *TGFB1* regulatory region and exon 1

During the typing of the 1,024 samples in the UD-HSCT patient-donor cohort a number of novel variants as well as rare *TGFB1* regulatory region and exon 1 alleles were discovered. The novel variant positions were caused by SNPs in all cases. If the polymorphism was confirmed and the presence of a confounding factor (such as excessive background noise in the sequence or a descaled individual fluorescence bleb), the sample was selected for further testing. In a first round of confirmation experiments, all potential new SNPs were confirmed by reamplifying the relevant sub-region anew, and sequencing this new fragment. Of all novel SNPs identified in a primary amplification, only 1 was not found in the fresh amplicon. In this case, this was deemed to be an artifact of the original sequence.

If all other positions in the sample were homozygous (i.e. *TGFB1* regulatory region and exon 1 allele homozygosity), then the SNP was deemed to be carried by this previously known allele. If the sample had a heterozygous allelic genotype (e.g. p001/p003), then further studies were carried out to define the phase of this novel variant. In order to set the phase of the SNP and to identify the context (i.e. the previously known allele) in which it was borne, the relevant sub-region or sub-regions were amplified with allele-specific primer combinations using the -2389dupAGG and the +29T>C-specific primers in a manner similar to that employed for allele ambiguity solving explained above. Most of the allelic phases could be defined using this strategy. However, in a small number of cases full (RI-RIV) amplification and subsequent cloning of the alleles was required to identify which one bore the novel SNP. The whole-region amplification, cloning and sequencing was carried out as explained in Chapters 2 and 3.

Overall, 16 rare SNPs were identified in 37 samples. Consequently, 3.6% of the samples in the cohort carried SNPs other than the basic 18 polymorphisms. Six of these rare SNPs were seen in more than 1 sample, and always in the context of the same allele, thus confirming their presence in the population, albeit at low frequencies. Of note, one of these rare SNPs, -1569C>T, had a frequency of 1.36% among the 1,024 samples.

One sample carried both a -1569C/T and a -328T/C. The 16 novel/rare SNPs are presented in **Table 6.7**. Their location in the context of *TGFB1*'s regulatory region and exon 1 is shown in **Figure 6.1**. Six novel SNPs were found to be borne in a p001 allele, while 7 were in the context of a p003 allele, and 2 were carried by a p014. Of note, one of the rare SNPs, -1189C>T, was seen twice in the context of a new *TGFB1* regulatory region and exon 1 allele (Z allele, see below), strongly confirming the existence of both.

Table 6.7 Novel (rare) SNPs within *TGFB1*'s regulatory region and exon 1 discovered during the typing of 1,024 patient and donor samples in the UD-HSCT cohort.

Novel variant	Times observed	Allelic context	Region	Coding effect
-2316C>A	1	p001	enhancer 2	N/A
-2219G>C	1	p003	enhancer 2	N/A
-2069G>A	4	p001	negative regulatory region 2	N/A
-2001C>G	1	p003	negative regulatory region 2	N/A
-1922C>T	1	p001	enhancer 1	N/A
-1569C>T	13	p003	negative regulatory region 1	N/A
-1189C>T	2	'Z'	promoter 1	N/A
-535C>T	1	p003	5'UTR	N/A
-498C>G	1	p003	5'UTR	N/A
-328C>T	3	p003	5'UTR	N/A
-265C>G	1	p001	5'UTR	N/A
-144G>A	1	p001	5'UTR	N/A
-73G>A	3	p014	5'UTR	N/A
+122T>G	1	p003	exon 1	codon 41, Val->Gly
+149G>C	1	p014	exon 1	codon 150, Arg->Pro
+348C>T	3	p001	exon 1	codon 116, silent

In addition to the discovery of new/rare SNPs, 3 rare recombinant alleles of the previously known 18 polymorphic positions were identified in this cohort. These samples showed genotypes that were incompatible with any of the possible combinations of known *TGFB1* regulatory region and exon 1 alleles. These samples were first typed again due to the possibility of a genotyping error at any of the stages. All 4 sub-regions for each sample were thus re-amplified and sequenced again. When the individual genotypes were confirmed, further studies were carried out. As was mentioned before, this was the case of 9 out of the 1,024 samples typed in this study.

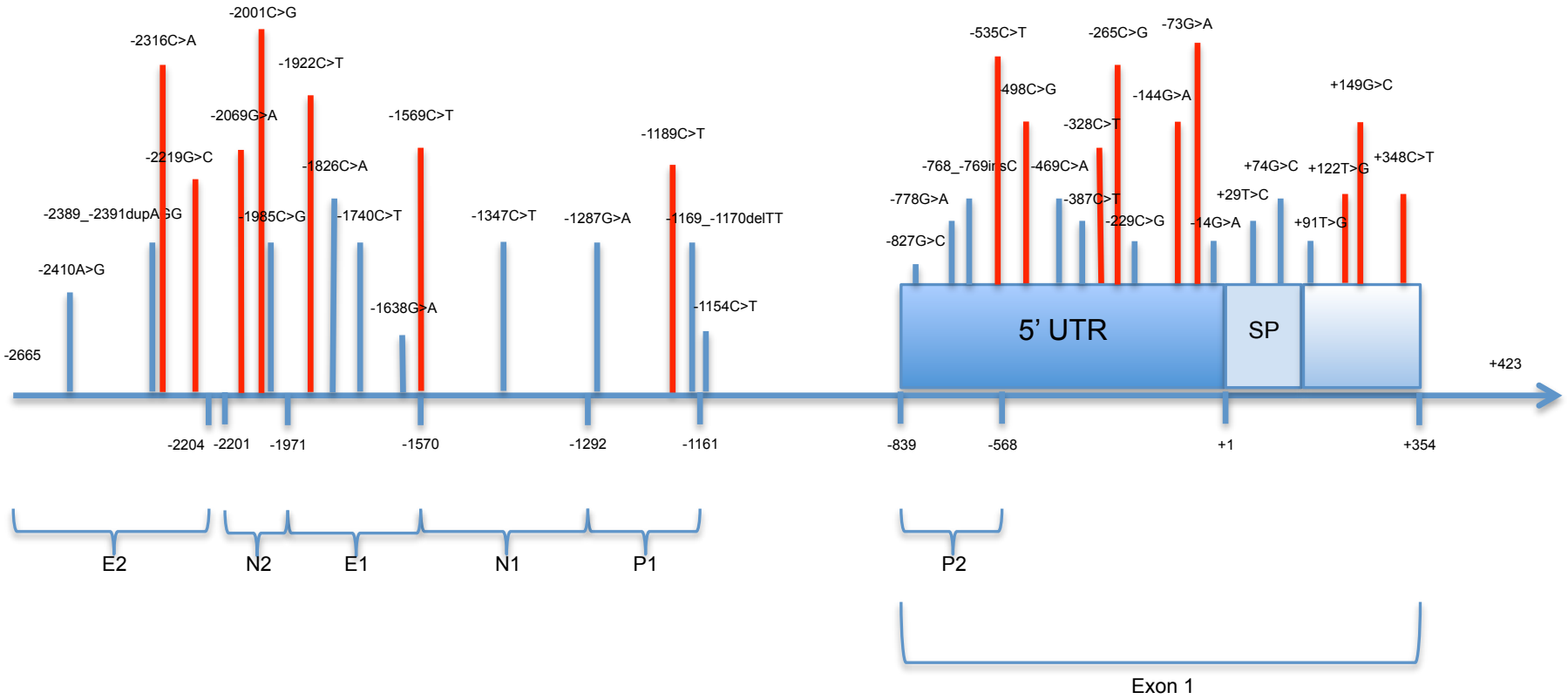

Figure 6.1 Novel variants in *TGFB1* regulatory region and exon 1. The gene's regulatory region and exon 1 analysed in this study is shown schematically and not to scale. The translation start site is indicated as +1. Previously known variants that define known alleles are shown in blue, while novel variant positions discovered in this study are shown in red. The locations of enhancers (E), negative regulatory regions (N) and promoters (P) are shown. 5'UTR, 5' untranslated region; SP, signal peptide.

These samples were then subject to allele-specific amplifications as explained above. Among these 9 samples (5 donors and 4 patients) two of the three recombinant alleles were seen in more than one sample. Rare allele 'X' was observed in 6 samples, while alleles 'Y' and 'Z' were seen in 1 and 2 samples, respectively. Their novel combinations are shown in **Table 6.8**. Of note, allele 'Z' is equivalent to that discovered in the healthy donor sample family study explained in Chapter 3, thus confirming the previous results in completely unrelated samples. As shown in **Table 6.8**, the 'X' allele is most similar to allele p001, except for its +29T. The 'Y' allele is also similar to p001, but shows a -2410G in addition to a +29T. The 'Z' allele seems to have an upstream half similar to p001, but its proximal half lacks the -768insC and the +74C variant.

6.5 Models of analysis of the effect of *TGFB1* regulatory region and exon 1 polymorphism on the outcome of haematopoietic stem cell transplantation with unrelated donors (UD-HSCT)

Prior to any statistical analysis being carried out, I devised the strategies that would lead to the study of the effect of *TGFB1* regulatory region and exon 1 polymorphisms on UD-HSCT outcomes. The rationale behind them was based on the study of the effect of alleles, single polymorphisms, and genotypes in the typed cohort. In this way, both the presence of combined effects of different polymorphisms, of effects due to a single polymorphism shared by various alleles, and the presence of combinatory effects of different alleles carried by any subject could be tested. The models were then classified in 4 major categories:

1. Allele-specific effects
2. SNP-specific effects
3. Genotype-specific effects
4. Reduced genotype effects

These ways of organizing the molecular data would be tested at different source levels: the patient effect, the donor effect, and a 'matching' effect. Patient and donor models would test the effect of the specific model when the variables were carried by either the patient or the donor. On the contrary, the 'matching' models would test the effect of the patient-donor pair having none or several copies of the same genetic variable within the pair.

In addition to the source models, the allele-specific and the SNP-specific categories would be tested in dominant and recessive models, in order to test the preeminence of one variable over the other. In the case of the 'matching' model, dominant models would mean that 2 or more copies of the allele or SNP would be carried by the patient-donor pair, while the recessive model would determine the potentially additive effect of having 4 copies of that allele or SNP in one patient-donor pair.

In the allele-specific models, the analysis would be reduced to the 4 major alleles (i.e. p001, p003, p006 and p014). This would mean 4 separate analyses for the dominant model (one for each allele) in which each analysis would test the presence of one allele, in a homo or heterozygous way, against the absence of it. However, for the recessive model, one analysis with 5 sub-groups formed by the samples having 2 copies of 1 of each of the 4 major alleles and the addition of a 'heterozygous' sub-group would be carried out.

In the SNP-specific models, a similar organization was followed, albeit with 2 subgroups for the dominant models (variant present, variant absent), and 3 for the recessive model (homozygous variant A, homozygous variant B, heterozygous). For the SNP analyses, I selected the -1347C>T, +29T>C for testing. These 2 SNPs were chosen because of their capacity to group alleles that bear the same variants at these positions. Also, the use of SNP-specific analyses would allow for the incorporation of samples bearing alleles p009, p013, 'X', 'Y' and 'Z'. Both recessive and dominant models for +29T>C would be then tested. However, only recessive models would be set up for the minor variant of the other SNP (i.e. -1347T) because dominant models would add no additional information since they would be equivalent to the dominant models for p001 (-1347T is nearly specific for p001). In turn, dominant models for the major variant -1347C would allow for the indirect testing of the allelic effect of p001 against the rest of the alleles together (i.e. one allele vs the rest), thus being a complement to the recessive allelic models that would test homozygosity for this allele against separate categories for the other alleles (i.e. allele A vs allele B vs allele C, etc). In all allele and SNP-specific recessive models, alleles p006 and p014 would be excluded because of their low homozygous frequency.

The genotype models would include those genotypes for which sufficient numbers occur in the cohort. The 7 most common genotypes would be selected, and the rest of them would be grouped in an 'other' category'. Again, patient and donor analysis would be carried out for the genotype models.

Finally, the reduced genotype models would classify the samples according to their -1347C>T and +29T>C genotypes. In this way, the alleles could be grouped in slightly more categories than those generated by the SNP analysis, and, once again, incorporating

samples bearing alleles p009, p013, 'X', 'Y' and 'Z'. **Figure 6.2** shows a schematic summary of the statistical analysis structure devised for this study.

The study plan was to initially analyse the potential effect of the aforementioned models in a univariate manner on OS as a primary endpoint. Any significant association of *TGFB1* regulatory region and exon 1 polymorphism and OS would then be further explored by analysing its effect on EFS, NRM and relapse incidence in an attempt to isolate any effect in terms of these mortality measures. Further significant associations with any of these secondary endpoints would then be analysed for specific cause of death such as aGVHD. In parallel, clinical patient, donor and transplant-related characteristics would also be analysed in univariate models for their association with the relevant outcomes. Finally, any *TGFB1* genetic associations would be tested in combination with significantly associated clinical variables in multivariate models.

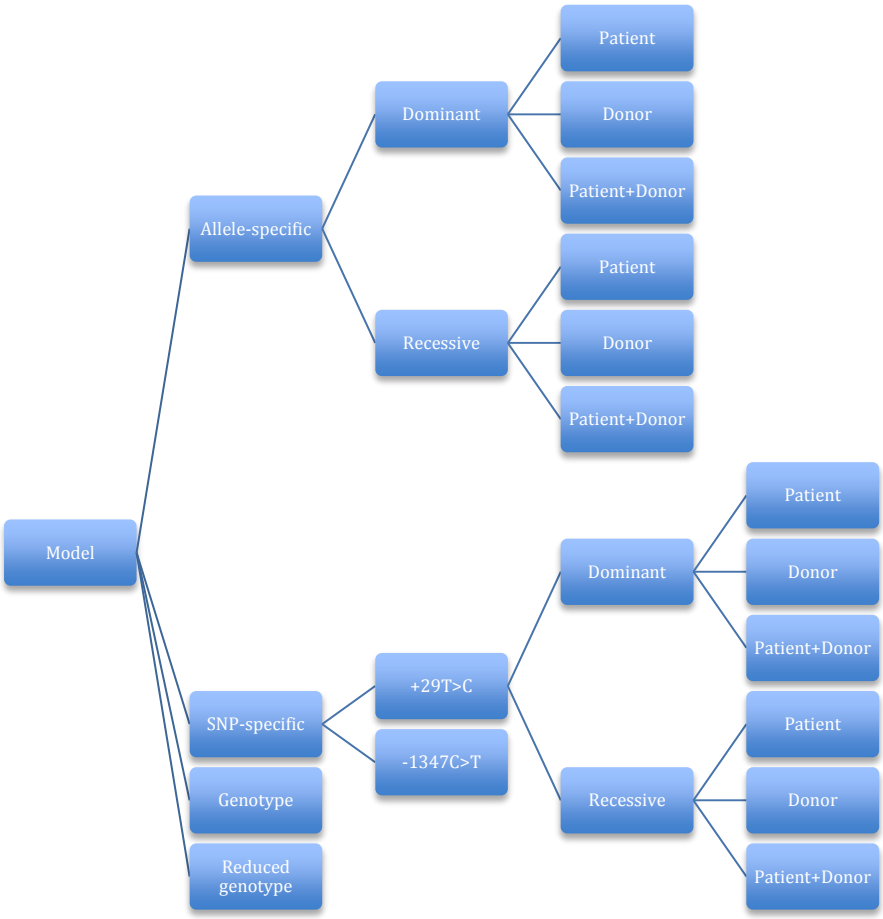

Figure 6.2 Schematic representation of the genetic model study plan. The effect of *TGFB1* regulatory region and exon 1 polymorphism would be tested in allele, SNP, genotype and reduced genotype-based models. For each of these, recessive and dominant models would also be tested. These recessive or dominant models would in turn be tested for patient, donor and 'matching' (i.e. patient+donor) effects on OS. Models for the -1347C>T SNP, genotype and reduced genotype models are not shown for space reasons.

Table 6.8 **Novel *TGFB1* regulatory region and exon 1 alleles discovered during the typing of 1,024 patient and donor samples in the UD-HSCT cohort.**

Allele[b]	Position[a]																	
	-2410	-2391dupAGG	-1985	-1638	-1347	-1287	-1169[c]	-1154	-827	-778	-768insC	-469	-387	-229	-14	+29	+74	+91
p001	A	-	C	G	T	G	TT	C	G	G	-	C	C	C	G	C	G	T
p003	G	AGG	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p006	G	AGG	-	A	C	-	-	-	-	-	-	-	-	-	-	T	-	-
p009	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
p013	G	AGG	-	-	C	-	del	-	-	-	-	-	-	-	-	T	-	-
p014	-	AGG	-	-	C	-	-	-	-	-	C	-	-	-	-	-	C	-
'X'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-
'Y'	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-
'Z'	-	AGG	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-

a. Nucleotide position relative to the major translation start site (+1) as described in Shah R, *et al*. Human Genet (2006) 119: 61-74 and Shah R, *et al*. Tissue Antigens (2009) 74: 50-6. Only known polymorphic positions are included.
b. Promoter allele defined by the sequence of *TGFB1* regulatory region and exon 1. Sequences are compared to allele p001 (GenBank accession no. AY871232).
c. Indicates deletion of nucleotide(s) (del).

6.6 Results

The results for the statistical analyses performed on the outcome of the UD-HSCT cohort are presented here. Of note, of the 522 patient-donor pairs included for typing of their *TGFB1* regulatory region and exon 1 alleles, clinical data for 18 pairs were not available for analysis. Consequently, the total number of pairs included in each of the models varies since these pairs had to be excluded from the analysis.

6.6.1 Analysis of survival

Median follow up in the cohort was 20.5 months (range 0.2-178.9). Five-year OS in the complete cohort was 40.9%. Median OS was 21.6 months (95%CI=11.5-31.6). Five-year EFS in the whole cohort was 30.4%. Median EFS was 9.9 months (95%CI=7.6-12.2 months). One-year cumulative incidence for NRM was 26.8% in the whole cohort. Five-year relapse cumulative incidence was 39.0%. Median time to relapse was 51.6 months (95%CI=9.5-93.8 months). The results for the analyses on OS, EFS, NRM and relapse according to *TGFB1* regulatory region and exon 1 polymorphism are presented in the next sections.

6.6.1.1 Allele-specific models

6.6.1.1.1 Recessive models

As explained before, when the recessive effect of *TGFB1* regulatory region and exon 1 alleles was tested on the whole cohort alleles p006 and p014 were excluded from analysis because of the reduced numbers of homozygous samples. The categories were then reduced to p001, p003 and heterozygous samples. The results for the patient, donor and matching models are shown below.

Patient *TGFB1* regulatory region and exon 1 had a significant effect on the OS of the entire cohort. When the homozygosity for p001, p003 and heterozygosity was compared, significant differences were found (n=486; p=0.04). The results showed that patients homozygous for p003 had the highest median OS (43.8 months), while patients homozygous for the p001 allele had the lowest median OS (7.9 months). When pair-wise comparisons were considered, there was a significant difference between patients homozygous for p001 and p003 (p=0.01), and a trend between p001 and the heterozygous group (p=0.07; median OS 19.5 months). The survival curve for this model is shown in **Figure 6.3**.

When the effect of donor alleles was tested, no differences in OS were found (n=491; p=0.47). The results for the donor effect in the recessive allele model are shown in **Figure 6.4**.

When the matching model was applied to the recessive allele model, pairs in which both patient and donor were homozygous for allele p001 appeared to fare the worst, but this was not confirmed statistically (n=484; p=0.42). However, the number of p001-matched pairs was very small (4 pairs). The results without the p001-matched pairs did not reach statistical significance either despite higher OS for those pairs matched for p003 (n=38; p=0.40).

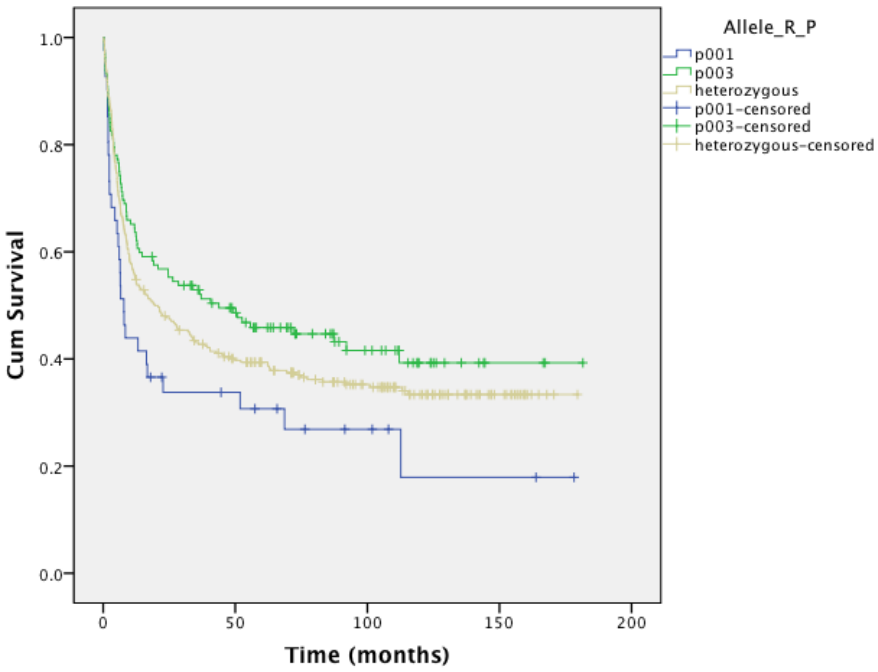

Figure 6.3 Survival analysis according to recessive effects of patient *TGFB1* regulatory region and exon 1 allele for the whole cohort. Patients homozygous for allele p001 show significantly worse overall survival (OS) when compared to patients homozygous for p003. A trend for lower OS in patients homozygous for p001 was also found when compared to heterozygous patients.

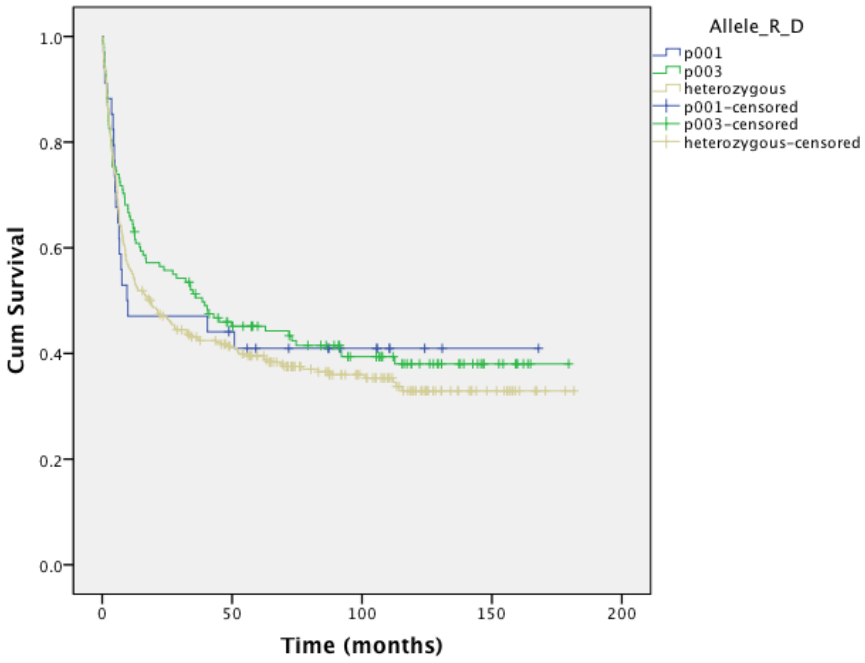

Figure 6.4 Survival analysis according to donor *TGFB1* regulatory region and exon 1 allele in a recessive model for the whole cohort. The genotype for donors does not impact on overall survival in this model.

6.6.1.1.2 Dominant models

When the effect of the patient *TGFB1* allele was tested in a dominant model, none of the alleles showed a significant effect on OS when compared to other allelic combinations lacking the allele of interest (n=493). The results for these analyses are shown in **Figure 6.5**.

When the dominant allele models were tested for the donor effect in the whole cohort, a significant impact of p001 was found on OS (n=495; p=0.04). Patients whose donors carried at least one copy of p001 had worse OS than patients whose donor lacked this allele (median OS 13.7 vs 39.5 months, respectively). Alleles p003, p006 and p014 did not have statistically significant dominant donor effects on OS in this cohort (**Figure 6.7**).

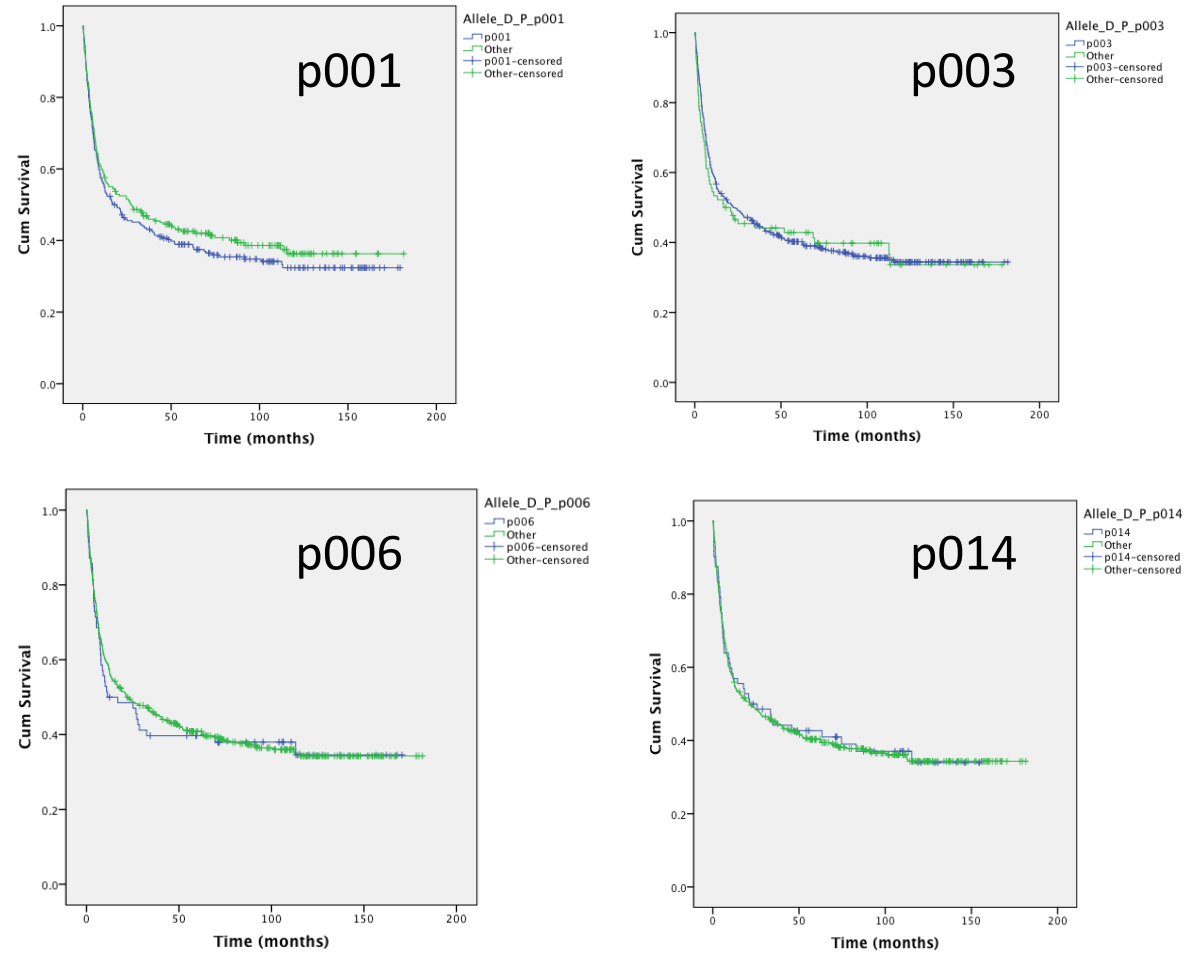

Figure 6.5 The effect of patient *TGFB1* regulatory region and exon 1 alleles on overall survival (OS) in a dominant model in the full cohort. The presence of alleles p001, p003, p006, and p014 in patients (blue curve) was tested in a dominant model (n=493). None of the alleles show an effect on OS when analysed in this manner. Top left plot: p001 (n=255), top right plot: p003 (n=402). Bottom right plot: p006 (n=71), bottom left plot: p014 (n=72).

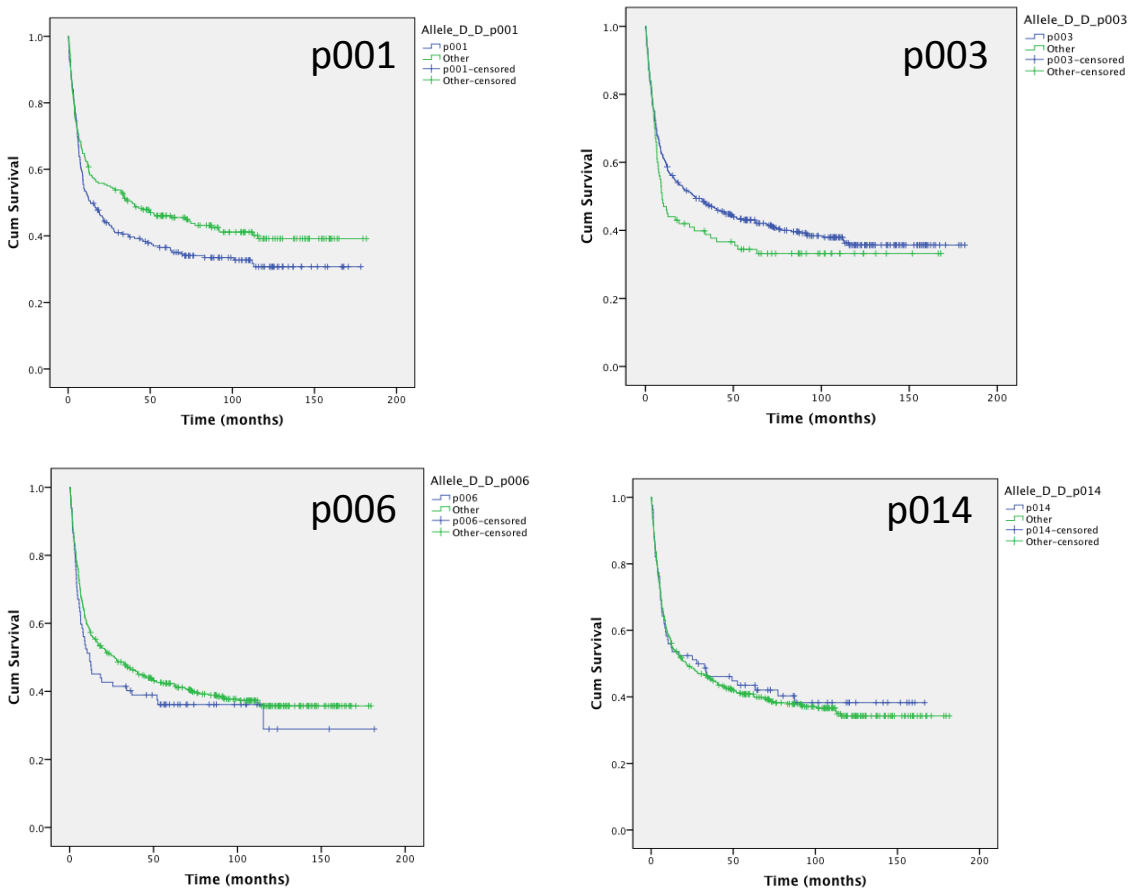

Figure 6.6 The effect of donor *TGFB1* regulatory region and exon 1 alleles on overall survival (OS) in a dominant model in the full cohort. The presence of alleles p001, p003, p006, and p014 in donors (blue curve) was tested in a dominant model (n=495). The presence of p001 in a dominant manner in donors significantly reduced the median OS of patients transplanted with these donors (median OS 13.7 months vs. 39.5 months for 'others', p=0.04). None of the other alleles show an effect on OS when analysed in this manner. Top left plot: p001 (n=245), top right plot: p003 (n=394). Bottom right plot: p006 (n=83), bottom left plot: p014 (n=84).

When the matching model was applied to the dominant allele analysis models, no significant impact on OS was found in the complete cohort. Matching in this dominant model would be equivalent to there being at least 2 copies of a specific allele in the patient donor pair (1 in the patient and 1 in the donor), while 'not matched' implies that there is only one copy of the allele within the pair. The 'other' category is equivalent to no copies of that allele within the pair. Despite statistical confirmation, there appears to be a gradual worsening of OS for patient-donor pairs that have increasing number of copies of allele p001 within the pair.

6.6.1.2 SNP-specific models

6.6.1.2.1 *TGFB1 +29T>C*

6.6.1.2.1.1 Recessive models

The effect of *TGFB1* +29T>C on OS was tested in the whole cohort. When patient effects were tested (n=493), patients homozygous for the C variant seemed to have lower OS, but this was not confirmed statistically (p=0.24). **Figure 6.7** shows the results for this analysis.

When donor effects were tested in a recessive model (n=495), no effect of the donor genotype for this SNP was found on OS in the complete cohort (p=0.38). **Figure 6.8** shows the result for this analysis.

When the matching model was applied to the recessive model for *TGFB1* +29 T>C, there was no difference in the OS in 'matched', 'non-matched' and 'other' groups (n=484; p=0.60). The 'matching' for the TT genotype (i.e. 4 copies of the T variant in the pair) seemed to have a better OS. The number of pairs matched for the CC genotype was very small (n=10).

6.6.1.2.1.2 Dominant models

When the effect of patient *TGFB1* +29T>C was tested in a dominant model in the whole cohort (n=493), the presence of a T variant (TT+TC) seemed to result in better OS in patients than those with a CC genotype. However, this was only a minor trend (p=0.10). When the opposite model was tested (i.e. +29 CC+TC vs TT), there was no indication of an effect of patient genotype on OS (p=0.37). The results for these analyses are shown in **Figure 6.9**.

When these dominant models were tested for donor effects in the whole cohort (n=495), transplants that had a TT donor seemed to have a higher OS than those performed with donors carrying a C variant (CC+TC vs TT), but this lacked statistical significance (p=0.16). The dominant model for +29T did not suggest any effect from donor genotype on OS (p=0.65).

When the matching model was applied for this polymorphism in a dominant model to the whole cohort (n=484), the lack of +29C variants in the patient-donor pair (i.e. TT vs TC+CC matching, n=79) seemed to confer a higher OS, but this did not reach statistical significance (p=0.29).

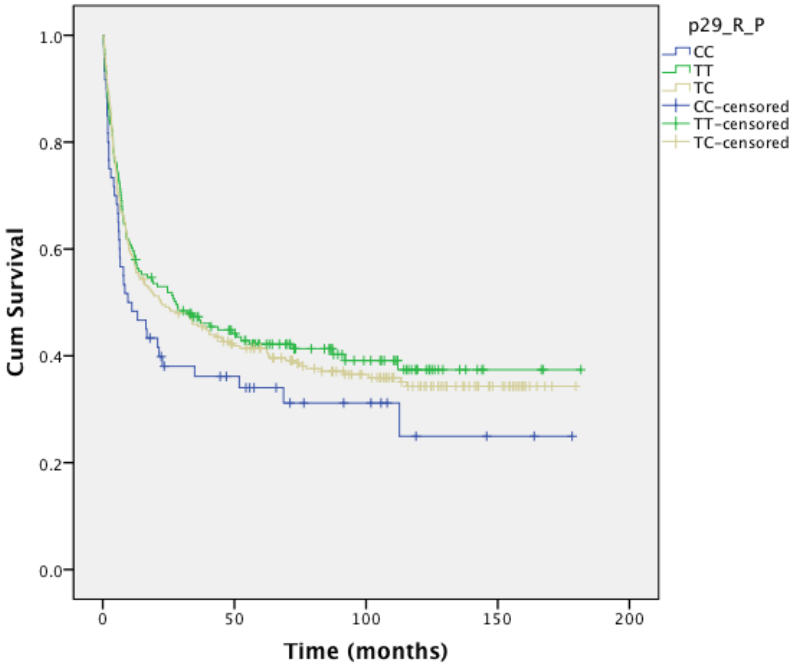

Figure 6.7 Survival analysis according to recessive effects of patient *TGFB1* +29T>C for the whole cohort. Patients homozygous for the C variant (n=60) show appear to have worse overall survival when compared to patients homozygous for the T variant (n=181) or heterozygous (n=252), but this is not confirmed statistically (p=0.24).

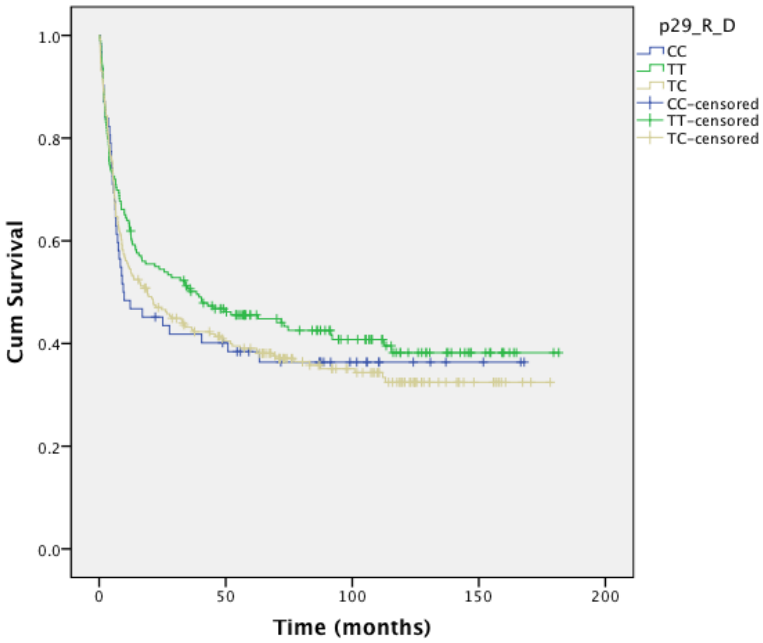

Figure 6.8 Survival analysis according to recessive effects of donor *TGFB1* +29T>C for the whole cohort. No individual genotype for *TGFB1* +29T>C has an effect on overall survival in the overall cohort (n=495; p=0.38).

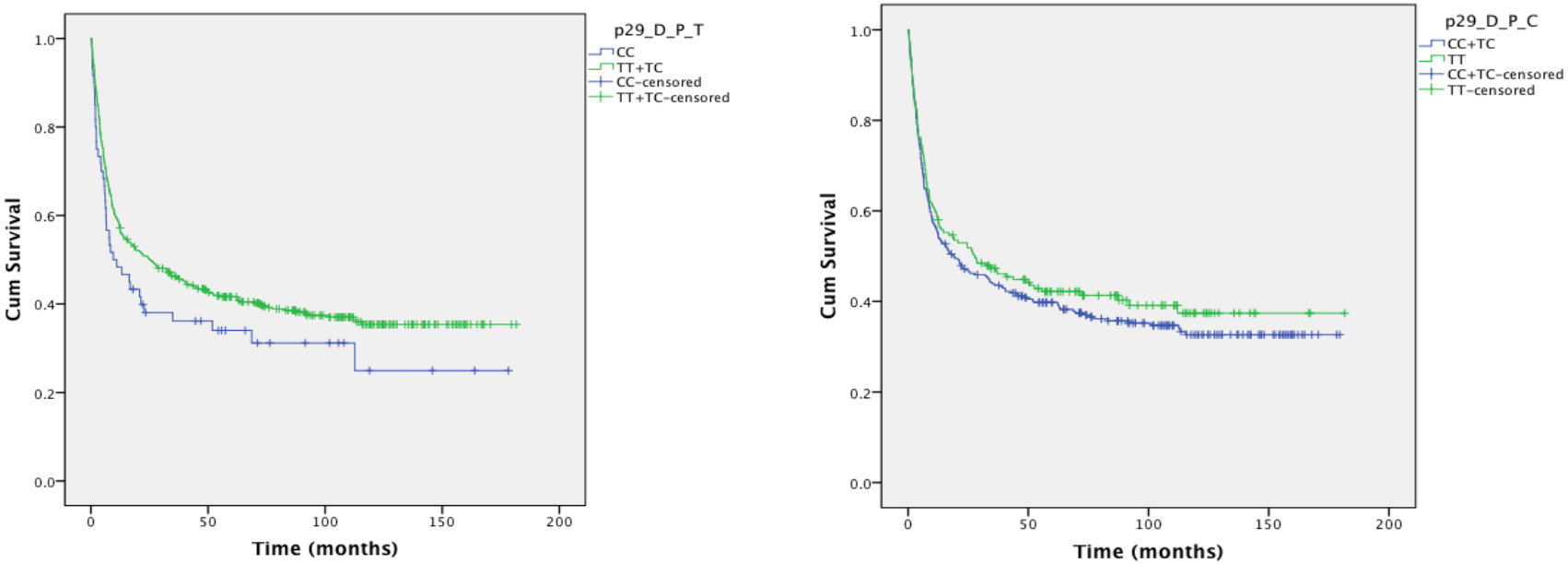

Figure 6.9 Survival analysis according to dominant effects of patient *TGFB1* +29T>C for the whole cohort. The left panel shows the dominant model for the T variant, while that on the right shows the dominant model for the C variant (n=493). Patients who bear a *TGFB1* +29CC genotype (n=60) seem to have worse overall survival than those that bear at least one copy of the T variant, but this is not statistically significant (p=0.10).

6.6.1.2.2 *TGFB1 -1347C>T*

The effect of *TGFB1* -1347 T>C on OS was tested in the complete cohort. When the recessive model was applied (i.e. TT vs CT vs CC), there was no statistical evidence for an effect of either patient (n=493; p=0.11) and donor (n=495; p=0.11) genotype on OS (**Figure 6.10 and 6.11**). However, patients with a TT genotype seemed to have worse OS than those with at least a C variant. When the matching model in a recessive model was tested (n=484), there was no statistical evidence of an effect on OS despite the CC-matching appearing to have higher OS than the other groups. Reduced frequency of matching for the -1347TT genotype (n=5) precluded further conclusions on this model.

When the dominant model for -1347C was tested (i.e. TT vs CT+CC), patients that had the -1347TT genotype (n=43) did indeed have significantly lower OS than that of patients bearing at least one C variant (median OS 7.9 vs 25.1 months; p=0.04). When the donor genotype was analysed in this model no evidence of an effect on OS was found (p=0.82). The results for these analyses are shown in **Figure 6.12**. When the matching model was applied to the dominant model for the -1347C variant, no suggestion of an effect in this model was present. The number of TT-matched pairs was very low (n=5) and this precluded further analysis of this model.

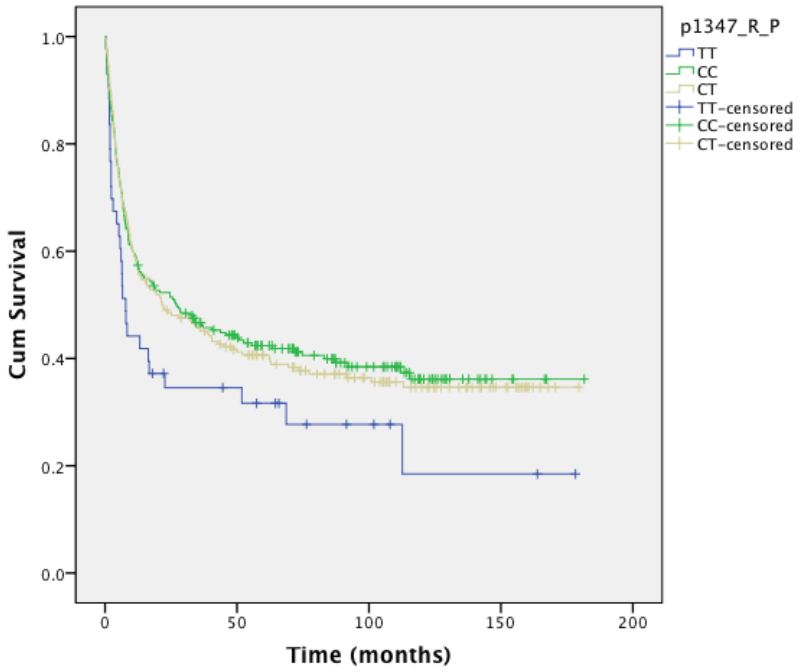

Figure 6.10 Survival analysis according to recessive effects of patient *TGFB1* -1347C>T for the whole cohort. Among the whole cohort (n=493), patients bearing a -1347TT genotype (n=43) seem to show lower overall survival. However, this was not confirmed statistically in this model (p=0.11)

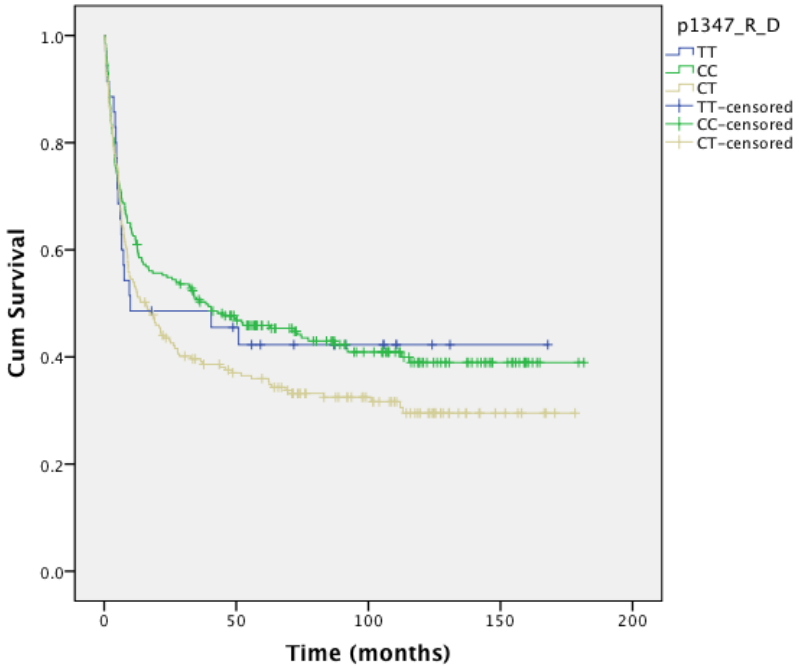

Figure 6.11 Survival analysis according to recessive effects of donor *TGFB1* -1347C>T for the whole cohort. Among the whole cohort (n=495), there was no suggestion of an effect of this SNP among donors on overall survival in this model (p=0.11).

6.6.1.3 Allelic genotype-specific models

When the patient genotypes were studied for their impact on OS (n=493), there was a wide variation in the survival curves between genotypes. The overall comparisons showed a trend towards differences between genotypes (p=0.06). Of note, as shown in **Figure 6.13**, the p001/p001 genotype (n=41) had the lowest OS. Interestingly, patients with a p003/p006 genotype (n=43) also had lower OS than the other six groups.

The genotype analysis was also done for donor genotypes (n=495). The results for these analyses are shown in **Figure 6.14**. Despite some stratification of the survival curves, there was no statistical evidence for an impact of individual donor genotypes on OS (p=0.29).

When the matching model was applied to the allelic genotype model, very few pairs were matched for p001/p001 (n=4), p001/p006 (n=1), p003/p006 (n=3) and p003/p014 (n=5). These pairs were thus excluded from the analysis. The results for the remaining pairs (n=471) therefore included matching for p001/p003, p003/p003 and non-matched. Despite pairs matched for p003/p003 showing higher OS than the other groups, this was not confirmed statistically (p=0.45).

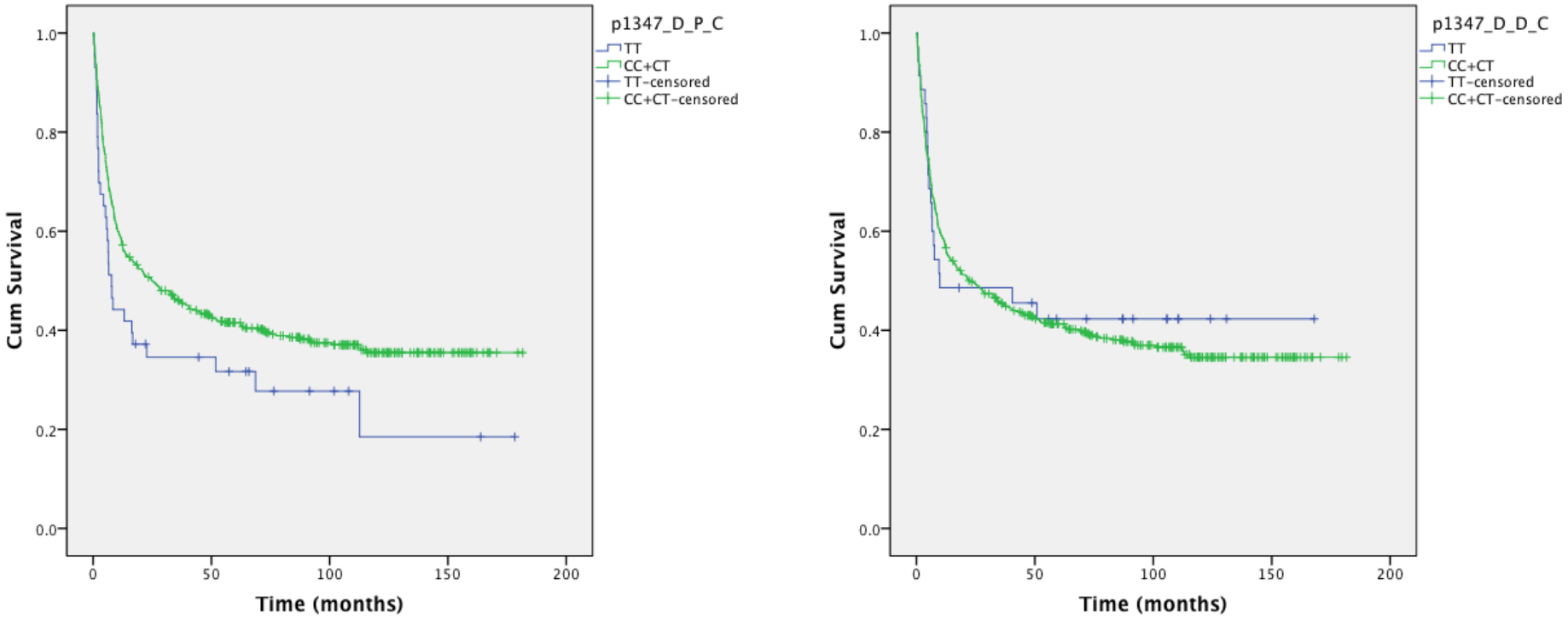

Figure 6.12 Survival analysis according to dominant effects of patient and donor *TGFB1* -1347C for the whole cohort. A dominant model for the major variant for the -1347C>T was tested in order to compare the TT genotype against the other genotypes together. The left panel shows the analysis among patients (n=493) while that on the right shows the results for the donors (n=495). The presence of a *TGFB1* -1347TT in patients (n=43) results in significantly lower overall survival (median OS TT vs CC+CT 7.9 vs 25.1 months; p=0.04). No effect of the donor genotype was suggested by this model (p=0.82).

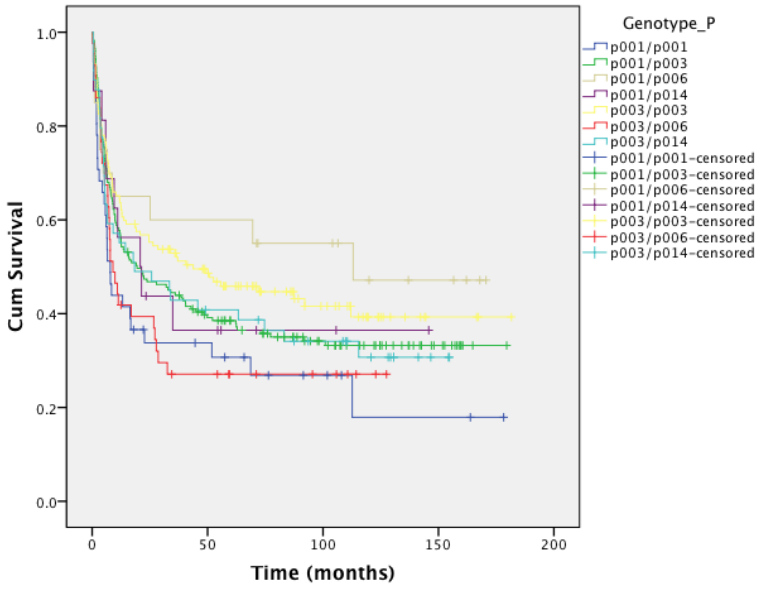

Figure 6.13 Survival analysis according to the effects of patient *TGFB1* regulatory region and exon 1 allelic genotype for the whole cohort. The analysis of patient genotype effect showed a statistical trend towards stratification of overall survival according to this variable in the complete cohort (n=493; p=0.06).

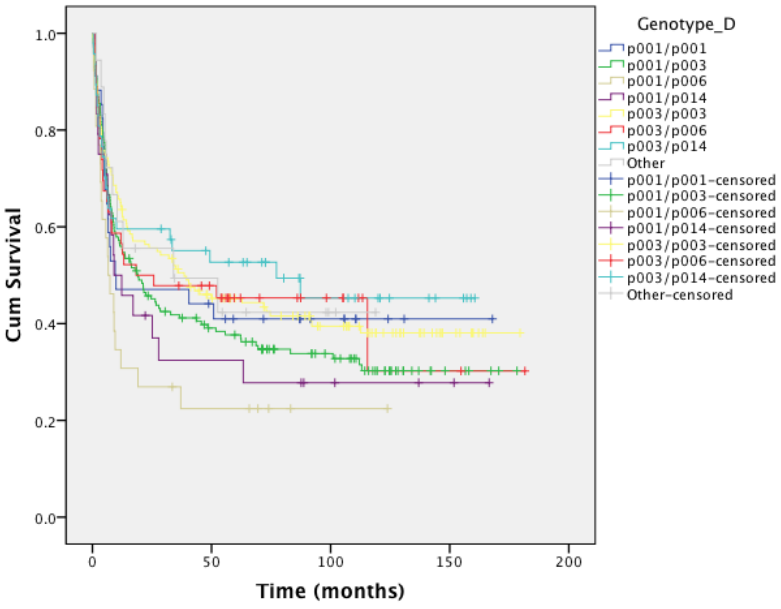

Figure 6.14 Survival analysis according to the effects of donor *TGFB1* regulatory region and exon 1 allelic genotype for the whole cohort. The analysis of donor genotype effect did not suggest any effect of individual genotypes on overall survival in the complete cohort (n=495; p=0.29).

6.6.1.4 Reduced genotype-specific models

In order to complement the allelic genotype models and to simplify them, I also tested the effect of patient, donor, and matching of the combined genotypes formed by *TGFB1* -1347C>T and +29T>C on OS. In these analyses 5 categories were included as variables.

The analysis of the effect of patient reduced genotypes (n=487) is shown in **Figure 6.15**. As shown in this figure, the genotype formed by TT-CC (n=41) seems to have lower OS, but this in not confirmed statistically (p=0.30).

When the analysis was performed on donor reduced genotypes (n=488) there was no clear effect on OS (p=0.32) and no suggestion of an individual effect of any specific combination **Figure 6.16**.

Finally, when the effect of matching for these reduced genotypes was tested (n=474), pairs that were matched for the CC-TT genotype (n=75) seemed to have higher OS, but this was not confirmed statistically (p=0.67). Very few pairs were matched for the TT-CC and the CT-CC genotypes, and thus these had to be excluded from the analysis.

6.6.1.5 Analysis of the effect of *TGFB1* allele p001 on overall survival (OS)

Taking into account that the models with significant effects on OS (41/43 *TGFB1* -1347TT patients were also *TGFB1* p001/p001), the previous results suggested that the main effect was exerted by p001 homozygosity. As a further way to confirm the previous effects on OS, the recessive model for patient *TGFB1* regulatory region and exon 1 alleles was repeated grouping all other genotypes and comparing their OS to that of patients who are homozygous for p001. The results for this model show that patients who are homozygous for this allele (n=41) have significantly lower OS than the rest of the patients (n=452; p=0.03) (**Figure 6.17**). When Cox regression was used to assess this effect, the results showed that the relative risk of dying for patients homozygous for p001 was 1.5 (95%CI 1.03-2.19) times that of other patients. The probabilities of survival at 5 years post-transplant were 30.7% for p001/p001 patients and 41.6% for the rest of the patients. Ten years after the transplant, the probability of survival for p001/p001 patients is approximately half of that for the rest of the patients (17.9% vs 35.5%, respectively).

In an attempt to clarify their effect, the models that showed consistent significant effects on OS (i.e. patient homozygosity for p001 and donors bearing at least one copy of p001) were further explored for their effect on EFS, NRM and relapse incidence.

The overall probability of EFS at 5 years in the whole cohort was 30.4%. The patient-related models (i.e. recessive models for *TGFB1* regulatory region and exon 1 alleles and -1347C>T dominant model for the C variant) and the donor-related model did not show any significant effect on EFS (**Figure 6.18**).

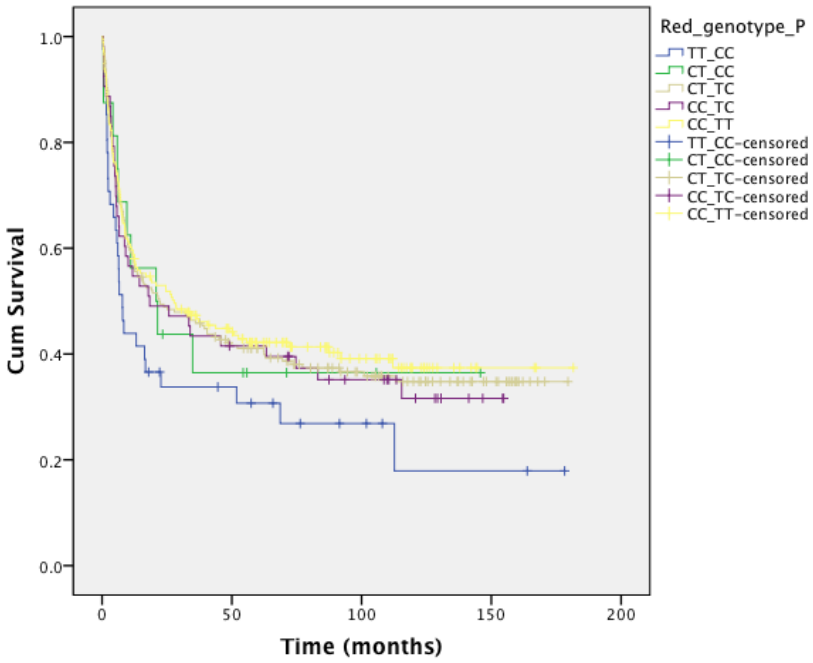

Figure 6.15 Survival analysis according to the effects of patient reduced *TGFB1* regulatory region and exon 1 genotype for the whole cohort. The analysis of reduced genotype patient effects on overall survival (OS) appeared to show lower OS for the TT-CC genotype (n=41) when compared to the other combinations. However, this did not reach statistical significance (p=0.30).

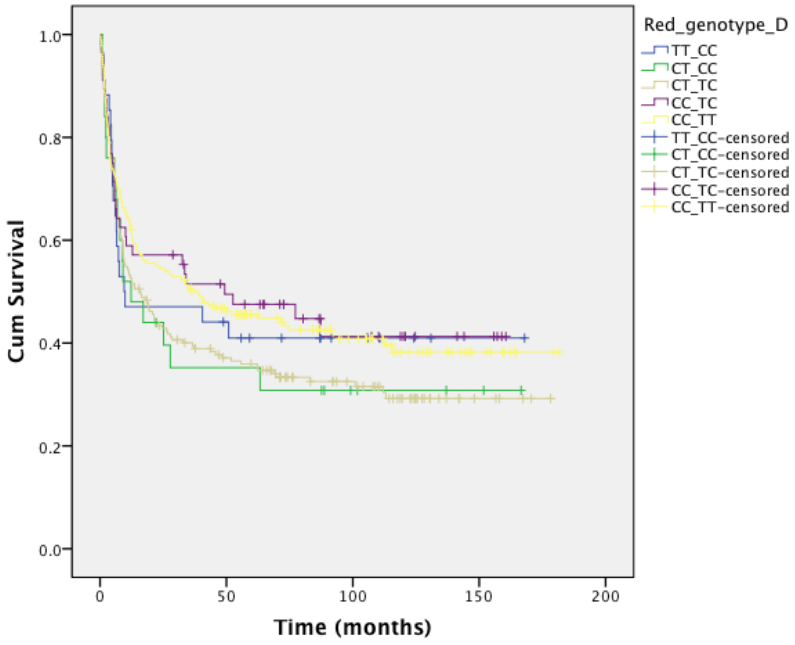

Figure 6.16 Survival analysis according to the effects of donor reduced *TGFB1* regulatory region and exon 1 genotype for the whole cohort. The analysis of donor reduced genotype effect did not suggest any effect of individual genotypes on overall survival in the complete cohort (n=488; p=0.32).

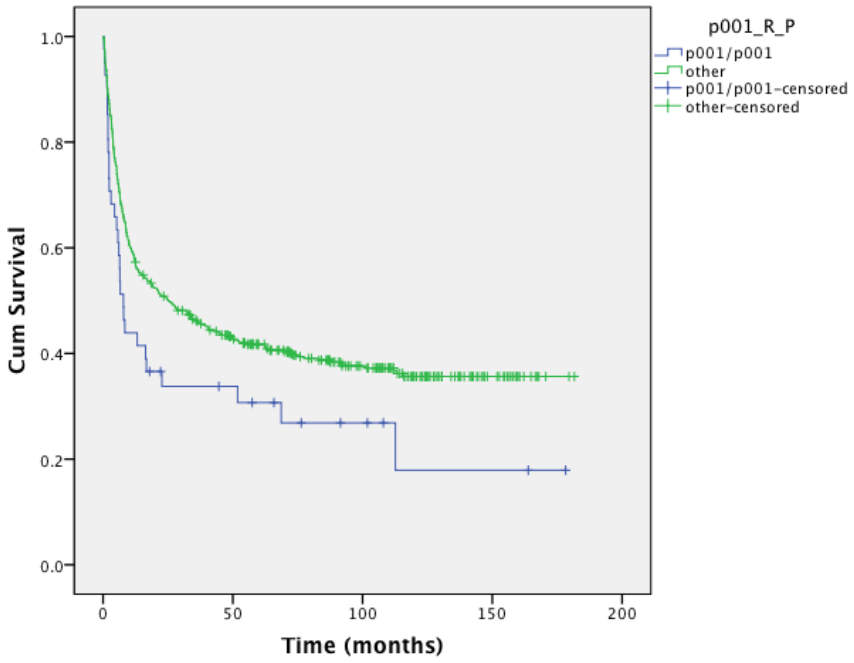

Figure 6.17 Survival analysis according to the effects of patient p001 homozygosity for *TGFB1* regulatory region and exon 1 genotype for the whole cohort. Patients homozygous for allele p001 show reduced overall survival when compared with other genotypes (n=493; p=0.03).

6.6.2 Analysis of non-relapse mortality (NRM) and relapse

The cumulative incidence of NRM at 1 year after transplant was 26.8%. As shown in **Figure 6.19**, when the effect of these models was studied for NRM, there was a significant increase in its incidence among patients that bear the p001 allele (1 year NRM: 39.0%; p=0.039) or the -1347T (1 year NRM: 39.5%; p=0.029) in a homozygous manner when compared to other genotypes (1 year NRM: 25.4% and 25.3%, respectively). There was no effect of the dominant presence of p001 among donors.

Finally, the cumulative incidence of relapse at 1 year post transplant was 27.6% in the whole cohort. When these models were tested for their effect on disease relapse there was no suggestion of a role of these variants in recurrence (**Figure 6.20**). Consequently, the patient models were selected for further study on multivariate models for OS and NRM. The donor model was discarded due to lack of consistency of its association.

Clinical patient, donor and transplant related variables were independently tested for their association with OS and NRM in the whole cohort. The results for these analyses are shown in **Table 6.9**. Factors that showed a p≤0.2 were selected for inclusion in the multivariate models for these outcomes.

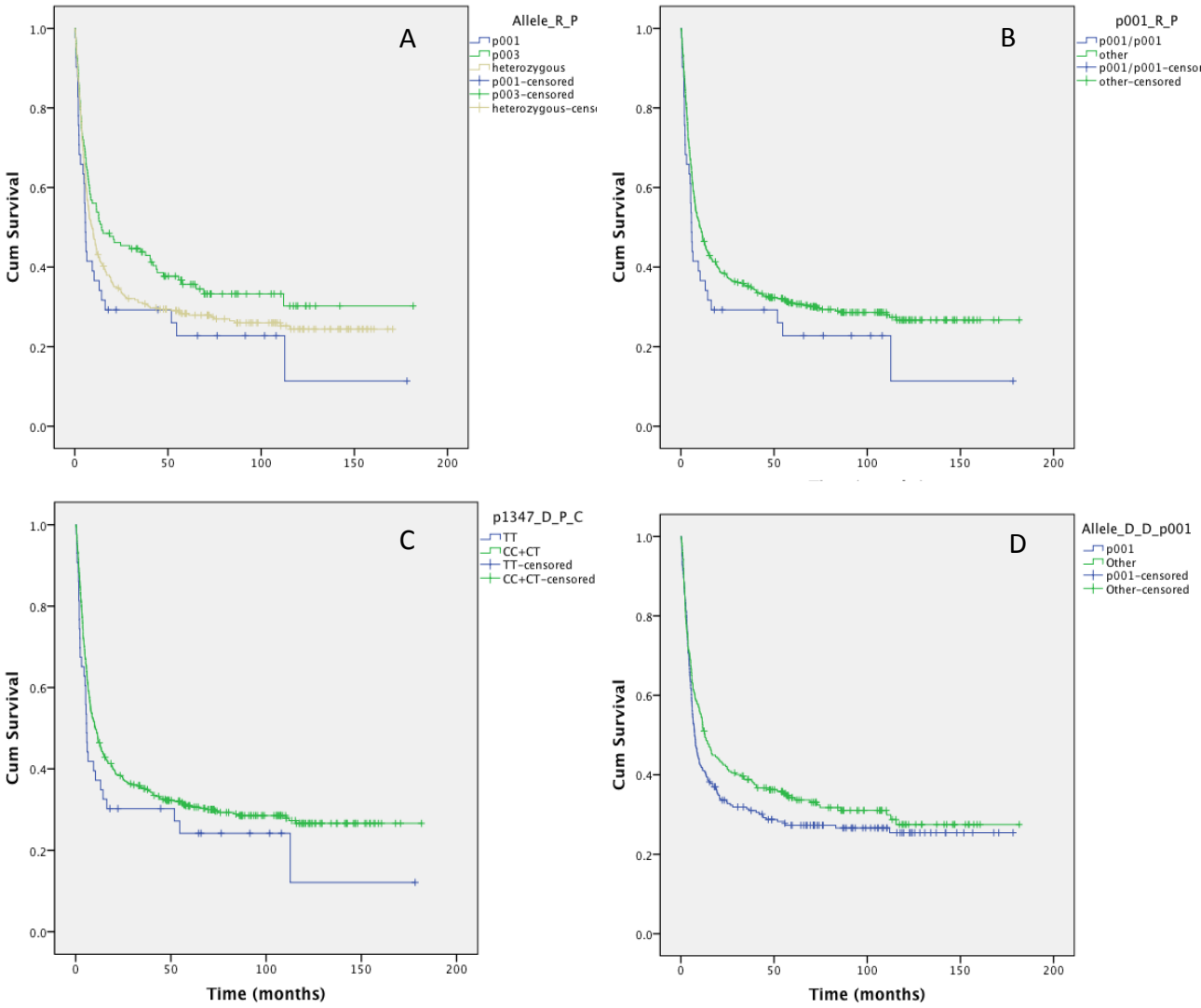

Figure 6.18 Survival analysis according to the effects of patient and donor *TGFB1* regulatory region and exon 1 genotype on event-free survival (EFS) for the whole cohort. The models that showed significant effects on overall survival (blue curve) were further studied for their effect on EFS. None of the models showed significant differences in EFS. Models: recessive patient allelic model (A); patient p001/p001 vs other (B); dominant patient -1347C (C); dominant donor p001 (D).

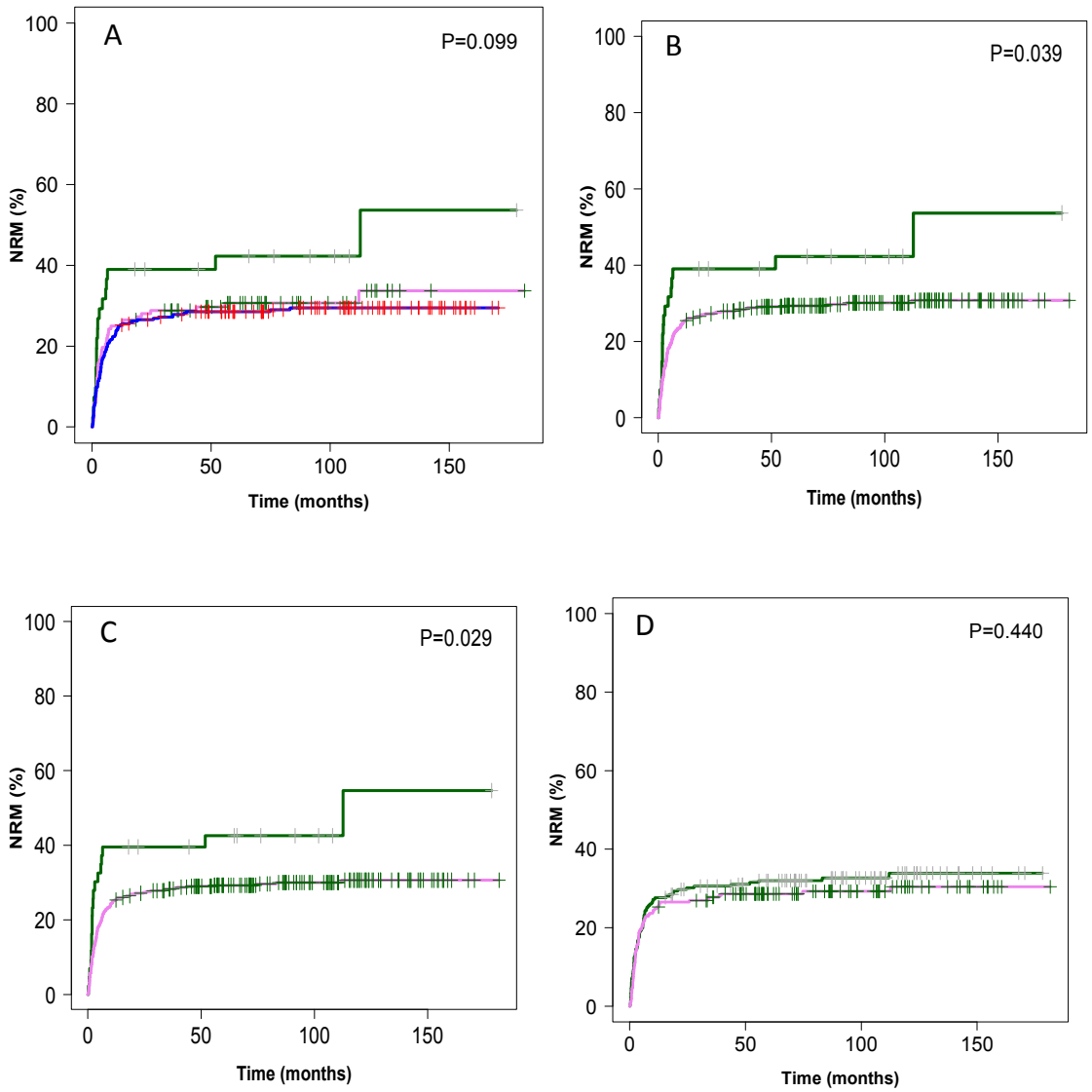

Figure 6.19 Survival analysis according to the effects of patient and donor *TGFB1* regulatory region and exon 1 genotype on non-relapse mortality (NRM) for the whole cohort. The models that showed significant effects on overall survival were further studied for their effect on NRM. Allele p001 and -1347T homozygosity in patients showed a significant increase in NRM in the whole cohort. No effect on NRM was observed for the donor p001 dominant model. Panels: recessive patient allelic model (A); patient p001/p001 vs other (B); dominant patient -1347C (C); dominant donor p001 (D). The green curve designates patient p001/p001 or *TGFB1* -1347TT or donor p001/-, as appropriate.

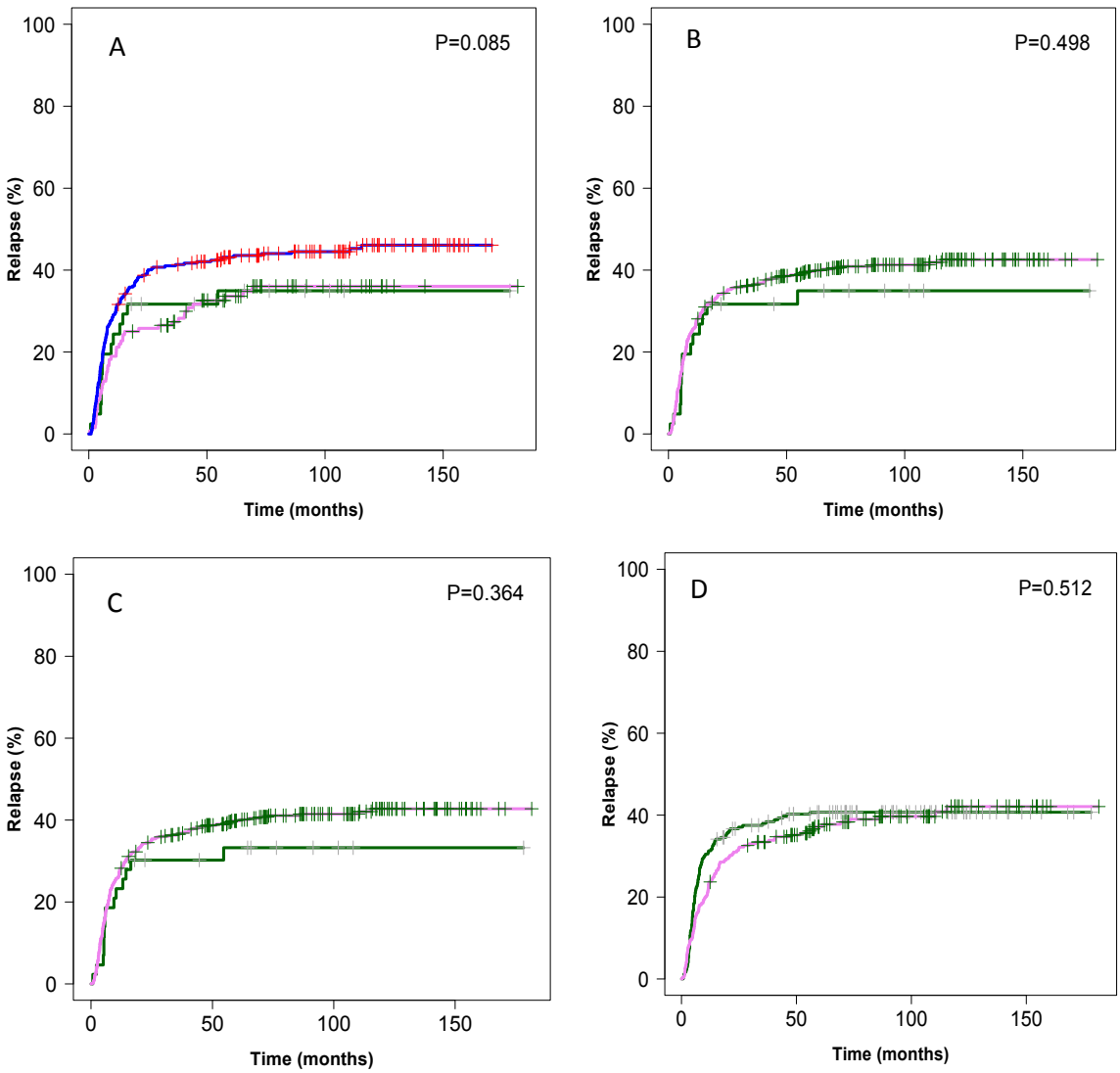

Figure 6.20 Survival analysis according to the effects of patient and donor *TGFB1* regulatory region and exon 1 genotype on the incidence of relapse for the whole cohort. The models that showed significant effects on overall survival were further studied for their effect on relapse. None of the models showed significant differences in relapse incidence. Panels: recessive patient allelic model (A); patient p001/p001 vs other (B); dominant patient -1347C (C); dominant donor p001 (D). The green curve designates patient p001/p001 or *TGFB1* -1347TT or donor p001/-, as appropriate.

Table 6.9 Analysis of the univariate association between clinical factors and OS and NRM in the whole cohort (n=504).

Risk factor		OS (p value)	NRM (p value)
Patient age > 40 years		<0.001	0.002
Donor age >30 years		0.08	0.38
Sex	Patients	0.15	0.37
	Donors	0.32	0.98
Sex matching	Overall matching	0.84	0.72
	Female donor – male recipient	0.59	0.69
HLA-matching	0 vs 1 vs >1 mismatch	0.05	0.05
Disease status[1]		0.004	0.44
CMV status	Patient	0.003	0.04
	Donor	0.30	0.63
	Matching	0.03	0.01
TBI presence		0.09	0.08
SC source		0.66	0.24
T cell depletion		0.95	0.18
GVHD prophylaxis		0.23	0.74

[1] Complete remission/chronic phase vs other.
CMV, cytomegalovirus; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; NRM, non-relapse mortality; OS, overall survival; SC, stem cell; TBI, total body irradiation.

6.6.3 Multivariate analyses

Based on the univariate analyses for the clinical factors, patient age, donor age, patient sex, HLA matching, disease status, CMV matching and use of TBI were selected for inclusion in the multivariate model for OS. Likewise, patient age, HLA matching, CMV matching, use of TBI, and use of T cell depletion were selected for the NRM model.

The multivariate models including the aforementioned clinical variables were performed for each of the patient genetic effects. The results for these analyses are shown in **Table 6.10**. As shown, when OS was evaluated, disease status at transplant and patient age together with the recessive allelic model were significant factors associated with this outcome. When the -1347C dominant and the 'p001/p001 vs other' models were examined, both were found to be significantly associated with OS together with patient age. Overall, being a patient older than 40 years of age, being transplanted not in complete remission/chronic phase and being homozygous for *TGFB1* p001 (or -1347T) were associated with decreased OS.

The multivariate analysis for NRM also revealed a significant effect of all the genetic

models in addition to patient age and HLA matching. Overall, patient homozygosity for *TGFB1* p001 (or -1347T), patient age older than 40 years and the presence of 1 or more allelic mismatches in any of the 5 main HLA genes were associated with increased probability of NRM.

Table 6.10 Analysis of the multivariate association between clinical factors and *TGFB1* regulatory region and exon 1 alleles and SNPs and their effect on OS and NRM in the whole cohort (n=504).

Outcome	Model	Factor[1]	OR [95%CI]	p value
OS	Model 1	Patient p001/p001 vs p003/p003 vs heterozygous	1.53 [1.02-2.27] (p001/p001) 0.75 [0.57-0.99] (p003/p003)	0.037 0.044
		Patient age < 40 vs > 40	0.59 [0.46-0.77]	<0.001
		Disease status (low risk vs high risk)	0.73 [0.53-0.99]	0.044
	Model 2	Patient -1347 TT vs CC+CT	1.65 [1.12-2.42]	0.011
		Patient age < 40 vs > 40	0.54 [0.42-0.70]	<0.001
	Model 3	Patient p001/p001 vs other	1.68 [1.13-2.48]	0.009
		Patient age < 40 vs > 40	0.54 [0.42-0.69]	<0.001
NRM	Model 1	Patient p001/p001 vs p003/p003 vs heterozygous	2.49 [1.47-4.22] (p001/p001)	0.001
		HLA matched 10/10 vs ≤ 9/10	0.55 [0.33-0.90]	0.016
		Patient age < 40 vs > 40	0.50 [0.35-0.72]	<0.001
	Model 2	Patient -1347 TT vs CC+CT	2.47 [1.50-4.07]	<0.001
		HLA matched 10/10 vs ≤ 9/10	0.52 [0.32-0.83]	0.007
		Patient age < 40 vs > 40	0.48 [0.33-0.69]	<0.001
	Model 3	Patient p001/p001 vs other	2.45 [1.47-4.08] (p001/p001)	0.001
		HLA matched 10/10 vs ≤ 9/10	0.52 [0.32-0.84]	0.008

	Patient age < 40 vs > 40	0.48 [0.33-0.69]	<0.001

[1] Factors are compared to the last one listed for their odds ratio (OR). NRM, non-relapse mortality; OS, overall survival.

The adjusted survival curves for the multivariate models for OS and NRM according to patient homozygosity allele p001 are shown in **Figure 6.21**.

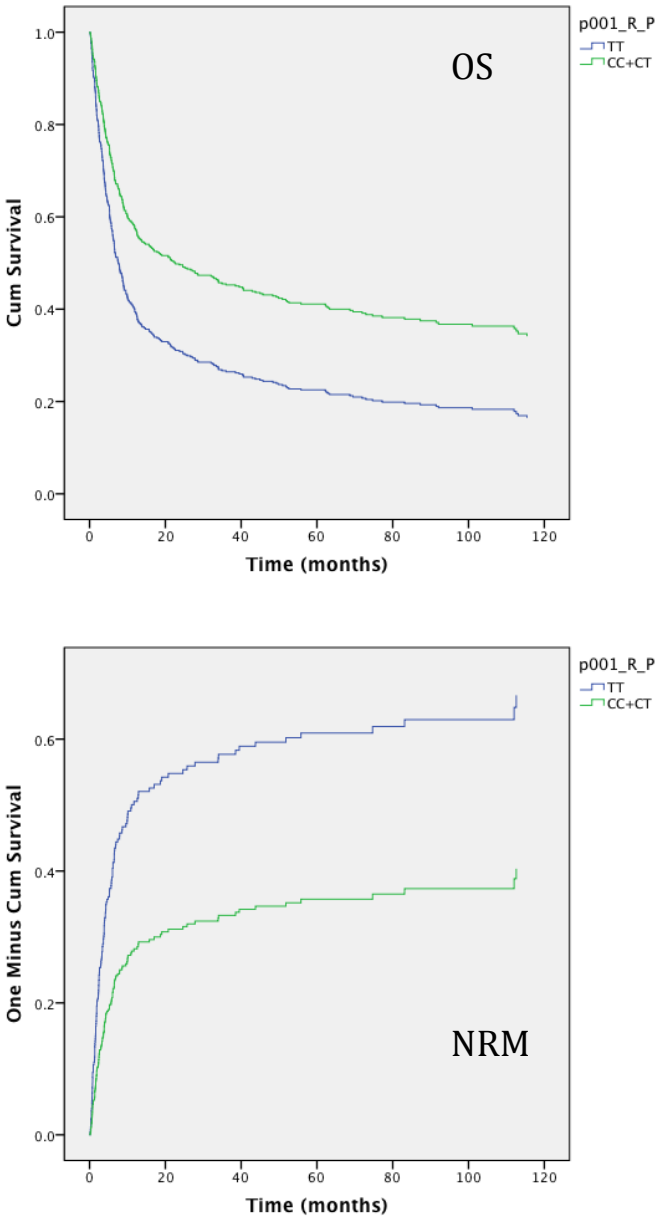

Figure 6.21 Adjusted survival curves for overall survival (OS) and non-relapse mortality (NRM) according to patient *TGFB1* regulatory region and exon 1 allele in the whole cohort. Multivariate regression analysis for OS (top) and NRM (bottom) including clinical factors was performed on patient genotype. Patients homozygous for allele p001 (blue line) show significantly lower OS and higher NRM than patients with other genotypic combinations adjusting for clinical factors.

6.6.4 Analysis of acute graft-versus-host disease (aGVHD)

In a final analysis, the genetic models for *TGFB1* regulatory region and exon 1 polymorphism that had a significant effect on OS and NRM were examined for their effect on the incidence of aGVHD. In univariate analysis, there was no suggestion of an association between homozygosity for p001 or for -1347T and overall (grades I-IV), clinically relevant (grades II-IV) or severe (grades III-IV) aGVHD. A multivariate analysis of clinical factors that showed univariate suggestion of relation (i.e. $p \leq 0.2$) and their effect on any grade aGVHD identified the lack of TBI in the conditioning regimen (OR=0.49 [95%CI: 0.27-0.89], p=0.021) and BM as a stem cell source (OR=0.65 [95%CI: 0.42-0.99], p=0.043) as a protective factors and patient age < 40 years as a risk factor (OR=1.66 [95%CI: 1.07-2.57], p=0.022) for the development of this complication. When this analysis was restricted to clinically relevant aGVHD (i.e. grades II-IV), only not using a female donor for a male patient was identified as a protective factor for this complication (OR=0.48 [95%CI: 0.27-0.84], p=0.011).

6.7 Discussion

In this chapter, I have presented my findings regarding the analysis of the role of *TGFB1* regulatory region and exon 1 alleles and SNPs in the outcome of UD-HSCT. This study is the largest study performed so far on the role of *TGFB1* polymorphisms in HSCT. Moreover, this is the first study to comprehensively examine the variation in a large region upstream of this gene. In contrast to previous studies, the analysis presented in this chapter focused mainly on the joint effect of various polymorphisms organised in defined alleles in a region of approximately 3 kb of the human genome. In fact, this is, to the best of my knowledge, the only genetic association study to use this approach in any medical field. Thanks to this approach, it has been possible to isolate a clear effect of the homozygous combination of one of these alleles, p001, in patients as a risk factor for lower OS and higher NRM after UD-HSCT.

6.7.1 Observed *TGFB1* regulatory region and exon 1 polymorphism

The typing of over one thousand unrelated samples revealed that only 9 previously known polymorphic positions were actually variant in this cohort. Among these, 2 positions showed very low minor allele frequencies. This means that out of the 18 polymorphic positions reported by Shah and collaborators (Shah *et al.*, 2006), only 7 were frequent in this sample of individuals. It is likely that the other polymorphic positions are not polymorphic in the population from which the patient and donors were extracted. In fact, many of the positions reported by this group were identified in specific ethnic groups. For example, 7 polymorphic positions, 5 of which were found to be associated in 1 allele (p005), were identified in persons of Sub-Saharan African descent (Shah *et al.*, 2006). There is no ethnicity information available for the patient and donor samples included in this study. However, since the transplants herein studied were performed in the United Kingdom, it is likely that the majority of these subjects are of Northwestern European descent. Their HLA genotypes also suggest a predominant European origin. Consequently, other known polymorphic positions in *TGFB1* might not be polymorphic in these populations.

Even though this was not a population study, the frequencies derived from the typing of such a large group of unrelated samples are likely to approach the frequencies in the populations from which these samples are taken. Of note, there is no report on population frequencies of *TGFB1* regulatory region and exon 1 alleles, making this study also the first glimpse on the comprehensive diversity of this genetic region in any population and a

potential reference for future studies. One study did report frequencies of alleles formed by some of the polymorphic positions in *TGFB1* regulatory region and exon 1. Awad *et al*. (1998) report that among 104 healthy controls, 65% of the alleles were formed by a -1347C~no ins-768~+29T~+74G, 24% were -1347T~no ins-768~+29C~+74G, and 10% were -1347C~no ins-768~+29T~+74G. Compared with the alleles observed in this study, these would be equivalent to p003+p006+p013 (61.92%), p001+p009 (29.40%) and p014 (8.25%), respectively. Consequently, the allele frequencies seem to be in accordance with these previous partial observations. Interestingly, an allele formed by -1347C~no ins-768~+29C~+74G, which would fit with the rare allele 'Z' found in the patient-donor cohort, had a frequency of 1% in this report.

Regarding previously reported frequencies for the polymorphic positions analysed in this study, all of them showed similar results to the ones presented in this chapter. In a twin study (n=340) the frequencies for the -1638A and the -1347T variants were 0.09 and 0.31, respectively (Grainger *et al.*, 1999). Hoffmann et al. (2002) studied the frequencies of variants at positions +29 and +74 in renal transplant patients and healthy individuals of different ethnic groups (n=329). The MAF for *TGFB1* +29C and +74C were 42-61% and 4-12%, respectively. Individuals of "White", "Black" and "Hispanic" ancestry showed similar frequencies for both polymorphisms. Only persons of "Asian" (i.e. Eastern Asian) ancestry had significantly different frequencies at position +29 when compared to "White" subjects (Hoffmann *et al.*, 2002). The +29~+74 genotype frequencies reported by this group for their "White" subjects were very similar to what their equivalents would be in the present study: TT~GG 35% vs 36.92%, TC~GG 40% vs 38.58%, TC~GC 12% vs 11.03%, CC~GG 10% vs 7.91%, CC~GC 4% vs 3.91%, and CC~CC 1% vs 0.68%, my results being the latter of the pair. Similar combined +29~+74 genotype results were reported for healthy Cuban and African Americans (Delaney *et al.*, 2004). Both variant and genotype frequencies for +29T>C and +74 G>C among healthy Brazilians (n=214, (Visentainer *et al.*, 2008)) were also similar to frequencies found in the patient-donor cohort. This study reported similar frequencies for *TGFB1* +29C (39.8% and 41.7%) and +74C (6.3% and 10.6%). Other report in Japanese population (n=48) found that the frequency for -1347C and +29T was the same, both at 41.7% populations (Watanabe *et al.*, 2002). Among Chinese controls for a cirrhosis study, the frequencies for -1347C and +29T were respectively 51.9% and 48.6%, while positions -1638 and -1826 were not polymorphic, also suggesting differences in Eastern Asian populations when compared to European or African ones (Wang *et al.*, 2008). A report from Iranian healthy subjects found slightly different frequencies for *TGFB1* -1347C>T an +29T>C when compared to European populations (Amani *et al.*, 2008). Dunning et al. (2003) examined *TGFB1* polymorphisms in a case-control breast

cancer study on subjects from the UK, Germany and Finland, virtually all of which were "ethnically Caucasian". The allele frequencies reported by them for *TGFB1* -1638~-1347~+29~+74 among the UK subjects also seem to agree with their equivalents based on the allele frequencies found in my study: G~T~C~G (p001+p009) 25% vs 29.4%, G~C~T~G (p003+p013) 53% vs 53.81%, A~C~T~G (p006) 7% vs 8.11%, and G~C~C~C (p014) 6% vs 8.25% my results being the latter of the pair (Dunning *et al.*, 2003). Again, one of their low-frequency alleles would agree with rare allele 'Z': G~C~C~G, although with higher frequency than in the present study (5%). Other studies performed in population from the UK show SNP and allele frequencies that agree with those found in the patient-donor cohort (Syrris *et al.*, 1998; Bayat *et al.*, 2002; Cotton *et al.*, 2002). Finally, a large study (n=19544, 97.4% "White/Caucasian") found a very similar frequency for *TGFB1* -1347T (30.8%) to the one observed in the patient-donor cohort. Overall, the frequencies for variant polymorphic positions and for *TGFB1* regulatory region and exon 1 alleles seem to agree widely with partial data previously reported by other studies.

The testing of HWE for those SNPs that were not extremely rare revealed a significant deviation for +29T>C. An excess of heterozygotes was found to be the cause of this deviation. However, when the allele distribution was evaluated, the observed genotypes were not significantly deviated from HWE in a 4-allele model. The reasons for the SNP deviation could be an accumulation of higher numbers of observed heterozygous combinations of different alleles that pushed the SNP frequencies to this excess. Nonetheless, this situation would not interfere with any of the analyses herein performed. Consequently, this deviation should be seen as a particularity of the patient-donor cohort studied in this project.

6.7.2 Novel single-nucleotide polymorphisms (SNPs) and alleles of *TGFB1* regulatory region and exon 1

A number of novel/rare SNPs and of rare recombinant alleles (i.e. rare haplotypes) of previously known polymorphic positions were found in this study. The fact that 3.6% of the typed samples bore a novel SNPs suggests the presence of a considerable degree of polymorphism only recognised when large groups of samples are studied. This becomes even more relevant if the fact that most of these samples are likely to belong to a Northwestern European population, and the increased diversity among other human populations such as Sub-Saharan Africans might hide several other novel variants. Regarding the consequences of these rare SNPs, 8 of 16 lie within a known regulatory region within *TGFB1* upstream region. Two of the remaining SNPs are predicted to cause amino acid changes in the mature protein. Altogether, these variants could have functional

consequences for those people bearing them. Nonetheless, as has been seen in this study and as other studies suggest, polymorphism in *TGFB1* seems to be concentrated in a handful of alleles with high or relatively high frequencies, with an underlying low frequency diversity.

6.7.3 *TGFB1* regulatory region and exon 1 alleles in the patient-donor cohort

Four *TGFB1* regulatory region and exon 1 alleles predominated in this cohort. Of these, alleles p006 and p014 had frequencies close to 8%. This situation caused that the analysis of homozygous individuals in statistical models was not possible. These frequencies would require a substantially larger study for it to be possible to assess these genotypes' role in the outcome of HSCT or any other medical condition or therapy. Of note, alleles p001 and p014 share a C variant at position +29 but have a T variant and a C variant at -1347, respectively. This situation is a clear example of how the study of a SNP as a marker for genetic diversity might not always be the right approach. *TGFB1* SNPs +29T>C and -1347C>T are the most studied in many medical fields. Consequently, if no allele analysis or, at least, a separate analysis of these two positions is performed, the presence of alleles p001 and p014 can potentially cause contradicting results in genetic association studies. As has been discovered in this study, a genetic effect might be caused by only one of the alleles that share a variant at any specific position. This becomes more likely if the shared variant is not the main effector of functional differences. Consequently, the use of SNPs for genetic association studies involving genes or genetic regions with important variation should only be done after validating that SNP as a specific marker for an allele, and not the other way around. For this reason, genetic association studies should follow population studies in which the clear picture of the genetic variation in the relevant regions is characterised, preferably by sequencing and the establishment of the phase of the set of variants. In this regard, the results from the present study reveal that the variant -1347T is close to a marker for allele p001. Among patients and donors, 98.69% of the samples that carried a -1347T did carry a p001 allele. Consequently, the -1347C>T could be used as a surrogate for the assessment of the presence of allele p001 in samples drawn from the population to which this clinical samples belong. However, as explained before, potential effects of other *TGFB1* regulatory region and exon 1 alleles in other settings cannot be assessed specifically with this SNP only.

6.7.4 *TGFB1* regulatory region and exon 1 polymorphism and the outcome of unrelated donor haematopoietic stem cells transplantation (UD-HSCT)

The analysis of the association of *TGFB1* alleles and SNPs and the outcome of myeloablative UD-HSCT revealed an effect on OS and NRM that could be shown statistically to be due to the presence of p001 (or -1347T) in the patients. The presence of one or two copies of this allele was associated with worse survival. This effect was clearly associated with death without relapse, suggesting that the relationship between *TGFB1* p001 and lower survival is transplant-related. Interestingly, the effect of p001 was more pronounced when clinical factors were taken into account, further supporting this finding.

When the findings from the present study are compared to previous ones, it becomes difficult to contrast them due to limitations and analytical differences. As has been discussed, most previous studies have a number of limitations and special characteristics that limit the generalization of their observations (see **Table 1.1**). First of all, most studies are very small, which reduces their power to identify potential effects. Seven out of 13 studies examined less than 100 patient-donor pairs. Moreover, most of the studies have focused their analysis on one or two SNPs. In addition, most of the previous studies have focused on the effect of these SNPs solely on GVHD. Two early studies have also used preconceived classifications of the genotypes in "high producer" and "low producer" groups, potentially introducing a bias in their analyses (Leffell *et al.*, 2001; Tambur *et al.*, 2001; Laguila Visentainer *et al.*, 2005). Other characteristics that hamper effective comparisons are the use of cohorts composed of both related and UD donors, and of myeloablative and reduced-intensity transplants. Small studies that report associations for +74 G>C genotypes (Leffell *et al.*, 2001; Rashidi-Nezhad *et al.*, 2010) are questionable since the +74CC genotype is very rare. Finally, most studies lack time-to-event data, and base their analyses on univariate Chi^2 tests, which is likely to impair thorough assessment of the outcome picture in those cohorts and lessens their power to identify meaningful effects.

The only previous attempt to analyse *TGFB1* regulatory region and exon 1 alleles in the context of HSCT outcome was that of Shah et al. (2009). However, this study was too small (n=38) to detect any genotype effect and was restricted to aGVHD incidence (Shah *et al.*, 2009).

Among those studies with larger cohorts, in the study by Laguila Visentainer *et al*. (2005) in Brazilian sibling transplants, no association for +29T>C and +74G>C was observed with OS or GVHD (Laguila Visentainer *et al.*, 2005). In this case, none of the combined +29~+74 genotypes showed any association, which could have potentially partially isolated the

effect of p001. A larger study by Berro *et al*. (2010) did test OS, NRM and aGVHD (as well as other outcomes). In this study, an association between the presence of +29C and reduced OS, increased NRM and no effect on relapse. Moreover, this study found increased risk of aGVHD in pairs with increasing numbers of +29C variants (Berro *et al.*, 2010). These findings partially resemble the ones found in my study. However, in my analyses, there was no statistical confirmation of a role of +29T>C despite +29CC individuals appearing to have lower OS in this cohort. An explanation for this difference could be the fact that, as has been mentioned before, +29CC genotypes could include both p001 and p014. The presence of p014 could not be analysed for a recessive effect in my cohort. However, since OS in +29CC patients was not statistically different from +29TT and TC individuals this could be evidence toward the lack of effect from p014. In another study performed in Chinese patients (n=240) *TGFB1* -1347C>T was associated with aGVHD incidence (Xiao *et al.*, 2011). The presence of both overall and clinically relevant aGVHD was reduced in patients that received a transplant from TT individuals, and also in patients who bore at least one copy of the T variant themselves. Only the association for donor genotype was significant among HLA-matched pairs. These results could potentially suggest a role of allele p001 in these transplants. However, because it is uncertain if *TGFB1* -1347T does correlate with allele p001 in the Chinese population, this might not be the case. In any case, there was no effect of this polymorphism on OS, NRM or relapse in this study's cohort. Another study performed on 102, mostly related donor, transplant patients found no association between *TGFB1*'s signal peptide polymorphisms and OS, engraftment and infections in patients transplanted for thalassemia major (Sellathamby *et al.*, 2012). In a large study performed in mismatched UD-HSCT there was no consistent association of *TGFB1* -1347C>T with OS, engraftment or GVHD despite initial findings in a discovery cohort in which it appeared to be associated weakly with aGVHD development (Harkensee *et al.*, 2012). Finally, in two recent reports analysing relatively large cohorts of mostly related donor transplants *TGFB1* -1347 TT and CT patients showed increased incidence of aGVHD (Kim *et al.*, 2012; Kim *et al.*, 2014). There was no association of this polymorphism with OS, EFS, or NRM. In the second report the authors identified the presence of a -1347T as a risk factor specific for skin aGVHD and as a protective factor against lung cGVHD (Kim *et al.*, 2014).

Overall, previous studies have more or less consistently associated *TGFB1* +29C and -1347T with increased risk of aGVHD, but there is limited evidence of their role in OS, NRM and relapse. One could hypothesize that these associations could be related to allele p001. However, this cannot be confirmed. In any case, in the present study, the effect seen for allele p001 on OS and NRM could not be explained by increases in the prevalence of

aGVHD, making these findings novel in terms of the role of *TGFB1* polymorphism in HSCT. The lack of association between the presence of allele p001 in recipients and aGVHD could also be less prominent due to the fact that most of the transplants included in our cohort were T cell depleted. Consequently, the incidence of aGVHD is not very high. Also, the fact that no cumulative incidence data for aGVHD was available impaired survival-like analysis of this complication. Alternatively, the genetic association with NRM could potentially be explained by another cause of death such as infection or organ toxicity. It would be necessary to obtain cause of death information in order to perform this analysis and so this remains unclear.

The fact that a strong detrimental effect of patient p001 was observed in this study could be related to differences in functionality among *TGFB1* regulatory region and exon 1 alleles or SNPs. Individuals bearing the -1347T variant were reported to have higher TGF-β1 plasma levels when compared to individuals with the -1347C variant (Grainger *et al.*, 1999). The -1347T variant has also been associated with a significant increase (30%) in TGF-β1 expression in reporter gene assays (Silverman *et al.*, 2004). In turn, this group also found that the -1347C>T polymorphism alters *TGFB1* promoter-reporter activity and promoter interactions with the transcription factor Yin Yang 1 (Silverman *et al.*, 2004). Luedecking and collaborators also studied the -1347C>T SNP for its expression profile and found that the T variant was associated with marginally higher transcriptional activity when compared to the C allele (Luedecking *et al.*, 2000). The differences were, however, modest.

Combining both the observations made for *TGFB1* -1347C>T and those made for +29T>C (Suthanthiran *et al.*, 2000; Dunning *et al.*, 2003), Shah *et al*. proposed that the cluster of *TGFB1* regulatory region and exon 1 alleles that share a -1347T and +29C would represent a high production phenotype (Shah *et al.*, 2006). In addition, Dunning and collaborators concluded that homozygosities of both -1347T and +29C, which were in strong linkage disequilibrium in their study, were associated with an increased incidence of breast cancer (Dunning *et al.*, 2003). Allele p001 would be the main representative of this cluster and the sole representative seen in the patient-donor cohort with significant frequencies. Of note, the study by Wang and collaborators among Chinese cirrhosis patients found opposite results and associated -1347T and +29C with lower plasma concentrations of this cytokine and lower reporter gene activities (Wang *et al.*, 2008). Another study found that a *TGFB1* promoter variant pattern congruent with allele p001 showed weaker promoter activity than another one congruent with allele p003 (Healy *et al.*, 2009). Consequently, the relative efficiency of *TGFB1* regulatory region and exon 1 alleles is still unclear. Reporter gene and secretion studies based on well-defined alleles and not single

polymorphisms or limited polymorphic positions could potentially help to elucidate any functional differences. In addition to a -1347T variant, a feature that is unique to p001 in this cohort is the absence of the -2389AGGdup. Healy and collaborators found that the absence of this duplication caused the gain of allele DNA-protein complexes in electrophoretic mobility shift assays rather than a loss, suggesting that the absence of duplication may lead to novel transcription factor binding site motifs (Healy *et al.*, 2009).

Due to TGF-β1's relevance in the immune system's development, homeostasis and response, this cytokine is likely to play a central role in both therapeutic and pathogenic immune processes associated with the different stages of HSCT, from conditioning to engraftment and the complications thereafter. Consequently, it is conceivable that genetic variation resulting in differences in its production and/or function can play a role in the way that this cytokine modifies these immune processes.

Several clinical factors are well known to modify the outcome of HSCT, and can be divided in disease, patient, donor, transplant and post-transplant-related categories. Not all of these previously known clinical risk factors were confirmed in the present cohort. Seven clinical factors showed significant associations with OS and 5 did so for NRM in univariate analyses. As shown above, the main factors that modified OS in addition to *TGFB1* regulatory region an exon 1 genotype in multivariate analysis were disease status at transplant (complete remission/chronic phase vs other) and patient age (older than 40 years vs younger than 40 years). Older patient age is a well-known factor that can have a detrimental impact on OS (Eissa *et al.*, 2011; Finke *et al.*, 2012). Likewise, transplants performed not in complete remission or chronic phase and in advanced stages are known to suffer higher mortality rates (Finke *et al.*, 2012; Khoury *et al.*, 2012; Thanarajasingam *et al.*, 2013; Yoshimura *et al.*, 2013; Della Porta *et al.*, 2014; Matsumoto *et al.*, 2014; Saad *et al.*, 2014).

For NRM multivariate models showed that patient age and HLA matching were also significant factors for death without relapse in addition to *TGFB1* alleles. Consequently, the conformation of study cohort in terms of these clinical variables should be taken into account when this study is repeated in other cohorts. Older patient age is known to have a detrimental effect on NRM (Della Porta *et al.*, 2014; Lee *et al.*, 2014). HLA mismatches have also been shown to affect survival mainly through NRM (Flomenberg *et al.*, 2004; Lee *et al.*, 2007).

The findings presented in this chapter are of clinical relevance. The effect of *TGFB1* regulatory region and exon 1 allelic genotype on the outcome of UD-HSCT was sustained when several potentially confounding clinical factors were considered, and it even became stronger than in univariate analysis. The fact that patients having a p001/p001 genotype

have significantly higher probabilities of dying early after the transplant could potentially allow for better preemptive measures to be applied by the clinical teams in order to improve the prognosis for these patients. The frequency of this genotype is relatively high (~8%) further enhancing its relevance for the clinical practice. Further studies on the exact effect on cause of death by this genotype would be of use in terms of identifying more precise causes that could potentially provide a window of action by the transplant team in order to prevent these deaths. Moreover, recent initiatives to create risk stratification algorithms based on genetic and clinical variables incorporating the findings of the present study are likely to benefit the outcome of the transplants.

This study is subject to the limitations of any retrospective study. Some of the potentially relevant clinical data were not available for analysis. For example, incomplete data for cGVHD and infection precluded a more detailed analysis for these outcomes. Also, aGVHD could not be analysed by cumulative incidence taking into account competing risks, and this limited the analysis to crude frequencies. In general, the lack of cause-of-death data made it impossible to define more precisely what the reasons for the elevated NRM and reduced OS in patients with the significantly associated genotypes were. Future availability of these data could potentially allow for the novel findings. Also, despite the large size of the study, the effect of *TGFB1* regulatory region and exon 1 alleles with lower frequencies such as p006 and p014 precluded the analysis of their effect in recessive models. The study cannot thus discard the possibility of potential effects from these alleles, and a much larger study will be needed for these analyses. Finally, despite there being a predefined study plan and a systematic approach to the genetic association testing, the possibility of a chance finding cannot be ruled out, especially in view of the several models that were tested.

Consequently, an independent confirmatory retrospective study in a similar patient-donor cohort is necessary to confirm the associations found in this study. Moreover, prospective studies based on the findings herein presented are necessary to further validate the associations of the retrospective studies. In addition, since this study was performed in myeloablative transplants, its findings must be tested in cohorts that used reduced-intensity conditioning regimes, which are likely to be subject to significantly different immune reconstitutive and inflammatory processes in which TGF-β1 and the cells that produce it might play a different role and have different outcomes. Likewise, as mentioned previously, the fact that two of the prevalent alleles could not be efficiently tested in recessive models of association due to low numbers of homozygous patients and donors impairs a thorough assessment of their role in HSCT outcome. The only solution for this limitation would be to assess a much larger number of transplants.

In conclusion, this study has found a strong association between the presence of *TGFB1* regulatory region and exon 1 allele p001 and worse OS and higher NRM after UD-HSCT. In addition, this is the largest study on *TGFB1* polymorphisms in HSCT performed so far, and the first to comprehensively compare the effect of ~3-kb alleles and their genetic association with transplant outcome rather than focusing on one or two SNPs. It is likely that this approach will influence the way in which these studies are carried out in this field and in other medical fields interested in the relationship between genetic variation and susceptibility to disease, its treatment or complications associated with it.

Chapter 7. General discussion and future work

In the studies presented in this thesis, I have found evidence that *TGFB1* polymorphisms have an impact on the expression of transforming growth factor-β1 (TGF-β1) by regulatory T cells (Treg) upon activation and also on the outcome of unrelated-donor haematopoietic stem cell transplantation (UD-HSCT), specifically on overall survival (OS) and non-relapse mortality (NRM). The following sections summarize my results and future projects that may arise from these findings.

7.1 The role of genetic variation in *TGFB1* in the production of TGF-β1 by regulatory T cells (Treg)

The functional experiments showed a trend towards differential levels of surface latency-associated peptide (LAP) expression on activated Treg from healthy individuals with respect to their *TGFB1* +29T>C genotype. Higher production of TGF-β1 by cells bearing the +29C allele has been observed previously (Dunning *et al.*, 2003), and the finding in the present study adds to the evidence of functional differences caused by this SNP or other polymorphisms associated with it. As mentioned previously however, these activation experiments should be repeated in order to include a sufficiently high number of subjects in each genotype or allele group so that this trend can be confirmed more soundly. The presence of substantial biological variation could have had an impact on the activation experiments despite the attempts to control for it with the experimental design. It is common that biologically significant differences need large experimental sets in order to become apparent.

The lack of correlation between TGF-β1 protein and mRNA levels in activated Treg, as well as the differences between Treg and effector cells with regards to the kinetics of mRNA induction upon stimulation suggest that there are differences in Treg and effector CD4+ cell TGF-β1 regulation. A hypothesis for these preliminary findings is explained in **Figure 7.1**. The failure to identify an induction of *TGFB1* mRNA in Treg upon activation of the cells as well as the discovery of preformed intracellular deposits of the small latent complex (SLC) both in Treg and in effector CD4+ cells open new questions regarding the origin of potential differences in production of this cytokine by T cells. For example, does the fact that no mRNA induction was observed in Treg mean that any functional

differences must modify post-transcriptional and transcriptional stages of TGF-β1 production? If this were the case, then a polymorphism at its signal peptide could bear more relevance than one associated with the efficiency of transcription of the gene (i.e. a regulatory region one). Alternatively, genetic variation in terms of gene activation could impact the amount of preformed TGF-β1 in the cells, and assessment of levels upon activation could only be a surrogate for the total differences in TGF-β1 production. One possibility that should be explored is to compare the levels of intracellular LAP both in Treg and effector cells of different *TGFB1* genotypes before and after activation and to couple this with analysis of secreted protein (if any is present) in order to better understand the previous observations. Either way, it is evident that the regulation of *TGFB1* expression implies a complex multi-level system of checks and factors that play a role in it, from transcription factors, through stem-like RNA loops to microRNAs, which could also have different efficacy in different cell types and in different contexts such as inflammation.

7.2 The role of genetic variation in *TGFB1* in the suppressive function of regulatory T cells (Treg)

The suppression experiments were inconclusive in terms of the effect of *TGFB1* polymorphism on Treg function. As was mentioned before, it is likely that the classical *in vitro* T cell suppression assays cannot give an answer to this question. Consequently, the possibility of functional consequences of *TGFB1* polymorphisms in Treg suppressive capacity could be explored in other systems. For example, NK cell suppression by Treg has been largely attributed to TGF-β1 (Bellone *et al.*, 1995; Ghiringhelli *et al.*, 2005; Ralainirina *et al.*, 2007), and hence it is an interesting option to consider. Another option would be to use cells that respond to TGF-β1, but this would require that these cells are able to activate TGF-β1 from the SLC attached to Treg surface. Treg control of DC through TGF-β1 is another possibility that could be tested. Phosphorylation of SMAD molecules within the target cells as a marker for TGF-β1 signaling could potentially aid in the assessment of Treg-mediated effects on either effectors or APC. Clearly, more research on this topic is needed.

It is possible that the functional observations can vary significantly in the context of an *in vivo* assay. The *in vitro* experiments have the obvious limitation of lacking many variables, contexts, molecules and cells that are present in a live organism. Consequently, *in vitro* experiments can only give a partial answer to any biological question. An *in vivo* system

for the testing of functional differences in Treg biology elicited by *TGFB1* polymorphism is something that should be explored as well.

7.3 The role of genetic variation in *TGFB1* in the outcome of haematopoietic stem cell transplantation (HSCT)

The findings of the clinical study are interesting in view of the strength of the associations that were observed and the important outcomes that were affected by *TGFB1* polymorphism. This study will hopefully set a standard regarding the approach that genetic polymorphism association studies should take in terms of how variation is defined and assessed. Despite interest in the field of non-HLA immunogenetics, and specifically in cytokine polymorphism, for some 15 years (Middleton *et al.*, 1998), one aspect that has hampered the introduction of this concept in clinical practice has been the lack of reproducibility among now hundreds of studies that have examined SNP association with HSCT outcome and complications. This lack of consistency can be traced down to many controllable and uncontrollable aspects related to study design, cohort diversity and size, statistical tests, thoroughness of the analysis, availability of complete data, etc. Consequently, if non-HLA immunogenetics is to be taken into account it must overcome this initial phase of almost random testing and enter another in which rational, validated and coordinated analysis of variants becomes the standard. Genetic variation must be assessed by techniques that allow for the incorporation of multiple variants and their combined effects on the way that a gene is expressed and/or its product is generated. In this sense the study of a large genetic region that controls *TGFB1* expression is an example of this novel approach. Hopefully, the findings of this study could be tested in other large and well-characterised independent cohorts for their reproducibility.

The associations found in the clinical study must also be tested in the context of other reproducibly tested genetic factors. Recently, a study evaluating candidate genetic risk factors for aGVHD used a large cohort of 1298 allo-HSCT patient-donor pairs (Chien *et al.*, 2012). The authors selected 40 previously reported candidate SNPs, of which 6 were genotyped and 10 were imputed in their study. Patient and donor genotypes were assessed for association with aGVHD, in univariate and multivariate allelic, recessive and dominant models. The authors found significant associations for only 6 of the genes, and 4 were inconsistent with original publications. The *TGFB1* -1347C>T was included in the original set of 40, but was unfortunately not genotyped nor imputed and was consequently not analysed in this study. In addition to these large validation studies, recent attempts to construct risk models for aGVHD, both for general (Kim *et al.*, 2012)

and organ-specific disease (Kim *et al.*, 2014), illustrate the way forward in non-HLA immunogenetics. The findings presented in this thesis should be evaluated in prospective studies considering not only clinical factors, but also other, potentially interacting, genetic risk factors for HSCT outcome for the construction of prognostic tools. In addition, because of potentially significant differences in immunobiologic and inflammatory conditions associated with this type of conditioning, a cohort of reduced-intensity UD-HSCT patients and donors should be tested in order to determine if the findings from the myeloablative cohort are similar in these transplants. Likewise, since the cohort analysed in this study was composed of BM and PBSC transplants only, a repeat study specific for transplants performed with cord blood as a source of stem cells must be performed.

From a technical perspective, the recent introduction of novel sequencing technologies could be an interesting alternative for the typing of *TGFB1* regulatory region and exon 1 alleles especially in the context of high-throughput typing. One of these novel approaches, next-generation sequencing, is already penetrating the HSCT field through the typing of HLA polymorphism (Bentley *et al.*, 2009; Wang *et al.*, 2012; De Santis *et al.*, 2013). One advantage of this approach would be that the phase of the different polymorphisms that make up the alleles in any given sample could be determined unambiguously without the need of further testing. The cost of the typing in transplant centers and tissue typing laboratories could potentially also be lowered by the use of these novel technologies if the typing of *TGFB1* and other non-HLA genes became a standard in clinical practice.

7.4 A combined interpretation of functional and clinical findings

The finding that the presence of allele p001 in patients confers worse OS and higher NRM after UD-HSCT could have different explanations. Interestingly, even though the levels of LAP expression after activation of Treg were analysed in terms of *TGFB1* +29T>C genotype, it is possible to argue that this effect is actually traced to allele p001. This is because all +29CC+TC healthy individuals that were tested in the activation experiments had at least one copy of *TGFB1* regulatory region and exon 1 allele p001. Consequently, there is a possibility that the increase in LAP expression is indeed due to this allele. This situation would give support to the potential high-expression phenotype associated with p001 in the context of the clinical HSCT study. Assuming that p001 is an allele that is characterised by higher production levels of this cytokine, then a detrimental effect of TGF-β1 after HSCT would be implicated in the association. The source of this TGF-β1 is still unclear. Since the effect was seen for patient genotype and not for donor genotype, one could speculate that the source of TGF-β1 could be cells/tissues other than the graft.

In this scenario, higher levels of TGF-β1 produced by endothelial cells for example could hamper early immune responses to infectious agents. Alternatively, higher levels of TGF-β1 in the context of an HSCT could mean that organ damage becomes exacerbated.

Interestingly, in a study using a mouse model for HSCT and murine gammaherpesvirus (Coomes *et al.*, 2010), transplanted mice showed reduced ability to clear virus from the lung. While this lack of control was not related to impaired leukocyte recruitment or defective APC function, transplanted mice suffered defective CD4 T cell proliferation, skewing of effector CD4 T cell phenotype from Th1 to a Th17 and overexpression of TGF-β1 with increasing numbers of Treg. The authors state that these results indicate that overexpression of TGF-β1 following myeloablative conditioning post-HSCT resulted in impaired effector T cell responses to viral infection (Coomes *et al.*, 2010). A subsequent report by the same group found that protection from pneumonitis in transplanted transgenic mice with double-negative deletion of *TGFBRII* was associated with decreased TGF-β1 produced by parenchymal cells (Coomes *et al.*, 2011). A similar study with a mouse model of HSCT found that the mice with elevated TGF-β1 showed severe pneumonitis but had lower viral load (Adler *et al.*, 1998). The authors also demonstrated that anti–TGF-β1 treatment decreased the histological evidence of pneumonitis in these mice. Consequently, there is a possibility that a higher production of TGF-β1 caused by polymorphism in its regulatory region could contribute to these infectious complications. Interestingly, a report studying the role of *TGFB1* +29∼+74 variants in CMV reactivation in cancer patients found that patients homozygous for the variants congruent with allele p001 (i.e. +29C∼+74G) had an increased risk of reactivation (Cano *et al.*, 2012).

Another example of a potential niche for TGF-β1-mediated detrimental effects on post-HSCT survival is hepatic veno-occlusive disease (VOD). VOD is a major complication of HSCT and can be highly lethal in its severe forms. The development of VOD has been associated with the use of busulfan as a conditioning agent. A study that examined the molecular mechanisms for this association found that patients treated with busulfan experienced a significant increase in their plasma levels of plasminogen-activator inhibitor-1 (PAI-1) (Reimer *et al.*, 2012). These investigators also found that the induction of PAI-1 expression and secretion by busulfan is mediated by TNF-α, TGF-β1, and activin A in an endothelial-like cell line. They found that a subset of genes that were regulated by busulfan exposure in this cell line and that were related to blood coagulation and hemostasis was controlled by TGF-β1. This finding was confirmed in primary endothelial cells. In view of this, the authors proposed that TGF-β1 might be a central mediator of busulfan gene activation, and might be a target in the treatment and prevention of VOD

(Reimer *et al.*, 2012). Consequently, a high-producer genotype for TGF-β1 could increase the risk of VOD even further.

Another possibility is that a high-expression phenotype for TGF-β1 could have a role in the plasticity of T cells after the transplant. It has recently been recognised that TGF-β1 plays a central role in the differentiation of Th17 cells (Mangan *et al.*, 2006; Zhu & Paul, 2008). TGF-β1 in addition to IL-6 induces the differentiation of naïve T cells into IL-17-producing pro-inflammatory cells (Veldhoen *et al.*, 2006). In fact, Treg and Th17 cells have been shown to arise from common precursors, the discriminating factor being IL-6 exposure (Bettelli *et al.*, 2006). Treg themselves have been proposed as a source of TGF-β1 for Th17 differentiation, specifically surface GARP-bound SLC (Edwards *et al.*, 2013). More interestingly, Treg have been shown to become Th17 cells given the right inflammatory conditions, both *in vivo* and *in vitro* (reviwewed by Afzali and collaborators (Afzali *et al.*, 2010)). Since the context of a myeloablative UD-HSCT implies the presence of a high degree of inflammation and a high level of IL-1 and IL-6, it is possible that this in combination with higher levels of TGF-β1 could produce a skewing of the T cell compartment toward the Th17 subset, which could be detrimental to patients.

Even though the results from the clinical study did not confirm an association between *TGFB1* regulatory region and exon 1 polymorphism and the incidence of aGVHD, there remains the possibility that this mechanism of organ damage does take place in a more pronounced way in patients with a high-producer genotype. As mentioned in the previous chapter this association could be masked by the lack of cumulative incidence data considering competing risks. There is increasing evidence that Th17 cells and related cytokines play a role in the development and/or the evolution of aGVHD post HSCT (Dlubek *et al.*, 2010; Zhao *et al.*, 2011). A recent report by Liu and collaborators found that both the percentage of Th17 cells and the expression of their signature transcription factor RORγt were significantly higher in patients with severe (grade 3 to 4) and mild aGVHD (grade 1 to 2) when compared to patients without aGVHD (grade 0) and healthy donors (Liu *et al.*, 2013). On the contrary, the authors found that the percentage of Treg and the expression of FOXP3 were significantly lower in the former when compared to the latter. In addition, the expression of IL-6, IL-1β, IL-17, IL-21, IL-23, and IL-23R were increased in aGVHD patients. Overall, the authors state that there was a reciprocal relationship between Treg and Th17 cells (Liu *et al.*, 2013). Mouse models have also found a role for Th17 cells and aGVHD (Yu *et al.*, 2011; Ma *et al.*, 2012; Jankovic *et al.*, 2013). Of note a report measuring the frequency of T cell subsets after allo-HSCT found low levels of reconstitution within the first 3 months after the transplant (Bahr *et al.*, 2013). The authors reported no association between aGVHD and changes in the size of inflammatory

T cell compartments including Th17. However, Th17 induction could be happening in the tissues and not be apparent from peripheral blood frequencies. The conversion of Treg into Th17 cells must be taken into consideration as Treg immunotherapy is increasingly being proposed as a therapeutic strategy to control inflammatory conditions, including GVHD (Edinger & Hoffmann, 2011). Consequently, it would be interesting to study the role of *TGFB1* regulatory region and exon 1 in the efficiency of generation of Th17 cells in an inflammatory context both *in vitro* and *in vivo* and to try to link this with complications and survival after myeloablative UD-HSCT, and also with potential risks of using Treg immunotherapy in these patients.

Granulocyte colony-stimulating factor (G-CSF) is administered to donors for the mobilization of stem cells for the harvesting of PBSC. Of note, clinical trials have indicated that BM priming with G-CSF results in comparable engraftment, reduced severity of aGVHD, and subsequent cGVHD, as compared with mobilized PBSC (Morton *et al.*, 2001; Elfenbein & Sackstein, 2004). There is evidence of quantitative and qualitative differences in immunological cells and type 1 and type 2 cytokines between G-CSF-mobilized PB and G-CSF-primed BM, the former showing a more Th1-prone profile (Jun *et al.*, 2005). In view of this, the effect of the use of G-CSF on the plasticity of T cells and the role of TGF-β1 and its polymorphism in this phenomenon could also be studied.

One interesting opportunity to complement the clinical study would be to explore other pathologic functional tests that could help to give support to the genetic associations. Several models could be considered for this purpose. One example is skin explants that seek to predict the development of GVHD reactions *in vivo* (Dickinson *et al.*, 1988). In would be interesting to determine if genetic variation in *TGFB1* can affect the generation of lesions in these models, and, if so, what is the role of this cytokine and what the sources for it are in this system. This has been done previously for other genes (Dickinson *et al.*, 2001), and systems for the study of the role of specific cell subsets such as Treg have been developed (Wang *et al.*, 2009; Mavin *et al.*, 2012). Something to take into account, however, is that the use of T-cell depletion in HSCT using unrelated donors seems to induce false-positive GVHD reactions and no correlation between the predicted skin GVHD and clinical GVHD (Wang *et al.*, 2006). Other potential alternatives could be models for other organs and tissues such as lung (Jager *et al.*, 2014), or even the aforementioned VOD-related *in vitro* assay (Reimer *et al.*, 2012).

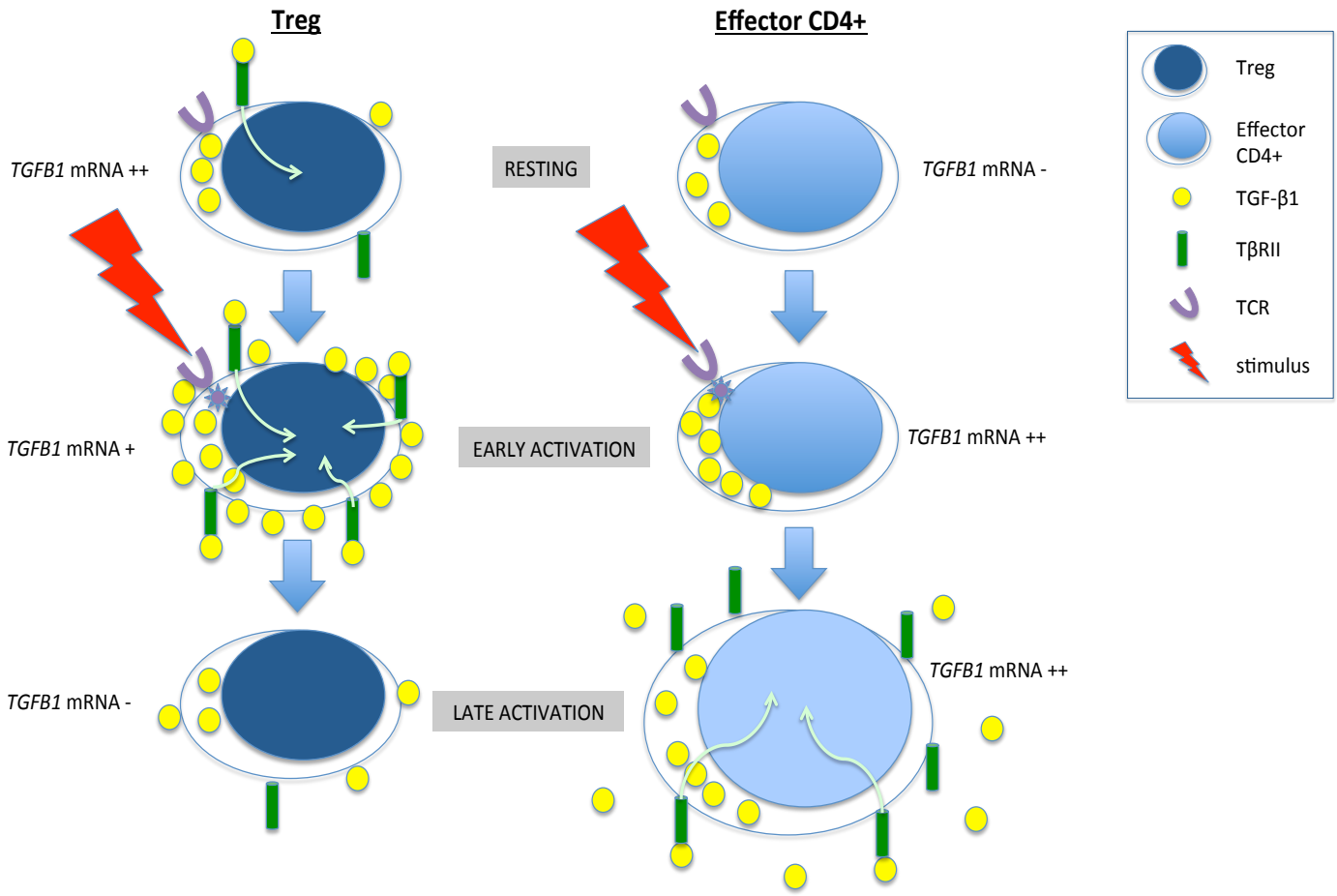

Figure 7.1 A hypothesis on the differential kinetics of transforming growth factor-β1 (TGF-β1) in resting and activated regulatory T cells (Treg) and effector CD4+ T cells. At a resting state, Treg express low levels of transforming growth factor-β1 receptor (TβRII) and of surface TGF-β1 (and low levels of autocrine signaling for the maintenance of the regulatory programme), whereas effector CD4+ T cells do not express either of these. Both cell subsets have intracellular deposits of latent TGF-β1. *TGFB1* mRNA is present at a resting state in both subsets, but levels are lower in effector cells when compared to those in Treg. Upon T cell receptor-mediated activation of these cells, TGF-β1 would be differentially regulated. Uniquely, Treg become highly positive for surface latent TGF-β1 and upregulate the expression of TβRII, increasing autocrine and paracrine signaling at early stages of activation. These events would strengthen the control of the immune response, but also the control of their regulatory programme. A consequence of TCR activation in these cells would be the reduction of *TGFB1* mRNA levels as a feedback loop in order to control Treg expression of this molecule. At later stages after activation, the levels of surface latent TGF-β1 are reduced, and the cell would be directed to a resting or proapoptotic condition, with further reductions in their levels of *TGFB1* mRNA. On the other hand, effector cells that become activated start to produce more TGF-β1 with increasing levels of *TGFB1* mRNA, in parallel to induction of the expression of TβRII. Eventually, this latent TGF-β1 starts to be secreted, as these cells cannot localize it to their cell surfaces. The secretion of TGF-β1 as well as the induction of its receptor would contribute to a later control of the immune response mediated by these cells.

7.5 Conclusion

In conclusion, this study has found further evidence of a role for *TGFB1* polymorphism in HSCT, and the production of this cytokine by a previously not studied cell subset such as Treg. Also, the finding of an association of *TGFB1* regulatory region and exon 1 polymorphism with the outcome of UD-HSCT could potentially help to modify the way in which patients are managed to improve their long-term survival. Interesting additional discoveries in human Treg and effector CD4 cell biology include the lack of induction of *TGFB1* mRNA upon TCR-mediated activation in Treg, the presence of preformed pre-activation intracellular SLC (LAP) both in Treg and effector CD4+CD25- cells, and the kinetics of transient LAP expression on the surface of Treg in the context of an *in vitro* suppression assay. The typing of *TGFB1* regulatory region and exon 1 alleles in more than 1000 unrelated samples gives the first large-scale glimpse of the diversity of *TGFB1* polymorphism in the population. Likewise, this study discovered the presence of novel polymorphic positions and novel alleles of this region of the human *TGFB1* gene, adding to the current knowledge on the diversity associated with this gene. From a technical perspective, additional contributions of these studies are also the design of a novel *TGFB1* regulatory region and exon 1 sequencing-based typing strategy and the generation of an informatics tool for the easy assignment of allelic genotypes for this ~3 kb region based on the individual variant genotypes at 18 polymorphic positions.

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Appendix A. Variant identification numbers and genomic location of polymorphisms in *TGFB1*'s regulatory region and exon 1

***TGFB1* polymorphic position**	**Variant ID**	**Position in chromosome 19[a]**
-2410G>A	rs4803457	41355454
-2389dupAGG	rs11466313	41355432
-1985C>G	rs3087453	41355029
-1638G>A	rs1800468	41354682
-1347C>T	rs1800469	41354391
-1287G>A	rs11466314	41354331
-1169delTT	rs56368056	41354213
-1154C>T	rs35318502	41354198
-827G>C	rs11466315	41353871
-778G>A	rs36185305	41353822
-768insC	rs1800999	41353812
-469C>A	rs35025994	41353514
-387C>T	rs11466316	41353431
-229C>G	rs61761350	41353273
-14G>A	rs9282871	41353058
+29T>C	rs1800470	41353016
+74G>C	rs1800471	41352971
+91T>G	rs61761349	41352954

ID, identification number. [a]Based on assembly GRCh38, build 106.

Appendix B. Consent form

ANTHONY NOLAN
BE A MATCH, SAVE A LIFE

ANTHONY NOLAN
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Version 6
Bes 050719

To: Potential bone marrow donors and recipients

From: Dr J Alejandro Madrigal
Research Director
Anthony Nolan Research Institute
020 7284 8324 (phone)

Information Sheet and Consent Form

Re: Analysis of genetic factors effecting outcome in unrelated haematopoietic stem cell transplants

At the Anthony Nolan Research Institute (ANRI) we are undertaking a research study that we would like you to consider joining. Before deciding, please read the following information carefully and ask any questions which you may have.

This study is investigating the importance of HLA matching (tissue typing) in unrelated stem cell transplants. We are also investigating other genetic factors that have been shown to influence the outcome of unrelated transplants. Recent advances in typing methods have made it possible to obtain very specific tissue types, but this in turn makes it even more difficult to find a 'perfectly' matched donor. As you will be aware, this is already a very difficult task and often only a partially matched donor is available. Many transplants done in these circumstances though, have very successful outcomes, leading us to suspect that some 'mismatches' may be acceptable, while others may be associated with increased complications.

The purpose of this study is firstly to use all available techniques to obtain genetic information (e.g. tissue types, cytokine gene polymorphisms) in the patient/donor pairs, and secondly, by following the outcome of transplant to correlate specific mismatches with a successful transplant outcome. Although this research will not directly alter results in this transplant, it is hoped that in the future this will enable us to advise which donor should be chosen in the event that no fully matched donor is available, but where there is a choice of partially matched donors.

We are asking all Anthony Nolan Trust donors and all patients who receive stem cells from an Anthony Nolan Trust donor to consider joining this research project. However, should you choose not to join, this will not affect your treatment/ donation in any way. **All that will be required from you will be a blood and/or a buccal swab sample (mouth swab).**

The DNA extracted from this sample will only be used for matching studies in our laboratory (i.e. only looking for factors to do with outcome in haematopoietic stem cell transplants). It will be stored within the Research Institute, with a unique coding number for the duration of the study (i.e. only the researchers will be able to link the sample to the person who provided it). After the study is completed we would like to store the donor/patient sample pairs in an anonymised form (i.e. the details cannot be traced back to an individual person). The purpose of this is to enable us to test these samples for any genetic factors related to stem cell transplantation that may be discovered in years to come. These samples will be owned by the Anthony Nolan Research Institute.

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Version 6
Bes 050719

To: Potential bone marrow donors and recipients

From: Dr J Alejandro Madrigal
Research Director
Anthony Nolan Research Institute
020 7284 8324 (phone)

Consent Form

Re: Analysis of genetic factors effecting outcome in unrelated haematopoietic stem cell transplants

1. I have read the information sheet and understand the details of the study. I have had an opportunity to ask any questions and have received satisfactory answers. ☐

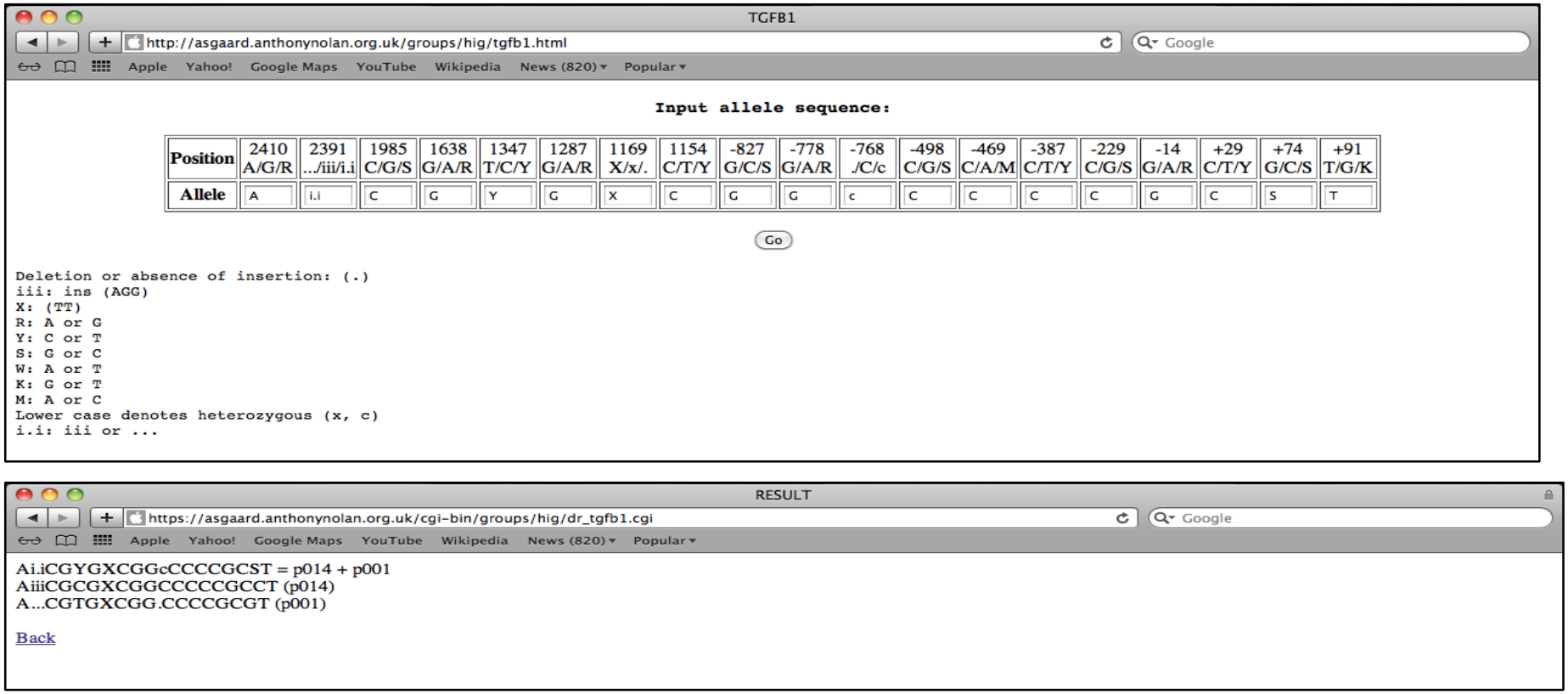
2. I understand that my participation is voluntary, and if I chose not to provide a blood and/or a buccal cell sample (mouth swab) my treatment/ donation will not be affected in any way. ☐

3. I agree to take part in the above study by providing a blood and/or buccal cell sample (mouth swab). ☐

4. I agree that my blood and/or buccal cell sample (mouth swab) can be retained after the study completes (in an anonymised form). ☐

Health Care Witness	Patient or Bone marrow Donor
Signed……..	Signed……
Name……..	Name……..
Position……..	Hospital……..

Appendix C. Bioinformatics tool interface and output



The upper panel shows the web interface of the bioinformatics tool. Here the user can type in the genotypes recorded for each polymorphic position using coding for heterozygosity and for insertion or deletion of bases as shown in the key. The lower panel shows the output generated for the example above. The tool produces the allelic combination that matches the genotypes that were input by the user and the variants associated with each allele.

Appendix D. Publications

Arrieta-Bolaños E, Madrigal JA, Marsh SGE, Shaw BE, Salazar-Sánchez L. The frequency of HLA-B*57:01 and the risk of abacavir hypersensitivity reactions in the majority population of Costa Rica. Hum Immunol. In press.

Cox ST, **Arrieta-Bolaños E**, Madrigal JA, Saudemont A. RAET1/ULBP alleles and haplotypes among Kolla South American Indians. Tissue Antigens. 2013; doi: 10.1016/j.humimm.2013.01.030.

Arrieta-Bolaños E, Alejandro Madrigal J, Shaw BE. Human leukocyte antigen profiles of Latin American populations: differential admixture and its potential impact on hematopoietic stem cell transplantation. Bone Marrow Res. 2012; 2012:136087. doi: 10.1155/2012/136087.

Arrieta-Bolaños E, Alejandro Madrigal J, Shaw BE. Transforming growth factor-β1 polymorphisms and the outcome of hematopoietic stem cell transplantation. Int J Immunogenet. 2012 Jun;39(3):192-202.

Arroyo J, Salazar-Sánchez L, Jiménez-Cruz G, Chaverri P, **Arrieta-Bolaños E**, Morera B. Prevalence and geographic distribution of haemophilia in Costa Rica. Hamostaseologie. 2010 Nov;30 Suppl 1:S28-31.

Arrieta-Bolaños E, Maldonado-Torres H, Dimitriu O, Hoddinott MA, Fowles F, Shah A, *et al*. HLA-A, -B, -C, -DQB1, and -DRB1,3,4,5 allele and haplotype frequencies in the Costa Rica Central Valley Population and its relationship to worldwide populations. Hum Immunol. 2011 Jan;72(1):80-6.

Appendix E. Manuscripts in preparation

Arrieta-Bolaños E, *et al*. Novel SNPs and alleles of the *TGFB1* regulatory region and exon 1

Arrieta-Bolaños E, *et al*. The production of transforming growth factor-β1 by human regulatory T cells and the functional consequences of *TGFB1* polymorphisms

Arrieta-Bolaños E, *et al*. *TGFB1* regulatory region and exon 1 alleles and their impact on the outcome of unrelated-donor haematopoietic stem cell transplantation

Arrieta-Bolaños E, *et al*. High-resolution HLA-A, -B, -C, -DRB1, and –DQB1 allele and haplotype frequencies in the Costa Rican Central Valley population

Arrieta-Bolaños E, *et al*. High-resolution HLA-A, -B, -C, and -DRB1 allele and haplotype frequencies in the population of Nicaragua

Arrieta-Bolaños E, *et al*. High-resolution HLA-A, -B, -C, and -DRB1 allele and haplotype frequencies in minority populations of Costa Rica

Arrieta-Bolaños E, *et al*. The HLA map of the world: an analysis of worldwide HLA variation

Appendix F. Awards

DNA Genotek 'Your Choice' Award, June 2013

British Society for Histocompatibility and Immunogenetics Education and Training Bursary, May 2013

Best Research Student Poster Award, UCL Cancer Institute 5th Annual Conference and the 3rd Annual UCL Cancer Research UK Centre Conference. London, UK June 2012

Life Technologies Best Young Scientist Poster Award, The Joint 16th International HLA and Immunogenetics Conference/26th European Immunogenetics and Histocompatibility Conference/23rd British Society for Histocompatibility and Immunogenetics Conference. Liverpool, UK, June 2012

British Society for Histocompatibility and Immunogenetics Education and Training Bursary, May 2012

Kitty Cookson Travel Award, University College London Cancer Institute, United Kingdom**,** April 2012

EFI Conference Travel Bursary, European Federation of Immunogenetics, April 2012

COST Training School Grant, European network of the HLA diversity for histocompatibility, clinical transplantation, epidemiology and population genetics (HLA-NET), February 2011

Graduate School Student Conference Award, University College London, United Kingdom**,** January 2011

Kitty Cookson Travel Award, University College London Cancer Institute, United Kingdom**,** January 2011

British Society for Histocompatibility and Immunogenetics Education and Training Bursary, November 2010

Global Excellence Scholarship, University College London, United Kingdom, April 2010