Development of *in vitro* procedures that can better predict the safety of therapeutic monoclonal antibodies

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A thesis submitted by
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I, Lucy Findlay 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. Some of these other sources are joint publications with colleagues at NIBSC, copies of which are on the enclosed CD. Information from this thesis constitutes most of the information presented in the publications Findlay et al. (2010) and Findlay et al. (2011a). However, for the publications Stebbings et al. (2007), Eastwood et al. (2010) and Findlay et al. (2011b), information from this thesis constitutes only part of the information presented. In the text of this thesis, references are made to these joint publications in cases where the information stated is not entirely my own work and is presented in the joint publication.

Abstract

Pre-clinical safety testing (*in vivo* and *in vitro*) of the therapeutic monoclonal antibody (mAb) TGN1412 (developed for the treatment of autoimmune diseases) failed to predict the life threatening adverse events that occurred during its Phase I Clinical Trial. The treatment of disease using mAb therapy is becoming increasingly common, so, to ensure the safety of mAbs, pre-clinical safety tests that can better predict the toxicity of immunomodulatory mAbs, such as TGN1412, are required. The aim of this study was to investigate the hypothesis that cytokine-driven adverse effects of therapeutic monoclonal antibodies and the mechanisms involved can be better predicted with novel *in vitro* procedures using human cells, given the failure of animal models to predict the toxicity of TGN1412. Consistent with the results from pre-clinical testing, aqueous phase TGN1412 incubated with human peripheral blood mononuclear cells (PBMC) failed to stimulate the "cytokine storm" suffered by the six recipients of TGN1412. In contrast, TGN1412 immobilised onto polypropylene microtitre plates by "air-drying" stimulated cytokine release from PBMC. This technique was superior to other mAb immobilisation techniques, investigated in terms of predicting cytokine release. Immobilisation of TGN1412 may mimic the immunological synapse formed between this mAb and target cells *in vivo*. In a more physiologically relevant procedure, TGN1412 incubated in aqueous phase with PBMC over a monolayer of human endothelial cells stimulated cytokine release. Endothelial cell to PBMC contact was crucial to these responses. Furthermore, interactions between lymphocyte function-associated antigen-3 (LFA-3) and intercellular adhesion molecule-1 (ICAM-1) expressed by endothelial cells with their counterstructures CD2 and LFA-1, respectively, expressed by T cells, mediated these TGN1412-stimulated responses. Both procedures developed in this study were capable of distinguishing therapeutic mAbs not associated with a significant incidence of cytokine-driven clinical infusion reactions from mAbs frequently associated with clinical infusion reactions.

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Abbreviations

3H-thymidine	tritiated thymidine
Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Akt	protein kinase B
ANOVA	analysis of variance
AP-1	activator protein-1
APC	antigen presenting cells
B-CLL	B cell chronic lymphocytic leukemia
bp	base pairs
BSA	bovine serum albumin
CDC	complement-dependent cytotoxicity
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining regions
C_H	constant heavy chain
C-HUVEC	primary endothelial cells from umbilical vein of fresh human cords
C_L	constant light chain
CM	culture medium
cSMAC	central supramolecular activation complex
Ct	number of PCR cycles to reach threshold fluorescence
CTLA-4	cytotoxic T-lymphocyte antigen-4
DAG	diacylglycerol
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotidetriphosphates
dSMAC	distal supramolecular activation complex
DTT	dithiothreitol
EBM	endothelial cell basal medium
ECGS	endothelial cell growth supplement
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGM-2	endothelial growth medium-2
ELC	endotoxin limit concentration
ELISA	enzyme-linked immunosorbent assay
ET	endotoxin
EU	endotoxin units

FACS	fluorescence-activated cell sorting
FcR	Fc receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Fyn	proto-oncogene protein tyrosine kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Grb2	growth factor receptor bound protein
hAB	human AB
HFF	human foreskin fibroblasts
HGF	human gingival fibroblasts
hi	heat inactivated
HSVEC	human saphenous vein endothelial cells
HuMAC	Human Materials Advisory Committee
HUVEC	human umbilical vein endothelial cells
IC	isotype-matched negative control
ICAM-1	intercellular adhesion molecule-1
ICOS	inducible T-cell costimulator
IFN	interferon
IFNGR	interferon gamma receptor
Ig	immunoglobulin
IL-	interleukin
IL-2R	IL-2 receptor
IP_3	inositol 1,4,5-triphosphate
IS	international standard
ITAMs	immunoreceptor tyrosine-based activation motifs
Itk	IL2-inducible T-cell kinase
IU	international units
KDa	Kilo Daltons
KLH	keyhole limpet hemocyanin
LAK	linker of activated T cells
LAL	Limulus amebocyte lysate
LBP	lipid binding protein
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	lymphocyte function-associated antigen-1
LFA-3	lymphocyte function-associated antigen-3
LICOS	ligand for inducible co-stimulator
LPS	lipopolysaccharide

mAb	monoclonal antibody
MABEL	minimum anticipated biological effect level
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
MAT	monocyte activation test
MHC	major histocompatibility complex
MHRA	Medicines and Healthcare Regulatory Authority
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
mRNA	messenger ribonucleic acid
MTOC	microtubule-organising centre
NF	nuclear factor
NFAT	nuclear factor of activated T cells
NHS	National Health Service
NIBSC	National Institute for Biological Standards and Control
NK cells	natural killer cells
NOEL	no observed adverse effect level
OD	optical density
OMCL	Official Medicines Control Laboratory
OPD	o-phenylenediamine dihydrochloride
PAF	platelet-activating factor
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PI3	phosphatidylinositol 3
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PLC-γ	phospholipase C
PP	polypropylene
PS	polystyrene
PSA	penicillin, streptomycin, amphotericin B
pSMAC	peripheral supramolecular activation complex
QEII	Queen Elizabeth II
RaCS	raft clustering during T cell surveying
RNA	ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
S.E.M	standard error of the mean

SLP-76	lymphocyte cytosolic protein
Syk	spleen tyrosine kinase
TBS	tris-buffered saline
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
Tm	melting temperature
TMB	3,3',5,5'Tetramethylbenzidine
TNF	tumour necrosis factor
TNFRI	TNF receptor I
TNFRII	TNF receptor II
Treg	regulatory T cells
Tris	tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylene-sorbitan monolaurate
Vav1	guanine nucleoside exchange factor
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
V_H	variable heavy chain
V_L	variable light chain
VLA-4	very late activation marker-4
vWF	von Willebrand factor
WHO	World Health Organisation
ZAP-70	zeta-chain-associated protein kinase

Chapter 1:
General introduction

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1.1 Brief

Infused or injected biological medicines (parenterals) can cause unwanted side effects as a result of an inherent property of the drug itself or as a result of contamination of the medicine with, for example, pyrogenic (fever-causing) substances such as endotoxin (Probey and Pittman, 1945; Steere et al., 1978). The potency and safety of parenteral medicines are predicted, as far as is practically possible, through *in vitro* tests or using animal models before their 'first time in man' clinical trial or before the release of a new batch of a biological medicine onto the market. Unfortunately, rare incidences can occur where the pre-clinical testing of a medicine does not predict unwanted *in vivo* effects. One of the best-known cases (because of the extensive press coverage) was the disastrous, life-threatening, first clinical trial of the immunomodulatory superagonistic anti-CD28 therapeutic monoclonal antibody (mAb) TGN1412 (Figure 1)

Figure 1. TGN1412 clinical trial disaster reported by the BBC. Taken from the BBC website, March 2006.

Monoclonal antibody (mAb) therapy for the treatment of disease is becoming increasingly popular since mAbs can be produced with high specific avidity for many therapeutic targets in, for example, cancer, autoimmune diseases and neurologic disorders (von Mehren et al., 2003; Novak et al., 2008; Shah and Mayer, 2010). This, taken together with the fact that pre-clinical tests failed to predict the toxicity of TGN1412, highlights the need for methods that can better predict adverse effects of therapeutic mAbs in the clinic if these medicines are to be used successfully for the treatment of disease.

1.2 Therapeutic mAbs

Therapy with mAbs is offering new ways to treat a variety of diseases and currently there are over 20 products that have been approved by the Food and Drug Administration (US) or the European Medicines Agency for therapeutic use (Kubota et al., 2009). A majority of these are intended for the treatment of cancer or inflammatory/autoimmune diseases. They therefore have the potential to modulate aspects of the immune system (i.e. innate, cell-mediated or humoral immunity) and so are "immunomodulatory".

1.2.1 Structure of therapeutic mAbs

Therapeutic mAbs are usually immunoglobulin class gamma (IgG) and their basic structure, as shown in Figure 2A, is a heterodimer containing two light chains (which can be either κ or λ) and two γ heavy chains connected by disulphide bonds within the flexible hinge region. The heterodimer can be divided into three independent regions: the Fc domain and two Fab domains, as shown in Figure 2B. Each light and heavy chain has a region in which the amino acid sequence varies from one mAb to the next, giving rise to its target specificity. These variable regions are shown in Figure 2 as V_L and V_H. Amino acid sequence variability is concentrated in hypervariable regions (3 on each chain) called complementarity-determining regions (CDRs) within V_L and V_H. The remaining regions of V_L and V_H are much less variable and are known as framework regions. The combined structure of all CDRs forms a site which

binds to target antigen. The V_L and V_H regions form the two Fab portions of the antibody. The remainder of the antibody molecule is known as the constant region and consists of a constant light domain (C_L) as it constitutes the light chain and a constant heavy domain (C_H). The latter is divided into CH_1, a hinge region, CH_2 and CH_3 and is responsible for the biological function of the molecule (Kuby, 1997).

Figure 2. Structure of a mAb (IgG). V_L: variable light chain; V_H: variable heavy chain; C_L: constant light chain; C_H: constant heavy chain. Adapted from Kuby, 1997.

Therapeutic mAbs have progressed from being entirely murine to chimeric mAbs in which murine variable regions (including framework regions) are connected to human constant regions, and then to humanized mAbs in which the constant and framework regions are human but the CDRs are murine in origin, and now to fully human mAbs (Hansel et al., 2010). Fully human mAbs of course are likely to be tolerated better by humans.

1.2.2 Modes of action of therapeutic mAbs

Therapeutic mAbs exert their effects by several different mechanisms. Through binding of the CDRs to target receptors, mAbs can activate signalling pathways, as is the case for TGN1412 (see below). Alternatively, CDRs can bind target ligands or receptors to block activation (by other molecules) of signalling pathways as is the case for Tysabri, an anti-α4 integrin therapeutic mAb for the treatment of multiple sclerosis and Avastin, an anti-vascular endothelial growth factor (VEGF) for the treatment of various cancers. Other mAbs exert their effects through binding of the Fc portion of target bound antibody to Fc receptors leading to antibody-dependent cell-mediated cytotoxicity (ADCC). *In vivo*, ADCC is initiated when antibodies produced by B lymphocytes during a humoral response bind to a pathogen-infected target cell labelling it for destruction. Binding of the antibody Fab region to its target exposes the Fc portion to Fc receptors expressed by effector cells; primarily but not exclusively natural killer cells (cytotoxic lymphocytes), which stimulates the release of pro-inflammatory cytokines and cytotoxic granules containing granzymes (serine proteases) and perforin from these cells. These granules are internalised by target cells and ultimately trigger programmed cell death, otherwise known as apoptosis (Buzza and Bird, 2006). Therapeutic mAbs can use this physiological immune effector function to destroy target cells and so when a therapeutic mAb binds to target receptors on cells it can label them for destruction through ADCC (von Mehren et al., 2003).

Complement-dependent cytotoxicity (CDC) is another effector function controlled by the Fc portion of mAbs. *In vivo*, in the classical complement activation pathway, antibodies produced in a humoral response bind to pathogens via their variable region rendering the Fc portion free to bind C1q, a component of a complex called C1 which contains two other proteins, C1r and C1s. This binding activates a cascade of reactions involving other complement proteins, ultimately leading to several effector functions. Some complement proteins act as opsonins, i.e. they bind to the immune complex (antigen expressed by pathogen:antibody) labelling it as a target for destruction. Phagocytic cells expressing complement receptors (such as macrophages and neutrophils) are attracted to the immune complex by release of small

complement peptides such as C3a and C5a which posses chemotactic and pro-inflammatory activity. The phagocytic cells internalise the opsonised pathogen (by phagocytosis) where it is destroyed by lysosomal enzymes. Furthermore, complement can form a membrane attack complex which also destroys bacteria. Therefore, not surprisingly, when therapeutic mAbs bind to target receptors on cells, this can also label them for destruction through CDC (von Mehren et al., 2003; Bugelski et al., 2009). Different IgG subclasses activate complement to different extents. CDC induction by IgG antibody occurs when one Clq molecule is bound to the Fc portions of multiple IgG molecules held in close proximity to each other as a result of their binding to antigen (Murphy et al., 2008). For this reason, the density of target antigen for which the antibody is specific is likely to affect the capability of the antibody to induce CDC. Cell destruction by ADCC and/or CDC is a common mechanism of mAbs used for the treatment of cancer, such as Campath-1H (lymphocyte neoplasms) and Herceptin (breast cancer). In addition to CDC and ADCC, mAbs can induce antibody-dependent cellular phagocytosis in which mAb-targeted cells are phagocytosed by cells such as macrophages expressing Fc receptors which bind the Fc portion of the therapeutic mAb (Awan et al., 2010).

The constant region of the gamma heavy chains of IgG antibodies can be one of four subclasses, i.e.: IgG1, IgG2, IgG3 or IgG4. The size of the hinge region and the position and number of disulfide bonds between the two heavy chains predominantly define the subclass of the antibody. Small amino acid differences between the subclasses dramatically affects the effector function of the molecule. Therapeutic mAbs with ADCC effector mechanisms are commonly of the subclass IgG1 as this has a high affinity for Fc gamma receptor III expressed by natural killer cells. However, if ADCC or CDC is not a requirement, mAbs may be of the subclass IgG4 as the Fc portion has a low affinity in general for Fc receptors and for Clq. Furthermore, modification such as glycosylation of a specific area within the heavy chain constant region can improve effector functions of mAbs (Kubota et al., 2009; Kaneko and Niwa, 2011). Unlike all other IgG subclasses, IgG4 molecules are capable of dissociating under reducing conditions and undergoing “Fab arm exchange” with other IgG4 molecules, that is, a heavy-light chain pair (half-molecule) can exchange with a half-molecule from another IgG4 antibody. This results in transient bi-specific antibodies incapable of cross-linking antigens and

this has been reported to have anti-inflammatory effects (van der Neut Kolfschoten et al., 2007). However the contribution of Fab arm exchange to therapeutic IgG4 mAbs is still largely unknown.

1.2.3 Clinical infusion reactions caused by therapeutic mAbs

The specificity and high affinity of therapeutic mAbs for their target reduces the likelihood of activation of non-target pathways. Moreover, as proteins, therapeutic mAbs are catabolised into their constituent natural amino acids and so do not form reactive or toxic intermediates (Brennan et al., 2010). However, despite these facts, therapeutic mAbs are associated with side effects, a significant number of which are 'clinical infusion reactions'. Depending on the antibody, the reactions may occur in a large proportion of patients and may be severe enough to constitute what has been termed 'cytokine release syndrome' (Breslin, 2007; Chung, 2008; Wing, 2008; Hansel et al., 2010).

Cytokines are potent mediators of immune responses (as described below) and thus play a pivotal role in fighting infection. Many cytokines are pro-inflammatory, i.e. they promote the accumulation of leukocytes (as immune effector cells), plasma proteins and fluid in tissues. Although this accumulation is critical in order to combat infection, many of these (activated) immune cells/proteins can cause extensive tissue damage. The production and effects of cytokine release during an immune response are usually regulated by physiological control mechanisms which keeps their tissue-damaging effects (described below) under control and localised to infected/damaged tissue. However, therapy with mAbs can stimulate a widespread and dysregulated overproduction of cytokines (cytokine release syndrome), usually in the absence of infection, leading to widespread tissue-damaging effects. This mAb-induced response is often very similar to that induced by an infection of systemic tissue (e.g. blood and lungs), leading to a systemic inflammatory response and septic shock.

Usually the exact mechanism for cytokine release stimulated by therapeutic mAbs is not known. It is possible that during the manufacturing process, mAbs (as with all medicines) may become contaminated with a pyrogenic (fever-causing) substance such as endotoxin, which can

stimulate the release of pro-inflammatory cytokines. However, in most cases, pyrogenic contamination is controlled using pre-clinical tests such as the rabbit pyrogen test or the bacterial endotoxins test. Usually, cytokine release syndrome is caused by the intrinsic nature of the mAb itself. This can be through an Fc-mediated mechanism such as ADCC; for example, first dose cytokine release syndrome stimulated by Campath-1H (IgG1) is a consequence of Fc ligation of target-bound (to CD52) antibody to FcγIII receptors (CD16) on natural killer cells (Wing et al., 1995; Wing et al., 1996). Cytokines release may also be a result of the Fab portion of the mAb binding to target receptors leading to stimulation of cells (as was the case with TGN1412). The mechanism underlying cytokine release can be the same as that required for the therapeutic effect of the mAb. Cytokine release syndrome is made worse when the mAb-targeted ligands are expressed on cells other than those essential for the therapeutic effect. For example, a mAb for cancer therapy that targets antigens not exclusive to tumour cells is likely to stimulate larger cytokine responses than a mAb targeted for a tumour cell-specific antigen. When this is the case, the effects can become systemic depending on the distribution of the cells expressing the target antigen (Brennan et al., 2010).

If the onset, nature and severity of cytokine release stimulated by therapeutic mAbs can be predicted through pre-clinical testing, the predicted effects may be controlled by medical intervention prior to/during therapy. This would result in a situation where the benefits of mAb therapy (such as treating cancer patients) far outweigh the risks. The use of good clinical management (use of corticosteroids) has enabled therapeutic mAbs Campath-1H and Rituximab to be marketed and used as successful treatments for lymphoma (Wing, 2008). This highlights the importance of pre-clinical testing procedures that can predict cytokine release syndrome stimulated by therapeutic mAbs.

Depending on the therapeutic mAb, elevated levels of a range of cytokines are often reported during cytokine release syndrome. It is the sustained production of large quantities of cytokine(s) that causes toxicity in man. The cytokines tumour necrosis factor alpha, TNFα, interferon gamma, IFNγ and interleukin-6, IL-6 are often associated with cytokine release syndrome (Wing, 2008) although others are involved. A brief summary of the roles of cytokines that are potential contributors to cytokine release syndrome is given below.

TNFα

TNFα is a 51 KDa homotrimer and is produced by a range of cell types including lymphoid cells (i.e. lymphocytes and monocytes), mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, neuronal tissue and some epithelial cells (Walsh et al., 1991). It is able to transduce its cellular effects through binding to two distinct TNF receptors; type I (TNFRI), expressed on all cell types or type II (TNFRII), expressed by cells of the immune system and endothelial cells (Aggarwal, 2000). There are many inducers of TNFα release from cells. A well-known inducer of TNFα release (and of IL-1β, IL-6 and IL-8 release) from monocytes and macrophages is bacterial endotoxin, a pyrogenic component of the cell wall of Gram negative bacteria. Other non-endotoxin pyrogens (which may contaminate biological medicines) are also capable of stimulating TNFα release from monocytes and macrophages, e.g. peptidoglycan (Nakagawa et al., 2002) and flagellin (Wyant et al., 1999). Pyrogen-stimulated cytokine release is through the interaction of the pyrogen with Toll-like receptors (TLRs) expressed by monocytes and macrophages. For example, the endotoxin lipopolysaccharide (LPS), binds to lipid binding protein (LBP) in the host's blood and, subsequently, to CD14 and TLR4 ligands which leads to cell-activation and the release of cytokines such as TNFα, required for an inflammatory response to combat infection (O'Neill and Dinarello, 2000). Monocyte activation by mycobacterium involves TLR2 (Le Bert et al., 2011). TNFα causes a rise in body temperature and so is described as an endogenous pyrogen (fever-causing agent), along with IL-1β and IL-6. The physiological function of this rise in body temperature is to create a less favourable environment for pathogens during infection.

TNFα (along with IL-1β and IL-6) stimulates the synthesis of acute phase proteins by the liver during an inflammatory response. C-reactive protein and mannose binding lectin are examples of acute phase proteins and they bind to pathogens and act as opsonins and activate complement-mediated effector functions as described above. TNFα also stimulates the release of IL-8 and IL-6 from monocytes and macrophages and can cause tissue necrosis through the activation of signalling pathways leading to apoptosis (Li and Beg, 2000). In addition, TNFα acts on vascular endothelial cells (which form microvessels and the lining of large blood

vessels) in several ways. It induces them to synthesise colony stimulating factors which themselves induce haematopoiesis, leading to an increased production of leukocytes for combating infections (Kuby, 1997). TNFα release also leads to vasodilation to increase blood flow and an increase in vascular permeability to proteins, cells and fluid.

TNFα upregulates endothelial cell surface expression of a number of cell adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and the selectins E-selectin and P-selectin. E-selectin and P-selectin bind to specific carbohydrates on glycoproteins expressed at the surface of certain leukocytes. ICAM-1 and VCAM-1 bind, respectively, to the integrins lymphocyte function-associated antigen-1 (LFA-1) and very late activation marker-4 (VLA-4) expressed by lymphocytes. The interaction of adhesion molecules with their ligands expressed by immune effector cells results in their adhesion and migration across endothelial barriers, into damaged/infected tissues (Muller, 2002; Murphy et al., 2008; Mazza et al., 2010).

IL-6

IL-6 is a 26 KDa protein released by many cell types including lymphocytes, monocytes, fibroblasts, endothelial cells, mesangial cells and keratinocytes. It is able to transduce its effects through binding to a cell surface receptor complex which has two chains, IL-6Rα (which can actually be soluble or membrane bound), and gp130. The latter is always membrane bound and is the signal transducing component of the IL-6 receptor complex (Mire-Sluis and Thorpe, 1998). Like TNFα and IL-1β, IL-6 is generally regarded as a pro-inflammatory cytokine. Its release from monocytes and macrophages is stimulated by micro-organism derived pyrogens (amongst other factors) and it too causes fever. However, IL-6 can also be anti-inflammatory as *in vitro*, IL-6 inhibited the release of TNFα and IL-1 from peripheral blood mononuclear cells or PBMC (Schindler et al., 1990). IL-6 is also an inducer of the synthesis of acute phase proteins by the liver (as described for TNFα). It has a regulatory role in haematopoiesis and T cell proliferation and differentiation. In addition, it promotes terminal differentiation of B cells into plasma cells and antibody secretion from these cells and also promotes the production of platelets (Kuby, 1997; Mire-Sluis and Thorpe, 1998; Naka et al., 2002).

IL-8 (CXCL8)

IL-8 is an 8 KDa protein released by many cell types including monocytes, macrophages, T cells, endothelial cells, fibroblasts and neutrophils. It is a chemokine (chemoattractant) that binds to the extracellular matrix and the surface of endothelial cells and provides a chemical gradient in order to direct the migration of leukocytes, in particular neutrophils (phagocytes), towards the site of infection . IL-8 also activates conformational changes in integrins, adhesion molecules expressed by leukocytes. These conformational changes promote leukocyte adhesion to endothelial cells and their extravasation across endothelial barriers towards sites of infection (Murphy et al., 2008). IL-8 therefore elicits pro-inflammatory effects by facilitating the recruitment of immune effector cells, which also have tissue-damaging effects, to sites of infection.

IFNγ

IFNγ is a 17 KDa protein released by several cell types including T lymphocytes, natural killer cells (Langer et al., 1994) and macrophages (Robinson et al., 2010). It is able to transduce its effects through binding to interferon gamma receptor (IFNGR) comprising two ligand binding chains; IFNGR1 and two signal transducing chains; IFNGR2. IFNγ possesses a broad range of functions. In general, it promotes immune responses of the Th (T helper) 1 phenotype which include the activation of macrophages and activation and cell-mediated immunity (as part of the adaptive immune response) as described below. Activation of macrophages by IFNγ promotes several effector functions of these cells, e.g. upregulation of lysosomal enzymes which mediate the destruction of phagocytosed micro-organisms. IFNγ also induces the synthesis of reactive oxygen species and reactive nitrogen intermediates by macrophages (Schroder et al., 2004) which, when released, are toxic to host cells as well as pathogens. Consequently, activated macrophages can cause extensive tissue damage (Murphy et al., 2008). IFNγ also activates similar mechanisms in neutrophils (Boehm et al., 1997). Furthermore, the proliferation of macrophages and cell survival is promoted by IFNγ (Schroder et al., 2004).

As part of the adaptive immune response, peptides from phagocytosed pathogens are expressed on the surface of dedicated APCs such as dendritic cells, in the context of self-

molecules major histocompatibility class I (MHC Class I) or MHC Class II. As described in more detail later in this chapter, it is only in this context that such peptides can activate T lymphocytes. IFNγ upregulates the expression of MHC Class I and II molecules to increase the quantity and diversity of peptides presented to T lymphocytes by APCs, enhancing T cell-mediated effector functions. It is believed that the release of IFNγ by APCs and natural killer plays a pivotal role in early immune responses to infection; however, when the adaptive immune response develops, T lymphocytes are activated by APCs (or, alternatively; therapeutic mAbs) and are responsible for releasing most of the IFNγ (Schroder et al., 2004). Furthermore, the expression of Fc gamma receptor I (FcγR1) by monocytes is upregulated by IFNγ promoting ADCC (Erbe et al., 1990). In addition, IFNγ can increase the secretion of complement proteins and upregulate the expression of receptors for complement proteins, promoting cell destruction through CDC (Strunk et al., 1985). As with TNFα, IFNγ upregulates the expression of endothelial adhesion molecules, promoting the adhesion and migration of circulating immune cells across endothelial barriers. IFNγ also has anti-viral properties as it induces a number of proteins which inhibit viral protein synthesis and replication (Mire-Sluis and Thorpe, 1998).

IL-2

IL-2 is a 15 KDa protein released by T lymphocytes (Savage et al., 1993). It is able to transduce its effects by binding to the IL-2 receptor complex as described later in this chapter. The primary functions of IL-2 include activation of T cell proliferation and differentiation into immune effector cells and the development of memory T cells, which, upon re-encounter with their cognitive antigen, are capable of undergoing expansion leading to antigen specific immune effector functions (Bachmann and Oxenius, 2007). IL-2 is also a growth factor for B cells and natural killer cells (Oppenheim, 2007). The role of IL-2 is described in more detail later in this chapter.

Given the role that cytokines play during an immunological response (as summarised above), it is easy to see how a dysregulated and widespread production of these cytokines, e.g. as can be induced by mAb therapy, could send the immune system in to a state of turmoil.

Common symptoms of cytokine release syndrome include fever, chills, nausea, hypotension, tachycardia, asthenia, headaches and myalgia. However, in more severe cases, cytokine release syndrome can lead to life-threatening complications (Breslin, 2007). For example, when TNFα is released by cells in systemic sites such as the pancreas and liver, it causes widespread vasodilation, and it increases adhesiveness of vascular endothelial cells for leukocytes and platelets and increases systemic vascular permeability to proteins, cells and fluids. The resulting loss of blood pressure and plasma volume leads to shock. High levels of systemic TNFα can also induce disseminated intravascular coagulation in which the generation of blood clots leads to an unsustainable consumption and consequent depletion of clotting proteins, causing individuals to lose their ability to form further blood clots leading to bleeding and multiple organ failure (Murphy et al., 2008).

Perhaps the most recently publicised incident of a therapeutic mAb-induced clinical infusion reaction was the "cytokine storm" stimulated by TGN1412. This resulted in devastating clinical consequences during its "first time in man" phase I clinical trial, clinical effects which were in no way predicted during pre-clinical testing of this molecule.

1.3 Therapeutic mAb TGN1412

TGN1412 was generated by TeGenero AG and subsequently manufactured by Boeringher Ingelheim Pharma GmbH & Co. It is a 148 kDa mAb of the IgG4 subclass and has a kappa light chain (IgG4κ). The antibody is humanised and so the CDRs from mouse mAb 5.11A1 (Luhder et al., 2003) heavy and light chain variable regions, specific for human CD28, were transferred into a human framework of light and heavy chain variable regions. These were then combined with IgG4 heavy chain and human kappa light chain. Humanisation of the antibody was important to reduce immunogenicity and to improve the half-life of the molecule as a therapeutic medicine. The engineered antibody was produced by expression in Chinese Hamster Ovary cells (TeGenero AG, 2005a; TeGenero AG, 2005b).

TGN1412 belongs to a group of antibodies called CD28 superagonists. CD28, the target receptor for TGN1412 is expressed by T cells and plays a pivotal role in T cell activation. In

order to understand the mechanism of action of CD28 superagonists (addressed later in this chapter), T cell activation and the immunological responses which may follow are first reviewed.

1.4 Review of T cell activation

1.4.1 Conventional T cell activation involving the co-stimulatory receptor CD28

T cells are leukocytes that belong to the sub-group lymphocytes (along with B cells and natural killer cells) and play an important role in humoral and cell-mediated immunity. Following their maturation in the thymus, T cells migrate around the body in the blood and the lymph and reside in the lymph nodes. T cells are distinguishable from other lymphocytes by the fact that they have a receptor on their surface known as the T cell receptor, or TCR. The activation of T cells as mediators of the immune response normally requires two signals, both of which are usually provided by APCs such as dendritic cells, macrophages and B cells (Sharpe and Abbas, 2006). Pathogens (amongst other things) activate APCs residing in tissue through the interaction of pathogen associated molecular patterns (PAMPs) with Toll-like receptors expressed by the APCs. Activated APCs engulf and destroy the pathogen through phagocytosis. Processed peptide from the pathogen is then expressed on the APC surface as antigen in the context of self MHC molecules. Activated APCs also migrate from the infected tissue to the lymph nodes. A large population of naive T cells (i.e. T cells that have never encountered their specific antigen within the periphery) reside in the lymph nodes and it is here that they are activated by mature APCs.

The TCR expressed by a T cell is antigen specific. Peptide derived from this antigen is required to be complexed with self-MHC molecules expressed by APCs for antigen recognition to occur by the T cell. CD4+ T cells recognise peptide in the context of self MHC class II molecules and CD8+ T cells recognise peptide in the context of MHC class I molecules (Murphy et al., 2008). Recognition of the MHC/peptide complex by specific TCRs is the first signal required for T cell activation and provides specificity to the response which may follow.

The second signal, known as the co-stimulatory signal, is provided by molecules CD80 and CD86 (otherwise known as B7-1 and B7-2 respectively), the expression of which on the surface of APCs is enhanced by cytokines produced in response to microbes (Sharpe and Abbas, 2006). CD80 and CD86 interact with the membrane-bound receptor CD28, a 44 KDa homodimer consisting of extracellular, transmembrane and cytoplasmic domains, the latter being rich in motifs involved in signalling (Sharpe and Freeman, 2002). CD28 is constitutively expressed on the surface of 50% of human CD8+ T cells and on 90% of human CD4+ T cells (Peggs and Allison, 2005). Usually, it is only when T cells receive both signals (via CD28 and the TCR) that T cell activation occurs. This is followed by cytokine production and proliferation and differentiation into effector and memory T cells. Effector cells then migrate from the lymph nodes to carry-out their immune effector functions. In the absence of CD28 co-stimulation, T cells recognising foreign peptide in the context of MHC, through TCR ligation, either enter a state of anergy (Jenkins, 1994) in which proliferation and effector functions are inhibited (Schwartz, 2003) or they undergo apoptosis.

1.4.2 Signalling events during CD28 co-stimulation

The TCR at the cell surface is composed of two subunits known as the α and β proteins. Also associated with the TCR is CD3 which consists of heterodimers εδ and γε, and a homodimer ζζ. One of the functions of CD3 is to mediate cell surface expression of the αβ heterodimer. The arrangement of these peptides to form the TCR-CD3 complex is shown in Figure 3. The αβ heterodimer is specific for foreign peptide expressed in the context of MHC by APCs but, after ligation, the TCR alone cannot transduce signals (Kuhns et al., 2006).

Figure 3. Structure of the TCR-CD3 complex. Adapted from Murphy et al., 2008.

Signalling events following TCR and CD28 ligation are shown in Figure 4. An early event following TCR ligation is phosphorylation of tyrosine residues located in conserved sequences called immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of ε, δ, γ and ζ. Phosphorylation of ITAMS is by Src family tyrosine kinases, Lck (lymphocyte-specific protein tyrosine kinase) and Fyn (proto-oncogene protein tyrosine kinase) which are constitutively associated with receptors CD4 or CD8 and cytoplasmic domains of ε, δ, γ and ζ. Ligation of CD4 or CD8 receptors by MHC class I or II molecules presenting foreign peptide, and which are expressed by APCs, co-localises the TCR with its co-receptor since the TCR ligates to the foreign peptide, bringing together Lck tyrosine kinases associated with the co-receptor and ITAMs of the cytoplasmic region of the TCR-CD3 complex. Phosphorylated

ITAMs provide binding sites for ZAP-70 (zeta-chain-associated protein kinase) and Syk (spleen tyrosine kinase) which themselves become enzymatically active after phosphorylation. This leads to a cascade of events (Isakov and Biesinger, 2000) including ZAP-70 phosphorylation of adaptor proteins LAK (linker of activated T cells) and SLP-76 (lymphocyte cytosolic protein), which in turn stimulates the recruitment of PLC-γ (phospholipase C), which is subsequently phosphorylated and activated by Tec kinases such as Itk (IL2-inducible T-cell kinase). PLC-γ activation leads to generation of IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol); breakdown products of PIP_2 (phosphatidylinositol 4,5-bisphosphate), a membrane lipid. Receptor binding of IP_3 stimulates the release of stored calcium (within the cell) into the cytoplasm and the opening of calcium channels that permit the flow of more calcium into the cell. This calcium flux and DAG are able to activate different pathways which lead to the activation of transcription factors NFκB (nuclear factor κB), NFAT (nuclear factor of activated T cells) and AP-1 (activator protein) in the nucleus. These transcription factors regulate the expression of genes involved in T cell differentiation, proliferation and T cell effector functions. One of these pathways involves a Ras (GTPase) induced kinase cascade which stimulates AP-1 production. Another involves the activation of NFκB by protein kinase-Cθ and the final pathway involves activation of calcineurin, a phosphatase which activates NFAT (Murphy et al., 2008).

Signalling stimulated by ligation of CD28 by CD80 and CD86 is believed to synergise with TCR-CD3 signalling which ultimately stimulates T cell effector function (Collins et al., 2002). At the molecular level, CD28 ligation results in phosphorylation of a non-ITAM motif leading to the recruitment of Grb2 (growth factor receptor bound protein), an adaptor protein, which can result in Ras activation. As described above, Ras activation leads to the induction of the kinase cascade, a result of which is the production of AP-1. Therefore, in this pathway, CD28 ligation enhances AP-1 activation stimulated by engagement of the TCR. In addition, it has been proposed that CD28 co-stimulation can activate the NFκB pathway involving protein kinase-Cθ (Dennehy et al., 2003). CD28 has a motif on its cytoplasmic domain that is proline-rich and which binds to kinase activity-inhibiting domains of Lck and Tec kinase, Itk. Binding of these domains to this proline-rich domain reverses the inhibitory action leading to activated

Lck and Itk. In this case, TCR signalling is promoted by CD28 ligation through enhancement of activity of kinases (Murphy et al., 2008). Independent signals from TCR and CD28 engagement have been reported to interconnect at the stage of the signalling cascade where Vav1 (a guanine nucleoside exchange factor) is tyrosine phosphorylated. CD28 signalling, therefore, must enhance TCR-stimulated effector functions resulting from Vav1 activation (Hehner et al., 2000). Vav1 has been reported to promote the stability and function of SLP-76 clusters (Sylvain et al., 2011) as mentioned above and regulate calcium-dependent signalling (Hehner et al., 2000).

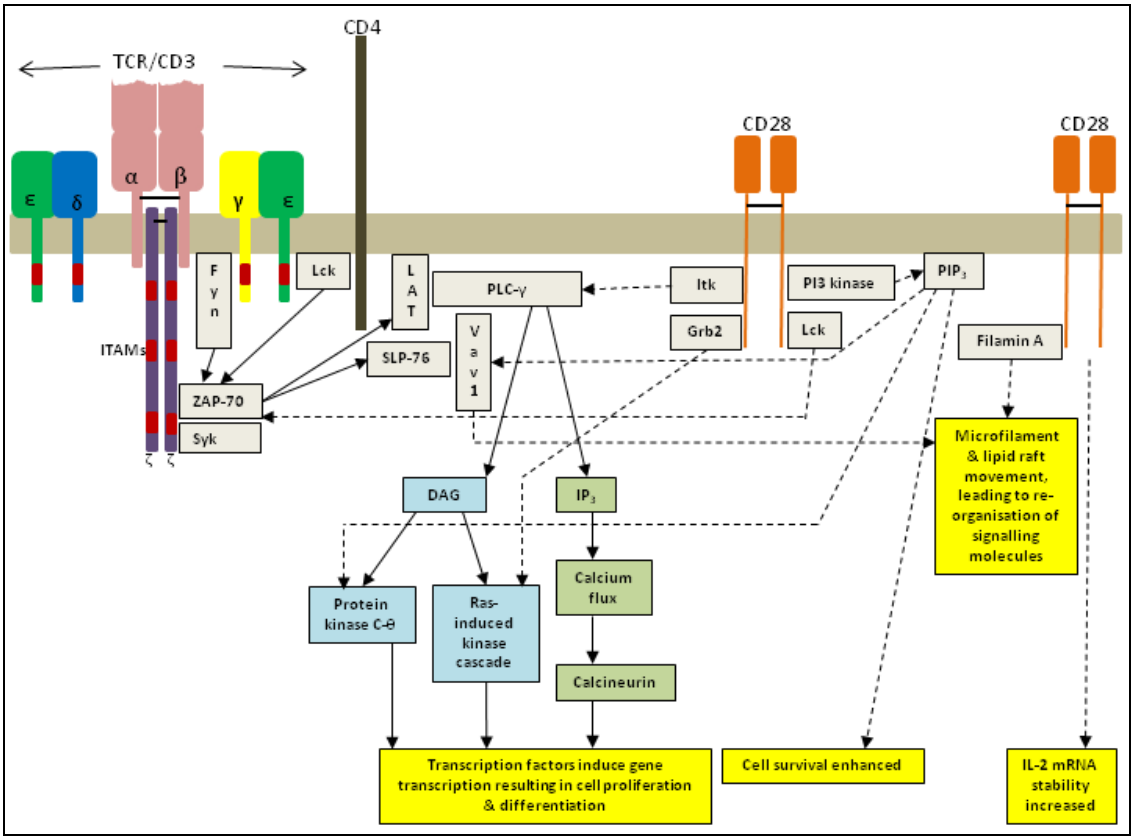


Figure 4. Summary of signalling pathways involved in T cell activation. Solid lines are signalling events resulting from ligation of the TCR. Dotted lines are signalling events resulting from ligation of CD28.

In addition to synergising with TCR signalling, CD28 ligation stimulates phosphorylation of tyrosine residues on a motif located on its cytoplasmic tail that is different from that of ITAMs. This activates recruited PI3-kinase (phosphatidylinositol 3) and subsequently leads to the production of PIP_3 (phosphatidylinositol 3,4,5-trisphosphate). Akt (known as protein kinase B) is then recruited to the membrane and activated leading to phosphorylation of other molecules involved in signalling, which in turn stimulates cell survival through upregulated

expression of antiapoptotic protein Bcl-xL (Boise et al., 1995; Murphy et al., 2008). T cell survival promoted by CD28 ligation may also involve a PI3-kinase independent mechanism (Collette et al., 1997).

1.4.3 The immunological synapse

The term "immunological synapse" was first coined by Norcross (Norcross, 1984) to describe the region where the APC membrane and T cell membrane interface which seems logical since "synapse" is derived from the Greek word s*unapsis* which means "together" or "joining" (Oxford English Dictionary). The immunological synapse is a highly organised array of proteins associated with the interfacing membranes and consists of three supramolecular activation complexes or SMACs which are 3-dimensional, spatially segregated distinct areas comprising many proteins, as shown in Figure 5. The central supramolecular activation complex (cSMAC) forms the inner circle and contains most of the signalling molecules important for T cell activation (Murphy et al., 2008). The cSMAC has been reported to be enriched in TCRs, protein kinase-Cθ and Src family kinases Lck and Fyn (Monks et al., 1998) as well as CD4 and CD8 co-receptors, CD28, and cell adhesion molecule CD2 (Dustin et al., 2010), the ligand for lymphocyte function-associated antigen-3 (LFA-3) expressed by APCs. Surrounding the cSMAC is the peripheral supramolecular activation complex (pSMAC) reported to be enriched with LFA-1 which promotes cell to cell adhesion. Also enriched in the pSMAC is talin, a ubiquitous protein in the cytosol involved in linking proteins such as integrin LFA-1 to the cytoskeleton, allowing their movement within the cell (Burn et al., 1988; Monks et al., 1998). The outermost region, called the distal supramolecular activation complex (dSMAC), is not fully characterised. One theory is that it contains molecules that have not specifically been recruited to the other two SMACs (Dustin et al., 2010).

Figure 5. Simplified illustration of an immunological synapse. The immunological synapse has 3 supramolecular activation complexes (SMACs); central (cSMAC), peripheral (pSMAC) and distal (dSMAC). Adapted from Janeway's Immunobiology (Murphy et al., 2008).

The specific functions of the SMACs within the immunological synapse are still not fully understood. The initial adhesion of T cells to their target cells is through the weak interaction of LFA-1 expressed by T cells and intercellular adhesion molecules 1 (ICAM-1) expressed by the target cell. Subsequent ligation of TCRs formed in microclusters with CD28 on the membrane, with peptide in the context of MHC molecules on target cells, i.e. APCs, increases the affinity of LFA-1 for ICAM-1, strengthening adhesion between the two molecules. TCRs, associated co-receptors (CD4 or CD8) and other molecules with co-stimulatory effects such as CD2 (which binds to LFA-3 on APCs) aggregate at the point of contact between the two cells, forming the immunological synapse. The high affinity interaction of LFA-1 and ICAM-1 enriched in the pSMAC forms a tight seal around cSMAC, stabilising the immunological synapse.

The formation of the immunological synapse mediates effector functions in two ways. It provides a highly organised array of proteins including surface receptors, kinases and adaptor proteins that favours the promotion of signalling events which ultimately lead to the expression of genes involved in regulating T cell responses, such as cytokine release. It also mediates the induction of polarisation of the Golgi apparatus (i.e. the Golgi apparatus moves within the cell to the immunological synapse), an important organelle for the processing and packaging of proteins, especially of those for secretion such as cytotoxins and cytokines. Polarisation of the Golgi apparatus is caused by the re-orientation of the microtubule-organising centre (MTOC).

The MTOC produces and organises microtubules which form the structural network (cytoskeleton) within the cell's cytoplasm responsible for structural support and the movement of molecules within the cell, owing to the microtubules' ability to grow and shrink through polymerisation of a major constituent; tubulin. The mechanism by which MTOC is re-orientated involves DAG. Polarisation of the Golgi apparatus towards the immunological synapse allows the concentration of effector molecules owing to the small area within the immunological synapse, and the targeted release of such molecules to the cell presenting antigen, to which the TCR is ligated (Huppa and Davis, 2003; Murphy et al., 2008; Yokosuka et al., 2008; Dustin et al., 2010). For example, cytokine release by T cells is stimulated by ligation of the TCR by antigen presented by an APC. Polarisation of the T cell allows cytokine secretion to be targeted at and confined to the target APC at the immunological synapse. Incidentally, effector molecules are not specific, i.e. they themselves do not target cells presenting antigen with a specific sequence of amino acids. This accentuates the importance of the TCR in recognising cells for which T cell effector functions are intended, i.e. through ligation of the TCR with antigen possessing a specific amino acid sequence. Without target specificity for T cell effector functions, effector molecules released by activated T cells could affect any cell within the vicinity of the activated T cell.

The exact role of CD28 in the immunological synapse is poorly understood. It has been reported that CD28 is involved in keeping protein kinase-Cθ within a sub-region of the cSMAC, prolonging signals required for T cell effector functions through the NFκB pathway (Yokosuka et al., 2008). In the absence of CD28 co-stimulation, protein kinase-Cθ is distributed throughout the immunological synapse and is not concentrated in the cSMAC (Huang et al., 2002). It has been suggested that the recruitment of PI3-kinase to the cytoplasmic tail of CD28 and subsequent activation of this kinase results in the production of PIP_3 in the immunological synapse which leads to protein kinase-Cθ localisation and activation (Sanchez-Lockhart and Miller, 2006) as shown in Figure 4. Sanchez-Lockhart and Miller also reported that CD28 engagement outside of the immunological synapse, i.e. away from the point of TCR/antigen ligation, enhances T cell activation by increasing the stability of IL-2 mRNA rather than through protein kinase-Cθ/NFκB-mediated IL-2 transcription (also shown in Figure 4).

Molecules are transported into the immunological synapse from around the cell through linkage with membrane microdomains called lipid rafts. Lipid rafts are moving platforms within the cell membrane consisting of sphingolipids and cholesterol, which are immiscible with the surrounding lipid bilayer. They are enriched with Src family tyrosine kinases such as Lck and Fyn and other molecules involved in early signalling events following ligation of TCRs and CD28 such as LAT (Tavano et al., 2004; Tavano et al., 2006). Lipid raft movement into the immunological synapse is induced by the simultaneous ligation of TCRs and CD28 and plays a pivotal role in organising molecules in the immunological synapse. Lipid rafts are linked to the microfilaments of the cytoskeleton by actin binding proteins (Rodgers et al., 2005). Microfilaments are the thinnest filaments forming the cytoskeleton and are produced by the linear polymerisation of actin subunits. Shrinkage of microfilaments at one end and elongation at the other causes movement of associated molecules and thus mediates aggregation of lipid rafts tethered by actin-binding proteins. TCR ligation and CD28 co-stimulation induces a re-organisation of actin microfilaments and the signalling pathway has been reported to involve Vav1 (Villalba et al., 2001). PIP_3, a product of the kinase P13K, activates Vav1 which, through activation of other signalling molecules, leads to the induction of actin polymerisation (Chichili and Rodgers, 2009). This is summarised in Figure 4.

Filamin A is a protein found in the cytoplasm which cross-links actin resulting in a 3-dimentional structure and so plays an important role in defining the structure of actin microfilaments in the cytoskeleton. It has been reported that during T cell activation, CD28 ligation leads to its own interaction with filamin A which stimulates a re-organisation of the microfilaments in the cytoskeleton. This in turn promotes the movement of lipid rafts linked to the cytoskeleton into the immunological synapse along with associated signalling molecules required for T cell activation (Tavano et al., 2006) as shown in Figure 4. The clustering of lipid rafts in T cells also occurs prior to TCR signalling but during initial contact of APCs with T cells. Others have called this “raft clustering during T cell surveying” or RaCS as it was thought to facilitate T cells in “surveying” APCs for antigen presented by MHC molecules. This is because raft clustering aggregates both TCRs and co-receptors such as CD4 at the point of contact between T cells and APCs, the purpose of which is to lower the threshold of

antigen/MHC molecules required for activation. The role of CD28 and other co-stimulatory molecules in RaCS formation is not yet clear however it has been reported to be PI3K and actin dependent (van Komen et al., 2007; Chichili and Rodgers, 2009).

1.5 Summary of T cell responses following activation

Activation of naive T cells in the lymph nodes induces the cells' entry into the first stage of the cell cycle. In addition, it stimulates the production of IL-2 and induces the synthesis of the α chain (CD25 or IL-2Rα) of the IL-2 receptor. Resting T cells only express the β and γ chains of the IL-2 receptor which can only bind to IL-2 with a moderate affinity which means that resting T cells can only respond to relatively high levels of IL-2. However, when the α chain associates with the cell surface β and γ chains (IL-2Rβ and IL-2Rγ), the resulting IL-2 receptor complex has a very high affinity for IL-2 which means that activated T cells can respond to relatively low levels of IL-2. When IL-2 (initially at low levels) binds to the high affinity receptor, it induces signalling and further progression through the cell cycle. Only IL-2-bound IL-2Rβγ or IL-2Rαβγ are capable of transducing signals. IL-2Rα alone cannot transduce signals as it does not have a signalling domain. Cell division continues, resulting in clonal expansion which creates a population of cells all with the same target antigen specificity. Activated T cells are able to synthesise IL-2 as a result of the activation of the transcription factors NFAT, AP-1 and NFκB which bind to the IL-2 gene promoter, inducing transcription of the IL-2 gene. Since CD28 co-stimulation leads to activation of AP-1 and NFκB and stabilises IL-2 mRNA (discussed previously), CD28 co-stimulation clearly plays an important role in IL-2 synthesis and cell proliferation (Appleman et al., 2000; Bachmann and Oxenius, 2007; Murphy et al., 2008).

The progeny of proliferating T cells in the lymph nodes differentiate into effector T cells which lose their ability to re-circulate through lymph nodes and migrate from the lymph nodes to the infected tissue. Effector T cells have a variety of functions. Cytotoxic T cells (expressing CD8) kill virus-infected cells through recognition of antigen associated with MHC class I molecules which is followed by the induction of apoptosis. Effector T cells expressing CD4 can

be T helper 1 (Th1), Th2, Th17 or regulatory T cells (Tregs). A principle function of Th1 cells is to promote macrophage activation and the subsequent killing of pathogens. Th1 and Th2 cells both co-ordinate B cell activation and antibody production and so promote the humoral response. Th17 cells enhance the recruitment of neutrophils to sites of infection and so promote the acute inflammatory response. Tregs, however, serve to limit immune responses because they produce inhibitory cytokines and suppress self-reactive T cells (illustrated in Figure 8).

The effector T cell subset that the progeny of proliferating T cells differentiate into is dependent on the nature of cytokines produced during the innate immune response. For example, in the presence of IL-12, expression of the transcription factor T-bet is promoted which commits cells to the Th1 type, characterised by their production of IFNγ and IL-12, but not IL-4 or IL-17. However, in the presence of IL-4, expression of the transcription factor GATA is promoted, committing cells to the Th2 type, characterised by their production of IL-4 and IL-15 but not IFNγ (Murphy and Reiner, 2002; Murphy et al., 2008; Pepper and Jenkins, 2011).

The differentiation process also alters the expression of specific homing receptors. Such receptors bind specific adhesion molecules on endothelial cells (for example LFA-1 to ICAM-1 or integrin α4:β7 to mucosal vascular addressin cell adhesion molecule-1 [MAdCAM-1] in the gut) and also chemokines. The differential expression of adhesion molecules by cytokine activated endothelial cells (e.g. by TNFα released by tissue macrophages) directs effector T cell subsets to sites of inflammation (along with chemokines). It is here where these cells produce specific cytokines and promote the clearance of antigens, for which the effector T cell subset is specific. Following this, approximately 90% of effector T cells die and 10% become CD45RO+ memory T cells which are quiescent in the absence of their cognitive antigen and long lived.

The activation of memory T cells upon again encountering cognitive antigen leads to an enhanced and rapid recall response to antigen. Memory T cells, like naive T cells, express CD28 on their surface which suggests that they are also susceptible to CD28 co-stimulation. Memory T cells can be effector memory cells or central memory cells. Effector memory T cells migrate to sites of inflammation in nonlymphoid tissue and can be found in large numbers in the lungs and the gastrointestinal mucosa (lining of the intestine) but do not re-circulate through lymph

nodes. Their migration route is facilitated by the expression of homing receptors. Once activated upon re-encounter with cognitive antigen, they produce microbicidal cytokines IFNγ, IL-4 and IL-5. The current theory is that Th1 effector cells become Th1 committed effector memory T cells, however, the role of Th2 and Th17 effector T cells in memory T cell formation is less clear. In contrast to effector memory T cells, central memory T cells are thought to migrate through lymph nodes owing to their expression of chemokine receptor CCR7 and L-selectin. It is here where they are thought to undergo a secondary response upon re-encounter with antigen. Initially, after activation, they produce IL-2 and proliferate. Only later do they acquire the function of effector memory T cells and lose their expression of CCR7, resulting in their migration to non-lymphoid tissue. As a result, central memory T cell-mediated responses are likely to be slower (Campbell et al., 2001; Sallusto et al., 2004; Lanzavecchia and Sallusto, 2005; Pease and Williams, 2006; Murphy et al., 2008; Eastwood et al., 2010; Pepper and Jenkins, 2011).

1.6 Superagonistic anti-CD28 antibodies

1.6.1 Mode of action at the membrane surface

Conventional agonistic anti-CD28 mAbs used in conjunction with antibodies which stimulate via the TCR-CD3 complex (for example anti-CD3 antibodies) are able to activate T cell proliferation but alone will not stimulate T cell responses (Acuto and Michel, 2003). However, 'superagonistic' anti-CD28 antibodies activate T cell proliferation and differentiation without concomitant stimulation via engagement of the TCR (Beyersdorf et al., 2005; Hunig and Dennehy, 2005). This is illustrated in Figure 6.

Figure 6. T cell activation by conventional and superagonistic anti-CD28 mAbs. Only the superagonistic mAbs are able to activate and stimulate T cell proliferation and cytokine production in the absence of a signal via the TCR. Adapted from Beyersdorf et al. (2010).

The mechanism by which superagonistic anti-CD28 antibodies are able to stimulate T cell responses without TCR/CD3 stimulation is unclear, however a number of theories, based on comparisons made between superagonistic and conventional antibodies, have been made. CD28 superagonists bind to a lateral, membrane-proximal extracellular region of the CD28 receptor termed the C''D loop (amino acid residues 43 – 70). It is thought that because superagonistic anti-CD28 antibodies can bind to this membrane-proximal region, they can bind bivalently to contiguous CD28 molecules, thus allowing them to cross-link CD28 receptors on the cell surface. This cross-linking leads to the formation of a complex of CD28 receptors, linked in a linear fashion by the antibody: this 'linear complex formation' is illustrated in Figure 7

(Beyersdorf et al., 2005). Conventional, i.e. not superagonist, anti-CD28 antibodies are unable to produce this linear complex because they bind to a membrane-distal region of the receptor where the natural ligands CD80 and CD86 bind, which only allows these antibodies to bind monovalently to CD28 molecules. Linear complex formation may lead to the aggregation of signalling molecules which favours the interaction of essential components required for subsequent T cell activation. This aggregation of signalling molecules may only occur upon stimulation of the TCR when conventional anti-CD28 antibodies are used (Luhder et al., 2003; Beyersdorf et al., 2005; Evans et al., 2005; Hunig and Dennehy, 2005).

Figure 7. Linear complex formation by superagonistic anti-CD28 mAbs. The capability of superagonistic anti-CD28 mAbs to bivalently attach to lateral membrane-proximal C''D loop regions of CD28 allows the formation of a linear complex, which may cause aggregation of other components involved in the signalling processes required for T cell activation. Adapted from Beyersdorf et al., 2005.

CD28 is monovalent for its natural ligands, i.e. *in vivo*, despite its existence as a homodimer, it has only one binding site for CD80 and CD86. This may be a mechanism to prevent activation of T cells without prior antigen recognition through the TCR since ligation of CD28 monovalent constructs only induced T cell responses if the TCR was engaged; in contrast, ligation of bivalent constructs did not require TCR engagement in order to stimulate responses (Dennehy et al., 2006).

Another theory on the mode of action of CD28 superagonists is that their capability to cross-link CD28 at close proximity to the membrane (compared with conventional CD28 agonists) due to the position of the epitope (C''D loop) favours formation of an immunological

synapse in which larger molecules such as phosphatases (which dephosphorylate molecules and thus are essentially antagonists of kinases) are excluded. This would allow domination by smaller molecules such as kinases which would favour the initiation of subsequent signalling events (Hunig and Dennehy, 2005). The close proximity of CD28 cross-linking to the membrane by superagonists may also influence the proximity of intracellular signalling molecules associated with the cytoplasmic tails of CD28, in a favourable manner for T cell activation. It has also been suggested that superagonistic antibodies preferentially bind CD28 molecules that have pre-aggregated on the membrane. Clustering of such molecules may render them competent in signalling due to a richness of signalling molecules such as kinases. Preferential ligation of pre-clustered molecules by superagonists may be due to the fact that only the epitope containing the C''D loop is accessible, eliminating the potential for conventional CD28 antibodies to bind. Another possibility is that superagonsists stimulate a conformational change which activates downstream signalling processes in a manner that conventional antibodies alone cannot stimulate (Luhder et al., 2003).

1.6.2 Signalling events following CD28 superagonist activation

Signalling pathways activated by strong signals elicited through the bivalent ligation of CD28 superagonists are not fully understood and are still being elucidated. Experiments using T cell hybridomas revealed that the presence of the TCR, ZAP-70 and LAT are required for CD28 superagonistic activation, despite the fact that ligation of the TCR is not required. It was suggested that superagonistic CD28 activation was reliant on low level constitutive "background" signals emanating from TCR (Hunig and Dennehy, 2005). The physiological function of these background signals is to regulate development of T cells and maintain T cell identity and such signals have been found to involve LAT and SLP76 (Roose et al., 2003). Further experiments aimed at identifying signalling targets undergoing tyrosine phosphorylation revealed that the point at which "background" signals emanating from unligated TCR merged with signals emanating from CD28 superagonistic activation was at the stage where signalling molecules SLP76/Vav1/Itk interact (Hunig and Dennehy, 2005). Interestingly, this is at the

same point where signals from conventional CD28 co-stimulation, including TCR ligation, have been shown to merge as described above. It is possible that signalling induced by either CD28 ligation by its natural ligand (in which CD28 is monovalent for the ligand) or CD28 ligation by conventional antibodies (in which the antibodies may be monovalent for CD28) is insufficient to enhance "background" signals emanating from unligated TCRs. However, stronger signalling from bivalent CD28 superagonist activation is sufficient to heighten "background" signals from the TCR. Other studies have shown that CD28 superagonist stimulation, as with conventional TCR/CD28 co-stimulation, leads to translocation of transcription factors NFκB and NFAT without phosphorylation of ZAP70 and TCRζ above "background" levels (Siefken et al., 1998; Bischof et al., 2000).

1.7 Intended therapeutic use of TGN1412

T cells undergoing development in the thymus are called thymocytes. During this development thymocytes undergo a process called positive selection which essentially only allows thymocytes that can recognise self antigen associated with self MHC molecules, expressed by other cells in the thymus, to undergo further development. The purpose is to select for thymocytes that, when mature, will only interact with antigen expressed in the context of self MHC molecules. The fact that thymocytes recognise self antigen associated with MHC at this stage is not important. Selected thymocytes continue to develop and become either CD4+ or CD8+ depending on their ability to interact with either self MHC class II molecules or self MHC class I molecules respectively. Thymocytes also undergo a process called negative selection in which cells that have the potential to recognise and strongly bind self antigens (through their antigen specific TCR), presented by thymic epithelial cells and bone marrow derived APCs such as dendritic cells, present in the thymus, are eliminated. This prevents the development of mature T cells capable of mediating immune responses against self-antigens (self-reactive T cells) expressed by healthy tissues. Negative selection has been termed central tolerance. However, some T cells are able to avoid negative selection in the thymus if they do not strongly bind self antigen during development. This may happen if the self antigen for

which the TCR is specific is not expressed by cells such as thymic epithelial cells during the process of negative selection.

T cells that have escaped negative selection in the thymus are kept in check by a number of peripheral tolerance mechanisms which operate outside of the thymus. Mature lymphocytes can be made tolerant to self antigen if such self antigens are expressed by all cells in the body or when expressed in high numbers by connective tissue and thus give strong and constant signals to TCRs. In addition, as previously discussed, T cell activation requires co-stimulatory signals in addition to antigen recognition via TCRs, thus self-reactive T cells recognising self antigens on APCs not expressing co-stimulatory ligands such as CD80 and CD86 are inactivated and enter a state of anergy or undergo apoptosis. Another peripheral tolerance mechanism is that imposed by CD4+ Tregs which result from positive selection in the thymus, but which are thought to have avoided negative selection and so weakly bind self antigens and so are self-reactive. However, in contrast to other CD4+ or CD8+ T cells, interaction of Tregs with self antigen does not activate their differentiation into effector cells that can damage tissues expressing the self antigen but, instead, activates their differentiation into suppressor cells of other self-reactive T cells (Walker and Abbas, 2002; Murphy et al., 2008). The mechanism by which Tregs suppress self-reactive T cells is yet to be elucidated but cell to cell contact between Tregs and self reactive T cells is thought to be required along with the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β).

Despite the fact that central and peripheral tolerance mechanisms are in place to avoid the detrimental effects that can be caused by self-reactive T cells, i.e. T cells with TCRs specific for self antigens expressed by healthy tissue, such events still occur resulting in autoimmune diseases such as rheumatoid arthritis. Defects in the genes involved in promoting central and peripheral tolerance mechanisms are thought to lead to autoimmune diseases. In addition, foreign antigens from pathogens can stimulate immune responses that are specific for the foreign antigen but, occasionally, cross-reactivity of immune responses occurs when a foreign antigen is similar to a self antigen. Several experimental studies suggested that CD28 superagonists showed potential as therapeutic antibodies for the treatment of autoimmune disease. Thus, it has been shown that CD28 superagonists were able to expand purified human

and rat Tregs *in vitro* and that these possessed enhanced suppressive activity compared with Tregs that had been freshly isolated and not stimulated with CD28 superagonists. The enhanced suppressive activity was exclusive to Tregs as other T cells were not suppressive when stimulated with CD28 superagonists (Beyersdorf et al., 2005).

Other *in vitro* proliferation assays were conducted involving the culture of TGN1412 (in solution) with either PBMC, purified T cells or T cell subsets including CD4+ and CD8+ T cells, and naïve (CD4+CD45RA+), memory (CD4+CD45R0+) and regulatory (CD4+CD25+) T cells from healthy donors. These assays showed that this CD28 superagonist was capable of stimulating activation and proliferation of T cell subsets including CD4+ cells, CD8+ cells, naive and memory T cells and, most notably, Tregs, unlike conventional CD28 mAb (TeGenero AG, 2005a). *In vivo* experiments revealed that CD28 superagonists administered to rats not only increased the number of Tregs, but activated these cells and led to an increase in their suppressive activity (Beyersdorf et al., 2005). Consistent with this finding, an anti-rat CD28 mAb; JJ316, inhibited the process of inflammation in adjuvant-induced arthritis, a model of autoimmunity, in rats (Rodriguez-Palmero et al., 2006). This inhibitory effect was associated with increased levels of Th2 cytokines such as the anti-inflammatory cytokine IL-10 and the expansion of Tregs (Rodriguez-Palmero et al., 2006). In addition, animal models of autoimmune encephalomyelitis (used to represent the autoimmune disease multiple sclerosis) showed that administered CD28 superagonists were able to greatly mitigate symptoms (Beyersdorf et al., 2005). Furthermore, CD28 superagonists were effective in a rat model of autoimmune neuritis (Schmidt et al., 2003) and arthritis in non-human primate models (TeGenero AG, 2005a).

It is not fully understood why CD28 superagonists preferentially expanded Tregs rather than other T cell subsets in pre-clinical studies although several theories have been proposed. The ability of Tregs to weakly bind self antigen may provide constitutive TCR signalling. The presence of this first signal could render the Tregs more sensitive to CD28 superagonist stimulation but not conventional anti-CD28 stimulation (Beyersdorf et al., 2005). Treg-imposed mechanisms have been found to be dependent on the interaction of CD28 expressed by Tregs with its natural ligand B7 (Tang et al., 2003). It is likely therefore that CD28 superagonists

"boost" pre-existing signals resulting from a physiological mechanism evolved to mediate Treg self tolerance mechanisms. An imbalance in the number and function of self-reactive T cells and Tregs leads to autoimmunity. CD28 superagonists are believed to resolve the imbalance by stimulating Treg differentiation and activation, as shown in Figure 8, and so have the potential to reinstate peripheral tolerance (Beyersdorf et al., 2005). Human rheumatoid arthritis is an autoimmune disease and so is mediated by self reactive immune responses and has been correlated with a deficiency in Treg cell numbers and/or function (Ehrenstein et al., 2004). Therefore, TGN1412 as a CD28 superagonist with the potential capability to preferentially expand Tregs was proposed for the treatment of this disease.

Figure 8. Anti-CD28 superagonist therapy restores a balance between regulatory T cell and self reactive T cell number and function in autoimmunity. Autoimmunity can occur when self reactive T cells are not suppressed due to a deficit in Treg number and function. Treatment with superagonistic anti-CD28 mAbs induces preferential expansion and activation of Tregs which suppress self reactive T cells and restore peripheral tolerance. Adapted from Beyersdorf et al., 2005.

Besides the treatment of rheumatoid arthritis, it was proposed that TGN1412 could be developed for the treatment of B cell chronic lymphocytic leukemia (B-CLL), a disease in which there is an accumulation of malignant B lymphocytes (or B-CLL cells) with an impaired ability to express MHC class II molecules and co-stimulatory ligands CD80 and CD86. This reduced expression of such molecules hinders their capability to function as APCs and to stimulate T cell responses. TGN1412 cultured as a solution with human PBMC from B-CLL patients up-regulated the expression of these molecules on the surface of B-CLL cells (Lin et al., 2004) arguing for its potential for treating B-CLL by restoring the antigen presentation properties of B-CLL cells, leading to improved T cell responses for combating infections and destroying the malignant B cells. In addition, CD28 superagonist treatment of rats in a model of T cell lymphopenia stimulated the expansion of T cells resulting in a re-population of T cells in which phenotypic diversity and function was maintained (Elflein et al., 2003); of course this would be an additional advantage to patients deficient in functional T cells, another clinical symptom of B-CLL (Cantwell et al., 1997).

The capability of TGN1412 to preferentially expand Tregs, justifying its potential for the treatment of rheumatoid arthritis, was originally thought to be dependent on its capability to induce the expansion of other T cell subsets (i.e. its justification for the treatment of lymphopenia). It was proposed that CD28 superagonist activation of T cells leads to IL-2 production which stimulates proliferation of these activated T cells and Tregs, which have also been activated by the CD28 superagonist. Activated and expanded Tregs may then suppress other activated T cells resulting in a "loop" that allows T cell tolerance to be maintained in the presence of functionally active T cells. Therefore, the two putative mechanisms of TGN1412 are not mutually exclusive (TeGenero AG, 2005a). In fact, it was thought that the overexpansion of Tregs by CD28 superagonists and their capability to induce anti-inflammatory cytokines gave these mAbs an advantage as a therapeutic means to reconstitute T cell repertoires in lymphopenia over other drugs that expand T cell populations through stimulation of the TCR-CD3 complex as these have resulted in toxic pro-inflammatory cytokine release in the absence of high Treg numbers to promote T cell tolerance (Elflein et al., 2003; TeGenero AG, 2005a).

1.8 Pre-clinical testing to predict TGN1412 toxicity

Findings from the experimental studies described above using CD28 superagonists were the basis for the proposed therapeutic applications of TGN1412. Other studies were conducted to evaluate the therapeutic potential of TGN1412 itself and to predict the clinical safety of the mAb to allow it to progress to a "first time in man" phase I clinical trial.

The specificity of TGN1412 itself for human CD28 was confirmed in various assay systems including Biacore analysis and flow cytometric analysis and the molecule did not cross-react with the receptors inducible T-cell costimulator (ICOS) or cytotoxic T-lymphocyte antigen 4 (CTLA-4) which are closely related to CD28.

As discussed earlier, therapeutic mAbs can elicit responses termed Fc effector functions, mediated through ligation of their Fc moiety as opposed to interaction of their variable region with target antigen. Such effector functions include ADCC and CDC. For some therapeutic antibodies, these effects are desirable/crucial to the treatment of the disease for which the antibody is intended. For other mAbs, these are undesirable side effects. The proliferation and expansion of functional T cell repertoires stimulated by TGN1412 was considered crucial to its capability to function as a treatment for rheumatoid arthritis and B-CLL, whereas destruction of these cells by TGN1412 Fc-mediated ADCC would reverse the effect. Of course, the destruction of all CD28 expressing cells through ADCC mediated by TGN1412 could also have devastating clinical effects. Due to the fact that TGN1412 is an IgG4 mAb, it was unlikely that ADCC events would occur but, to confirm this, several different *in vitro* assays were conducted. TGN1412 was incubated with modified human Jurkat cells and although the exact experimental details are not given, no evidence of ADCC activity was observed. In contrast, TGN1112, the IgG1 variant of TGN1412 (a subclass that is commonly associated with ADCC), did mediate ADCC against all CD28+ Jurkat cell lines tested along with Campath-1H, an IgG1 therapeutic mAb used for the treatment of cancer (TeGenero AG, 2005a; TeGenero AG, 2005b; Expert Group on Phase One Clinical Trials, 2006) owing to its capability to induce ADCC-mediated destruction of malignant cells expressing Campath-1H's target receptor; CD52 (Waldmann and Hale, 2005). In addition, pre-clinical tests were conducted to determine the capability of

TGN1412 to induce CDC. Although details on exactly how this was determined are not given, the incubation of TGN1412 and TGN1112 in solution with human PBMC showed no evidence to suggest that CDC was induced by these mAbs (TeGenero AG, 2005b).

In order to identify a suitable species for *in vivo* toxicology and safety studies, TGN1412 was incubated in solution with T cells isolated from humans, rodents and non-human primates, including rhesus and cynomolgus macaques and marmosets, expressing CD28. TGN1412 was able to bind to T cells from humans, cynomolgus and rhesus macaques but not with rodent or marmoset T cells. Furthermore, sequence analysis of the extracellular binding domain of TGN1412; the C''D loop, in different species revealed exact sequence homology between humans and cynomolgus macaques, which differed from rhesus macaques by one amino acid and marmosets by two amino acids out of a total of 6. For this reason, cynomolgus macaques were chosen for further *in vivo* studies. A 28-day repeat-dose pharmacological safety study was conducted in 16 cynomolgus macaques (8 male and 8 female) to assess the effects of TGN1412, at doses of 5 and 50 mg/kg, given 4 times, on the cardiovascular system, the respiratory system and the central nervous system. TGN1412 did not cause any significant changes in measurements such as heart rate or any histological abnormalities of cardiovascular tissue. No clinical symptoms were found that would suggest an adverse effect of TGN1412 on the respiratory system, although it was found that it did bind to lymphocytes in human and cynomolgus macaque lung tissue, which is not surprising given the distribution of the target receptor. Although TGN1412 did bind to brain, spinal cord and pituitary gland tissue, from both human and cynomolgus macaque donors, this was considered a low risk effect since the 28-day toxicology study in macaques showed no clinical adverse effects on the central nervous system. Furthermore, as an IgG mAb, in man it was not likely to pass the blood brain barrier. Taken together, these data suggested that TGN1412 was not likely to cause adverse affects in man on the cardiovascular system, the respiratory system or the central nervous system (TeGenero AG, 2005a).

Other toxicological studies in cynomolgus macaques revealed that TGN1412 was well tolerated and no adverse effects were observed at doses up to and including 50 mg/kg/week over a period of 4 weeks. TGN1412 administered to cynomolgus macaques did cause an increase in

the number of CD4+ and CD8+ T cells 13 – 17 days post infusion. This was considered an expected effect given the mode of action of CD28 superagonists and so further validated the use of cynomolgus macaques as an animal model for predicting the clinical effects of TGN1412. In addition, the serum levels of IL-2, and Th2 cytokines IL-5 and IL-6 were moderately elevated but pro-inflammatory cytokines TNFα and IFNγ remained unchanged following treatment, and there was certainly no evidence to suggest that TGN1412 would cause a "cytokine storm". In addition, intravenous administration of TGN1412 in rabbits did not indicate any clinically significant effects.

The starting dose of TGN1412 to be administered to the volunteers in the trial was based on the findings from *in vivo* experiments with Cynomolgus macaques. The No Observed Adverse Effect Level or NOEL is a method that has often been used to identify a safe starting dose in humans (US Food and Drug Administration: Center for Biological Evaluation and Research, 2005) and it is the highest dose at which no statistically significant and/or biologically relevant adverse event is observed (Stebbings et al., 2009). The maximum dose of TGN1412 infused into Cynomolgus macaques was 50 mg/kg and this did not cause any clinical symptoms, therefore 50 mg/kg was considered as the NOEL. The human equivalent of the non-human primate NOEL was 16 mg/kg, calculated by applying a correction factor of 3.1 which corrected for the difference in body surface area between the two species. The default safety factor of 10 was applied and an additional safety factor was included leading to a starting dose in humans of 0.1 mg/kg which actually allowed a 160-fold safety margin on the NOEL (TeGenero AG, 2005a).

In summary, pre-clinical testing of TGN1412 involved experiments conducted using TGN1412 and TGN1412 orthologues such as the mouse anti-rat CD28 mAb JJ316 (Tacke et al., 1997), the mouse anti-human CD28 mAb 5.11A1 (Luhder et al., 2003) and the IgG1 variant of TGN1412 with an identical variable domain; TGN1112 (TeGenero AG, 2005a). These studies involved the use of either animal models (rodents and macaques) or *in vitro* experiments using PBMC, purified T cells and T cell subsets in which the CD28 superagonists were incubated in solution and showed no evidence to suggest that TGN1412 could cause any clinical effects if

used in human therapy, despite its capability to induce T cell proliferation and the production of anti-inflammatory cytokines.

1.9 Phase I clinical trial of TGN1412

In the UK, regulatory approval was granted by the Medicines and Healthcare Products Regulatory Agency (MHRA) for a clinical grade batch of TGN1412 to progress to a Phase I "first time in man" clinical trial after pre-clinical tests showed no evidence to suggest that this mAb would cause any toxic effects in humans. The Paul Ehrlich Institute also granted regulatory approval for TGN1412 to enter a Phase I trial in Germany, scheduled to take place soon after the UK trial (Expert Group on Phase One Clinical Trials, 2006). Phase I trials are usually conducted early in the development of a drug and are carried out to identify a safe starting dose for subsequent studies and to assess the toxic side-effects, action, metabolism, distribution within the body and excretion of a drug. The UK trial took place on March 6th 2006 and was carried out by the contract research organisation Parexel on behalf of TeGenero, in leased space at Northwick Park Hospital. The aim of the study was to assess the effect of single doses of TGN1412 administered to human healthy males intravenously on cytokine release, T cell subsets and the generation of anti-TGN1412 antibodies. Females were not included in the trial since effects on reproduction had not been established in pre-clinical testing. Healthy subjects were used as opposed to patients suffering from B-CLL or rheumatoid arthritis, i.e. the two diseases for which TGN1412 was intended as a therapy. Healthy subjects were also used because pre-clinical tests were conducted in healthy animals which showed no adverse effects and this may not have been the case if subjects were not healthy. Moreover, the level of CD28 expression was comparable between human subjects and those suffering from B-CLL and rheumatoid arthritis thus pharmacokinetic behaviour was likely to have been comparable in patients with these diseases. In addition, TGN1412 activity was unlikely to have been affected by other medications in healthy subjects. The starting dose was 0.1 mg/kg body weight and it was intended that doses of 0.5, 2 and 5 mg/kg would follow, i.e. a dose escalation study was intended. Eight consenting males were enrolled in the study, six receiving the starting dose of

TGN1412, and two receiving a placebo. The mAb or placebo was infused into the volunteers at 10 minute intervals (Expert Group on Phase One Clinical Trials, 2006).

Within 60 - 120 minutes of infusion, all six volunteers given the TGN1412 suffered a number of clinical symptoms including severe headaches, lumbar myalgia, nausea, vomiting, diarrhoea, amnesia, severe pyrexia and restlessness. A systemic inflammatory response, which included peripheral vasodilation and erythema, was observed in all patients. After 240 minutes, all patients had tachycardia, hypotension and elevated body temperatures ranging from 39.5 to 40°C. Volunteers given TGN1412 were initially treated within the clinical trials unit and showed signs of recovery. However all volunteers were transferred to the nearby NHS Intensive Care Unit within 16 hours of infusion due to concerns that they might all develop respiratory distress after this had occurred in one of the volunteers. Indeed, the condition of all 6 patients worsened and all showed symptoms of respiratory distress. Other clinical effects suffered were disseminated intravascular coagulation leading to necrosis of the fingers of both hands and of all toes in one patient; (see Figure 9) and multiple organ failure in all patients. All six individuals survived but suffered subsequent clinical effects such as muscle weakness, myalgia, headaches, difficulties with concentration, peripheral numbness (Suntharalingam et al., 2006), loss of fingers and toes (one patient) and, not surprisingly, psychological effects.

Figure 9. Clinical effect of TGN1412: tissue necrosis in fingers of the most seriously affected TGN1412 clinical trial volunteer as a result of disseminated intravascular coagulation (BBC, 2006).

The adverse effects of TGN1412 were mediated by a rapid and dramatic increase in cytokine concentrations: TNFα concentrations in the serum of the volunteers increased within an hour after TGN1412 infusion. This was followed by increases in Th1 and Th2 and T regulatory cell cytokines including IL-2, IL-6, IL-8, IL-1β, IL-10, IL-4 and IFNγ within 4 hours post-infusion. Concentrations returned to normal within 2 days (which was likely to have been a result of medical intervention) except for the two patients who suffered the worst effects, whose elevated IL-4 and IL-6 serum concentrations were prolonged by a further 1 to 2 days. The term "cytokine storm" was coined to describe the cascade of pro-inflammatory cytokine release which evoked fever, pain and multiple organ failure in the trial volunteers (Suntharalingam et al., 2006).

In addition to the cytokine storm, a reduction in the number of lymphocytes and monocytes in the blood of the volunteers within 8 hours post-infusion was observed resulting in severe lymphopenia and monocytopenia. It is not clear whether this was a result of cell death or the migration of the cells from blood across endothelium to other tissues such as the lymph nodes. Despite the overlap with the cytokine storm, it is not known if this depletion of cells was a response to the infusion of the T cell agonist or a response to the cytokine storm alone. Following lymphopenia, the numbers of CD3+, CD4+ and CD8+ T cells measured in peripheral blood increased and peaked at day 5 and again at day 15 after infusion. The cytokines produced in the reaction were those associated with various T cell subsets (e.g. Th1, Th2 and Treg cells) and not just those of Treg cells, as predicted by the pre-clinical tests.

In summary, clinical events following infusion of TGN1412 were separated into four phases. The rapid increase in serum cytokine concentrations and lymphopenia and monocytopenia occurred in phase 1, which began approximately 60 minutes post-infusion. This lasted between 2 to 3 days depending on the volunteer. Phase 2 was considered as the reactive phase during which volunteers developed multiple organ failure including renal and respiratory failure. This occurred from day 1 through to day 8 but varied amongst the volunteers. Phase 3 was considered the recovery phase during which patients recovered from multiple organ failure, lymphopenia and monocytopenia and occurred between days 3 and 15, depending on the

volunteer. Phase 4 was considered as the steady-state phase during which measured responses returned to normal. This occurred from day 15 to 20, again depending on the volunteer.

Phase I clinical trials are usually conducted with the aim to confirm the findings from pre-clinical testing (Bhogal and Combes, 2006). Clearly the TGN1412 trial did not confirm the findings of pre-clinical testing as such tests did not predict that TGN1412 would induce a cytokine storm or any other serious adverse events.

1.10 Events following the TGN1412 clinical trial

Following the clinical trial, at the request of the MHRA, TGN1412 used in the clinical trial was subject to a broad range of analytical techniques conducted at the National Institute for Biological Standards and Control (NIBSC). The results of these tests confirmed that the batch infused into the trial volunteers complied with its specification, was of clinical grade and that no errors had been made during its quality control testing (Expert Group on Phase One Clinical Trials, 2006; Stebbings et al., 2007). Included in this range of confirmatory tests was a bacterial endotoxins test, also known as a Limulus amebocyte lysate or LAL test (Mascoli and Weary, 1979a; Mascoli and Weary, 1979b) and a rabbit pyrogen test, which can detect endotoxin and non-endotoxin pyrogenic (fever-inducing) contaminants (Probey and Pittman, 1945). These tests were carried out since the initial pro-inflammatory cytokine storm suffered by the volunteers was characteristic of a response to endotoxin or other pyrogenic contaminants of medicines. Nevertheless, the material inside the syringes intended for use in the trial passed the rabbit pyrogen test and the bacterial endotoxins test, as well as a test for abnormal toxicity using mice and guinea pigs. The results of these tests were not consistent with the notion that TGN1412 was contaminated with a pro-inflammatory/pyrogenic agent such as endotoxin or a non-endotoxin pyrogen to which rabbits are sensitive.

1.11 Aims of study

The disastrous events of the TGN1412 clinical trial clearly reflect the limited capability of testing procedures available at present to predict the toxic effects of novel biological medicines such as mAbs. It is essential, however, that the development of new therapeutics for the treatment of disease continues. To allow this to happen, pharmaceutical companies and regulatory authorities require better testing procedures capable of predicting toxic effects of therapeutic mAbs to be confident that such medicines are safe prior to their being given in man. The use of animal models for testing therapeutic mAbs has been questioned from an ethical point of view, especially as it cannot be certain that the chosen species will lead to accurate predictions about the toxicity of the medicine, as was the case for TGN1412. It is an opinion shared by many that more emphasis should be placed on developing alternatives to testing on animals (Bhogal and Combes, 2007; Liebsch et al., 2011), highlighted by the fact that some governments now endorse the "3Rs" principle of Replacement, Refinement and Reduction of tests involving animals, originally proposed by Russell and Burch in 1959 (Flecknell, 2002). *In vitro*, the use of human tissue itself is likely to be the most physiologically relevant means for predicting human responses to infused medicines though currently it is limited by the lack of methods available that can reliably predict the clinical effects of therapeutic mAbs. Therefore, the hypothesis of this study is as follows:

Cytokine-driven adverse effects of therapeutic monoclonal antibodies and the mechanisms involved can be better predicted with novel in vitro procedures using human cells.

The hypothesis of this study is tested by pursuing the following three experimental objectives:

1. To develop in vitro procedures using human cells capable of predicting the clinical effects of TGN1412

The basic format of cytokine release assays often successfully used to evaluate the capability of medicines to stimulate cytokine release (Taktak et al., 1991; Wing et al., 1995;

Gaines Das et al., 2004) can be summarised as follows. First, human white blood cells are incubated with a range of doses of the medicine under investigation in microtitre plates in a medium containing human or bovine plasma/serum. During the incubation, cells accumulate at the bottom of the wells of the microtitre plate under gravity permitting the isolation of the "cell-conditioned medium" from the cells using a multi-channel pipette. The term cell-conditioned medium is used to describe the cell-free culture medium that may contain cytokines (or other soluble factors) released by cells in response to the medicine. The incubation period is sufficient to allow for the accumulation of cytokines. Second, cytokine responses are measured by assaying the cell-conditioned medium in cytokine-specific enzyme linked immunosorbent assays (ELISAs). This basic format described will be used as a starting point for the development of *in vitro* procedures that can predict clinical responses to TGN1412. In the first instance, methods which result in a "solid phase" presentation of TGN1412 to human white blood cells during the incubation period will be investigated and compared with the conventional method of presenting the mAb in "aqueous phase". The term "solid phase" is used to describe a mAb that is immobilised by a solid support and is discussed in more detail in the Introduction of Chapter 4. In the second instance, the notion that TGN1412 activated target blood cells in the presence of endothelial cells in the trial volunteers is considered. This is highly likely given that endothelial cells form microvessels (including the high endothelial venules which facilitate the movement of lymphocytes from the blood into lymph nodes) and the lining of large vascular blood vessels. Furthermore, endothelial cells play a fundamental role during an immunological response (Pober and Sessa, 2007) as discussed in more detail in Chapters 5 and 6. Therefore, a method in which a monolayer of human endothelial cells is co-cultured with human white blood cells in the presence of TGN1412 (aqueous phase) is also investigated.

A number of different experimental conditions will be explored for both of the adaptations (i.e. the "solid phase" presentation method and the endothelial/blood cell co-culture method) of the basic format for cytokine release assays mentioned in the paragraph above. These are discussed in more detail in the relevant chapters. Fresh whole blood/PBMC isolated from donors at NIBSC will be used so that the cells are in their natural state as far as is

practicable. Cryogenic storage was not considered an option since it can often lead to altered phenotypes and cell damage (Tollerud et al., 1991).

To evaluate whether or not the procedures developed are capable of predicting the clinical effects of TGN1412, several responses or "readouts" will be measured which are representative of the responses stimulated by TGN1412 in the clinical trial volunteers. This includes the cytokines TNFα, IL-6, IL-8, IFNγ and IL-2 which, in most instances, will be measured in the cell-conditioned medium by cytokine specific ELISAs. Indeed, these cytokines were all elevated in the blood of the TGN1412 recipients (Suntharalingam et al., 2006). In addition, given that TGN1412 stimulated T cell proliferation during pre-clinical testing (TeGenero AG, 2005a) and in the trial volunteers (Suntharalingam et al., 2006), the proliferative response of cells remaining in the wells following the removal of cell-conditioned medium will also be measured.

2. To use these procedures to gain a better understanding of the mechanism of action of TGN1412.

After optimising the methods developed under the first objective, these procedures will be used to explore potential mechanisms for TGN1412-stimulated cytokine release in the trial volunteers. The mechanisms to be explored are described in the relevant chapters. Most notably, the role of PBMC in endothelial cell contact in TGN1412-stimulated responses is investigated together with the role of endothelial cell/PBMC adhesion molecule interactions.

3. To validate the use of the procedures developed for predicting cytokine release syndrome caused by therapeutic antibodies other than TGN1412 that are associated with a significant incidence of clinical infusion reactions.

A selected panel of therapeutic mAbs which are/are not associated with a significant incidence of clinical infusion reactions resulting in cytokine release (according to the literature) will be tested using the procedures developed in this study. The pattern of *in vitro* cytokine release stimulated by the panel of mAbs will be compared with their reported associated

incidence of pro-inflammatory clinical infusion reactions in order to evaluate the predictive value of the novel methods that are developed.

Chapter 2:
General methods

CHAPTER 2: General methods

This chapter describes the general methods used during the course of this study. Procedures developed as part of the aim of a practical chapter or that are specific to one chapter are described in the Methods section for the relevant chapter.

2.1 General information

Aseptic technique and materials that were sterile and free from detectable pyrogens were used for all procedures involving cell culture. Buffers/solutions referred to in the text were prepared as described in Appendix I unless otherwise stated. Bovine serum albumin (BSA) and ovalbumin used were from Sigma (A7888 and A5503 respectively). All concentrations of samples and controls were tested in quadruplicate in each assay unless otherwise stated in the specific Methods section for each chapter. In all cases, each value in a data set for a given treatment is the mean of 4 replicates. The number of independent assays carried out as repetitions of a particular experiment and the total number of donors providing cells for each experiment are given in the legends to each of the figures in the Results section for each chapter. Methods used for statistical analyses are also described in each chapter. Differences were considered significant where $p<0.05$.

2.2 Blood donation and isolation of PBMC

The use of donated blood for this project was approved by NIBSC Human Materials Advisory Committee (HuMAC). Human whole blood was donated by consenting employees of NIBSC and was taken by a qualified Phlebotomist. Donors were healthy males or females self-diagnosed to be free of symptomatic viral and bacterial infections who had not taken steroidal anti-inflammatory medicines during the 7 days prior to giving blood or non-steroidal anti-inflammatory medicines during the 3 days prior to giving blood or any other drug known to influence immunological responses. PBMC and donor plasma were isolated from heparinised

(Fragmin Dalteparin Sodium, Pharmacia, 10 IU [international units]/ml blood), freshly donated (<30 min after withdrawal) whole blood by density gradient centrifugation using Lymphoprep (Axis-Shield Diagnostics 1114545) or Histopaque (Sigma H8889) layered beneath whole blood diluted 1 in 2 with PBS (phosphate buffered saline) B when using Lymphoprep or undiluted whole blood when using Histopaque. After layering, the blood/Histopaque or Lymphoprep-containing centrifuge tubes were centrifuged at 340 x g for 45 min at room temperature. After centrifugation, a serological pipette was used to aspirate plasma forming the uppermost layer in the tube as shown in Figure 10 and stored for further use in assays where donor's own plasma was required.

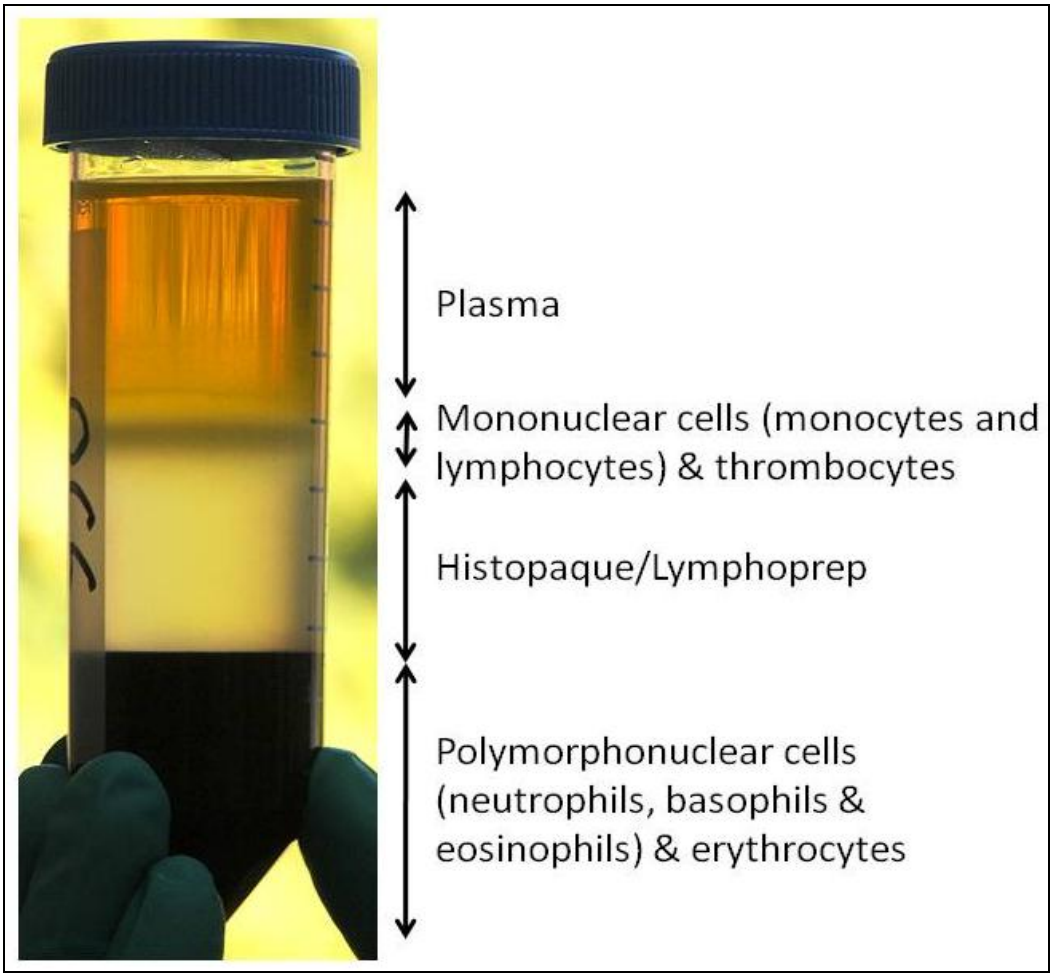


Figure 10. Separation of PBMC from human blood by density gradient centrifugation. Mononuclear cells (monocytes and lymphocytes) and thrombocytes have a lower buoyant density than erythrocytes and polymorphonuclear cells (neutrophils, basophils and eosinophils) and so whilst the polymorphonuclear cells sediment through a solution with a density of 1.077 g/ml (Histopaque or Lymphoprep), mononuclear cells are retained at the sample/solution interface when tubes are centrifuged. Image taken by NIBSC photographer.

PBMC forming the next layer (as shown in Figure 10) were aspirated and stored in a separate centrifuge tube. Tubes containing PBMC were filled with PBS B (50 ml final volume) and centrifuged at 340 x g for 10 -15 min at room temperature. After discarding the supernatant, PBMC were washed 1-2 more times prior to re-suspension in culture medium (as described in the Methods for each chapter of this study). PBMC were stored in a humidified incubator at 37°C, 5% CO_2, and were used in experiments within 4 h of blood collection. If used, donors' own plasma was stored at room temperature until required, i.e. also within 4 h of blood collection.

2.3 Cell counts

A sample of the cell suspension for which the cell concentration was to be determined was diluted 1 in 2 with culture medium and 95 µl of cell suspension was mixed with 5 µl trypan blue (0.4% w/v solution, Sigma T8154). Cell counts were conducted using an Improved Neubauer haemocytometer. The coverslip was fitted such that coloured concentric rings (Newton's rings) could be seen at the coverslip/counting chamber interface prior to loading the sample until the counting chamber was full but not overflowing. Cells were visualised under phase contrast using a microscope (200x magnification). Viable cells (i.e. those not stained blue due to their capability to exclude trypan blue) were counted in 1 square containing 16 smaller squares. This was repeated in different squares (each containing 16 smaller squares) to obtain 4 counts, a mean of which was calculated and multiplied by 1 x 10^4 and sample dilution factor (when diluted) to give a concentration in cells/ml. Counts were repeated using a fresh 95 µl aliquot of cells on the very rare occasions when the number of dead cells (stained blue) was greater than 5%.

2.4 Measurement of TNFα, IL-6, IL-8, IL-2 and IFNγ by Enzyme Linked Immunosorbent Assay (ELISA)

Antibodies for the detection of TNFα, IL-6 and IL-8 were generated within NIBSC except for clone 16 anti-human IL-6 mAb which was a generous gift from Dr. L. Aarden, Sanquin Research, The Netherlands. Monoclonal anti-human IL-2 capture antibody (MAB602) and biotinylated polyclonal anti-human IL-2 detection antibody (BAF202) were from R & D Systems. Monoclonal anti-human IFNγ capture antibody (551221) and biotinylated anti-human IFNγ detection antibody (554550) were from BD Biosciences. Capture antibodies (clone 16 anti-human IL-6 mAb, clone 101-4 anti-human TNFα mAb, anti-human IL-8 polyclonal antibody S333, clone 5355 anti-human IL-2 mAb and clone NIB42 anti-human IFNγ mAb) were coated in either Coating Buffer A (IL-6 capture mAb) or Coating Buffer B (all other capture antibodies), onto walls of wells of 96-well microtitre plates (Immuno MaxiSorp, NUNC) at 2.5 µg/ml (200 µl/well) for anti-IL-6, 2 µg/ml (100 µl/well) for anti-TNFα and 0.5 - 1 µg/ml (100 µl/well) for anti-IL-8, 1 µg/ml (100 µl/well) for anti-IL-2 and 2 µg/ml (100 µl/well) for anti-IFNγ. The anti-IL-6 coated plates were covered and left for 16 – 24 h at room temperature. The anti-TNFα, anti-IL-8, anti-IL-2 and anti-IFNγ coated plates were covered and left for 16 – 24 h at 4°C. Anti-IL-6 antibody coated plates were washed 3 times in demineralised water. Anti-TNFα, anti-IL-8, anti-IL-2 and anti-IFNγ coated plates were washed 3 times with Wash Dilution Buffer. Plates coated with IL-6 capture mAb were blocked with 200 µl of Blocking Buffer A. All other capture antibody-coated plates were blocked with Coating Buffer B containing 0.5 – 1% ovalbumin or BSA. All plates were washed 3 times with Wash Dilution Buffer and once with demineralised water prior to the addition of samples.

The following World Health Organisation (WHO) international standards (IS) were used as calibrants for the cytokine ELISAs: WHO IS 89/548 for IL-6, WHO IS 89/520 for IL-8, WHO IS 88/786 for TNFα, WHO IS 88/606 for IFNγ and preparation 86/564 for IL-2, all from NIBSC. The standards were prepared in cell culture medium (specific for the experiment) supplemented with plasma or serum as specified in the individual Methods section for each

chapter. For all ELISAs, 50 µl cell-conditioned medium or each concentration of cytokine standard was assayed in wells coated with the capture antibodies. Culture medium alone (specific for the assay) was used to give a reading for 0 pg/ml cytokine. Concentrations of standard and culture medium alone were added to every ELISA plate in duplicate wells. For the IL-6 ELISA, 200 µl sheep anti-human IL-6 polyclonal antibody S2446 conjugated to horseradish peroxidise (HRP) diluted 1 in 800 with Detecting Antibody Dilution Buffer was added to each well and plates were incubated for 2 – 3 h at room temperature. Plates were washed 3 times with Wash Dilution Buffer and once with demineralised water, prior to the addition of 200 µl substrate solution (0.5 ml TMB Substrate Solution in 10 ml Substrate Buffer A) and, 5 – 10 min later; 50 µl Stop Solution. The absorbance values were calculated by subtracting the OD (optical density) values measured using a corrective filter (540nm) from the OD values measured using a 450nm filter, using a spectrophotometer (Molecular Devices) and SoftmaxPro software. For the TNFα, IL-8 IL-2 and IFNγ ELISAs, after the addition of the cell-conditioned medium/cytokine standards, 50 µl sheep anti-human TNFα polyclonal (H91) biotinylated antibody (diluted 1 in 4000) or sheep anti-human IL-8 polyclonal (S333) biotinylated antibody (diluted 1 in 2000) in Wash Dilution Buffer containing 1% normal sheep serum (NIBSC) or 50 µl goat anti-human IL-2 polyclonal biotinylated antibody (diluted 1 in 1000) in PBS A containing 1% BSA or 50 µl clone 4S.B3 biotinylated anti-human IFNγ mAb (diluted 1 in 250) in PBS A containing 0.5% BSA was added and the covered plates incubated overnight at 4°C. Plates were washed 3 times in Wash Dilution Buffer prior to the addition of 100 µl avidin-HRP (Dako P347) or streptavidin-HRP (Jackson ImmunoResearch S7973-97A) in Wash Dilution Buffer; plates were incubated for 15 min at room temperature and then washed 3 times in Wash Dilution Buffer and once in demineralised water. For the TNFα and IL-8 ELISAs, the addition of 100 µl substrate solution (o-phenylenediamine dihydrochloride, OPD [Sigma P-7288], dissolved in Substrate Buffer B containing 30% hydrogen peroxide [Sigma H-1009]) was followed by the addition of 50 µl Stop Solution after a 5 – 15 min incubation. Absorbance was measured at 490nm using a spectrophotometer (Molecular Devices) and SoftmaxPro software. For the IL-2 and IFNγ ELISA, 100 µl substrate solution (as for the IL-6 ELISA) was added and, 5 – 10 min later; 50 µl Stop Solution. The absorbance values were

calculated as described for IL-6. For all ELISAs, a 4-parameter fit was assigned to the cytokine standard curve given by each ELISA plate and cytokine concentrations in the cell-conditioned media were calculated from this curve.

2.5 Measurement of cell proliferation by 3H-thymidine incorporation

Cell proliferative responses were quantified by measuring the amount of radioactively-labelled thymidine incorporated into "new" strands of DNA in proliferating cells. After removal of the majority of the cell-conditioned medium, cells remained in 50 µl of cell-conditioned medium in the wells of the microtitre plates. 3H-thymidine (Amersham Bioscience TRK300) was added to each well (0.5 µCi/well in 50 µl fresh culture medium supplemented with plasma or serum as specified in the Methods section of each individual chapter) to give a final concentration of 5 µCi/ml. Cells were incubated with 3H-thymidine for 18-19 h in a humidified incubator at 37 °C, 5% CO_2. Using a Micro 96 harvester (Molecular Devices), cells were harvested onto filtermats (Perkin Elmer 1405-421) which were dried and sealed in clear plastic bags with scintillant (Perkin Elmer 1205-440). The amount of 3H-thymidine incorporated into DNA was quantified (in counts per minute, cpm) using a 1450 Microbeta Trilux (Perkin Elmer) radioactivity counter. Counts per minute are directly proportional to the amount of cell proliferation.

2.6 Monoclonal antibodies

The mAbs used in this study were all of clinical grade. TGN1412 was originally from TeGenero AG, Würzburg, Germany. Avastin (Bevacizumab, Roche) is a humanized IgG1 anti-VEGF for the treatment of cancer; Herceptin (Trastuzumab, Roche) is a humanized IgG1 anti-Her2, used for the treatment of breast cancer and Campath-1H (Alemtuzumab, Genzyme), is a humanized IgG1 anti-CD52 for the treatment of lymphocyte neoplasms, transplant rejection and autoimmune diseases. The isotype-matched (to TGN1412) negative control antibody used in experiments was either Tysabri (Natalizumab, Biogen IDEC), a humanized IgG4κ anti-α4

integrin used for the treatment of multiple sclerosis or a humanised IgG4κ antibody of clinical grade with an irrelevant (to this study) specificity. This antibody had failed its phase III clinical trial on grounds relating to its efficacy and was provided as a gift for use as a control provided that details regarding its manufacturer and specificity are not disclosed. (For those wishing to repeat any of the work carried out as part of this study Tysabri was shown to be an adequate negative control as can be seen from the data presented below.)

An unavoidable consequence of incubating therapeutic mAbs in aqueous phase with human cells (as is the case for some of the methods investigated in this thesis) is that the mAbs are transferred in the cell-conditioned medium to the cytokine specific antibody-coated ELISA plates. Therefore, all of the therapeutic mAbs used in this study were evaluated for their capability to interfere with the detection of cytokine in each of the cytokine-specific ELISAs described above. A dilution series (3 concentrations minimum) of each cytokine standard was assayed in the absence and presence of each of the therapeutic mAbs, by ELISA. Each cytokine standard was diluted in supplemented culture medium typically used for cell-based assays (RPMI 1640 [Sigma R0883] supplemented with 2 mM L-glutamine [Sigma G7513], 100 units/ml penicillin, 0.1 mg/ml streptomycin [Sigma P0781] and non-essential amino acids [Gibco 11140]) containing 2% donor plasma. The final concentration of mAb added to each concentration of cytokine standard tested was 1000 µg/ml for TGN1412, 40 µg/ml for the isotype-matched (to TGN1412) control and 400 – 500 µg/ml for Tysabri, Avastin, Herceptin and Campath-1H for cytokines TNFα, IL-6, IL-8, IL-2 and IFNγ. These concentrations reflect the highest doses incubated with cells in the methods investigated in this study, i.e. the maximum doses transferred to ELISA plates. Concentrations of cytokine standard tested in the presence and absence of mAbs spanned the range of the concentrations used for ELISA standard curves in assays to quantify the amount of cytokine in cell-conditioned medium (typically 31.25 pg/ml – 4000 pg/ml). The cytokine standard curves were tested in ELISAs as described in section 2.4 above. A comparison of the optical density values given for each cytokine standard curve tested in the absence and presence of each therapeutic mAb was made. The results of these comparisons are shown in Appendix II and in Figure 12. It was concluded that none of the therapeutic mAbs interfered with the detection of any of the cytokines by ELISA.

Chapter 3: Conventional in vitro procedures are not predictive of the clinical effects of TGN1412

CHAPTER 3: Conventional *in vitro* procedures are not predictive of the clinical effects of TGN1412

3.1 Introduction

The rapid increase in pro-inflammatory cytokines (TNFα, IL-6, IL-8 and IL-1β) in the blood of the TGN1412 trial volunteers following infusion of this mAb is characteristic of the response induced by pyrogenic contaminants of medicines (Poole et al., 1988; Nakagawa et al., 2002). It was conceivable that the cytokine storm suffered by the volunteers was caused by a non-endotoxin pyrogen or contaminant that was not detected in either the bacterial endotoxins test or in the rabbit pyrogen test but which was very potent in man. Over a number of years, NIBSC, Novartis and Baxter Healthcare have developed a cell-based *in vitro* test system (Taktak et al., 1991; Gaines Das et al., 2004) that is better able to detect non-endotoxin pyrogenic contaminants of medicines as described in US patent 7736863B2. The test system has been termed a 'monocyte activation test' (MAT) and is compliant with the method described in the European Pharmacopoeia (published April 2010). It is more accurately described as a 'cytokine release test' since cells other than monocytes can play a part in responses to contaminants. The test involves the culture of the medicine (in aqueous phase) with either human PBMC or cells of a monocytic cell line (e.g. Monomac 6 or THP-1) or with human whole human blood. Usually after 16–24 h of cell culture, pro-inflammatory cytokines in the cell-conditioned medium are quantified by cytokine specific enzyme-linked immunosorbent assays (ELISAs). Although cell-based tests completed by TeGenero during the pre-clinical evaluation of TGN1412 provided no evidence that the antibody might be capable of evoking a cytokine storm, the test systems applied were not optimised for detecting pyrogenic contamination. It was therefore a suitable starting point to use the cytokine release test for contaminants developed at NIBSC to investigate whether or not the cytokine storm was caused by a pyrogenic contaminant of TGN1412 that was undetected in the bacterial endotoxins test and in the rabbit pyrogen test. Failure of TGN1412 to stimulate cytokine release in this assay would suggest an absence of pyrogenic contamination of this mAb and would suggest that it

was TGN1412 itself which was responsible for the cytokine storm in the trial volunteers. The cytokine readouts chosen were TNFα, IL-6 and IL-8 because elevated concentrations of these pro-inflammatory cytokines were measured in the blood of the trial volunteers' post-infusion of TGN1412. IL-1β is also a pro-inflammatory cytokine that is produced in response to pyrogens and was elevated in the blood of the trial volunteers. Its biological functions are similar to those of TNFα and together these cytokines can have synergistic effects. However, it is produced in relatively small amounts by PBMC compared with TNFα, IL-6 and IL-8 making it a poor marker for measuring the pro-inflammatory effects of medicines *in vitro*. It is for these reasons that IL-1β was excluded as a readout in this study.

3.2 Methods

3.2.1 Rationale for TGN1412 Doses Tested

At its clinical trial, TGN1412 was injected into volunteers at 0.1 mg/kg (Suntharalingam et al., 2006). An average human male weighs 70 kg, and has approximately 5 litres of blood. TGN1412 dissolves in the plasma which is approximately 60% of the total blood volume and so the effective dilution volume is 3 litres. Therefore, 0.1 mg/kg (human dose) x 70 kg = 7 mg TGN1412 per 3 litres plasma, which is approximately 2 µg TGN1412/ml plasma. It should be noted that 2 µg/ml is the maximum *in vitro* concentration equivalent to 0.1 mg/kg and does not take into account antibody half-life and preferential distribution in one body compartment rather than another (Stebbings et al., 2007). The maximum dose given to macaques was 50 mg/kg, i.e. 500x more than that given in man (Expert Group on Phase One Clinical Trials, 2006). This larger dose is approximately equivalent to 1000 µg/ml in an *in vitro* test system. So, in the experiments described in this chapter, aqueous phase TGN1412 was tested *in vitro* at concentrations between 2 and 1000 µg/ml. TGN1412 formulation buffer (alone) was tested at 4 dilutions that corresponded to the volumes of buffer contained in the 4 largest concentrations of TGN1412 tested.

3.2.2 Cytokine release assay using PBMC

PBMC were isolated from fresh blood as described in section 2.2. Media used with PBMC was RPMI 1640 (Sigma R0883) supplemented with 2 mM L-glutamine (Gibco 25030), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco 15140) and non-essential amino acids (Gibco 11140-035) and is termed culture medium in this chapter. Aqueous phase TGN1412, at a range of concentrations, endotoxin IS (94/580, NIBSC) as a positive control, TGN1412 formulation buffer and culture medium alone (as a negative control), were incubated with 50,000 – 200,000 PBMC/well in 250 µl culture medium and the donor's own plasma (2%), in wells of 96-well, round-bottom, polypropylene plates (Corning 3790). Cultures were incubated for 16 – 18 h in a humidified incubator at 37°C, 5% CO_2. Cell-conditioned medium was withdrawn from the PBMC cultures and concentrations of TNFα, IL-6 and IL-8 were measured by ELISA as described in section 2.4. Cytokine standards for ELISAs were diluted in culture medium containing 2% pooled donors' plasma (i.e. plasma from the donors of PBMC used in the assay). Cell-conditioned medium was diluted accordingly with culture medium containing 2% donor's own plasma.

3.2.3 Cytokine release assay using whole blood

The cytokine responses of whole blood to TGN1412, its formulation buffer and endotoxin were measured as for PBMC described in section 3.2.2, except that 20% (v/v) blood (diluted with sterile, pyrogen-free saline from Fresenius-Kabi 31-58-736) was substituted for PBMC. Samples were diluted with saline and cytokine standards used for ELISAs were diluted in saline containing 1% BSA.

3.3 Results

Figure 11 shows that TGN1412 (2.0–1000 µg/ml) and its formulation buffer alone (diluted 1 in 10, 1 in 20, 1 in 40 and 1 in 80), added as an aqueous solution and cultured for 16 –

18 h with human PBMC (50,000–200,000 PBMC/well) or human whole blood (20% v/v final concentration), stimulated the release of TNFα, IL-6 and IL-8 to concentrations barely above basal values. In contrast, endotoxin positive control stimulated the dose-dependent release of all cytokines measured. The endotoxin limit concentration (ELC) for TGN1412, i.e. the permitted maximum concentration of endotoxin in order for this product to comply with its specification, was 0.5 IU (= EU [endotoxin units])/ml for 10 mg/ml TGN1412. For a 1000 µg/ml solution of TGN1412, the ELC would be 0.05 IU (5 pg)/ml. All responses to 1000 µg/ml TGN1412 and its formulation buffer diluted 1 in 10 were below the cytokine concentrations released in response to endotoxin at 0.05 IU (5 pg)/ml. This shows that TGN1412 and its formulation buffer did not contain concentrations of pyrogenic contaminants that could have given rise to the profound pro-inflammatory responses that occurred in the volunteers in the clinical trial.

When TGN1412 (1000 µg/ml) was "spiked" with 0.075 IU (7.5 pg)/ml endotoxin, IL-6 responses of PBMC were similar to those stimulated by 0.075 IU (7.5 pg)/ml endotoxin in the absence of TGN412. IL-6 values were 12,730 ± 2667 pg/ml for TGN1412 spiked with endotoxin and 10,953 ± 2115 pg/ml for endotoxin alone (values are means ± S.E.M [standard error of the mean] of 4 donors of PBMC). This shows that TGN1412 did not inhibit the detection of endotoxin in the assay. In a separate experiment, TNFα, IL-6 and IL-8 ELISAs were carried out on serial dilutions of recombinant cytokine standard in the presence and absence of 1000 µg/ml TGN1412. There was no detectable difference between the absorbance values for the dilution series with and without TGN1412 for TNFα and IL-6, indicating that the presence of TGN1412 in these ELISAs did not interfere with the detection of these cytokines. TGN1412, at the largest dose tested perhaps enhanced the detection of IL-8 since absorbance values were higher for the dilution series prepared in the presence of TGN1412. This result is shown in Figure 12. Therefore, the absence of cytokine responses to TGN1412 shown in Figure 11 could not have been the result of inhibition by TGN1412 of the cytokine detection methods.

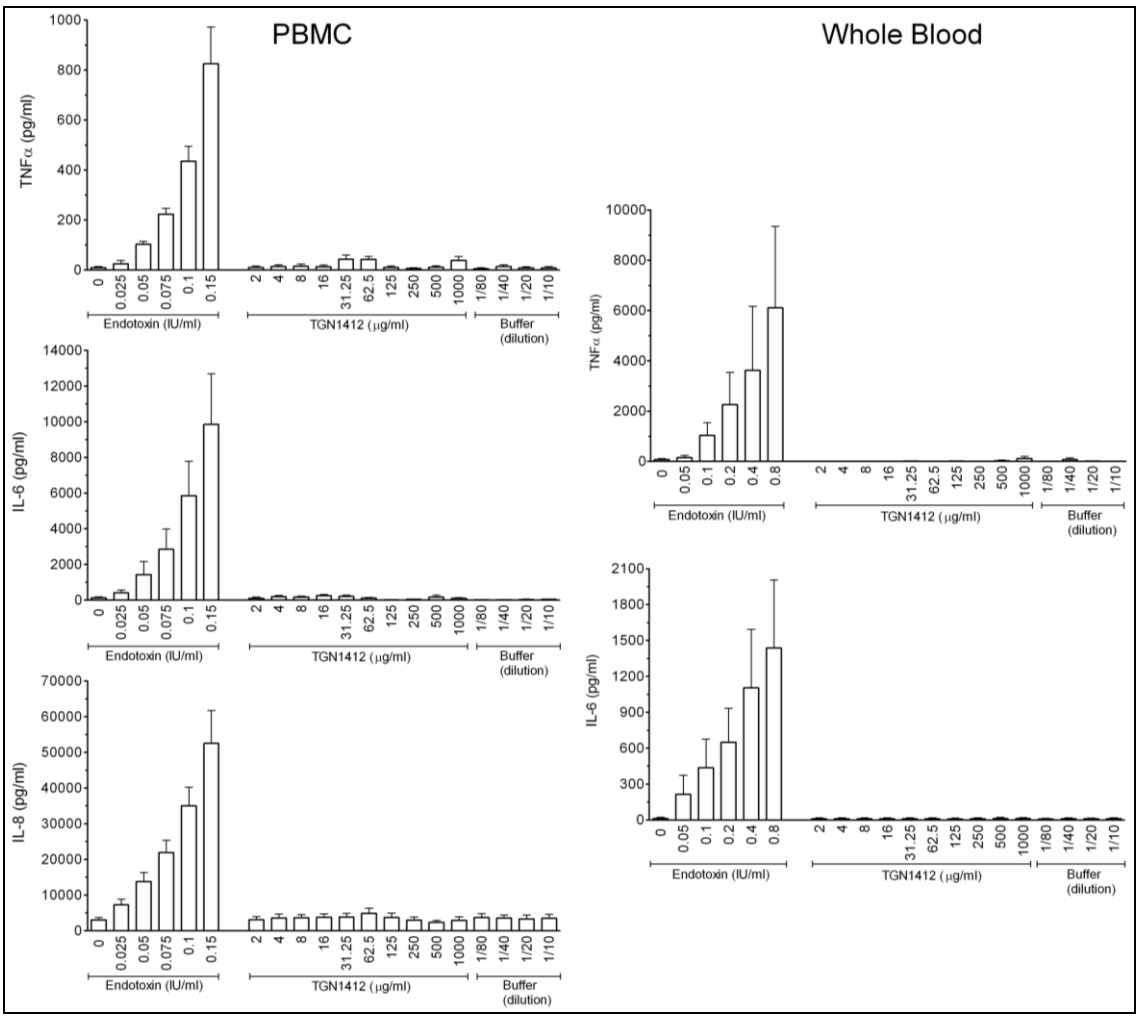


Figure 11. Cytokine responses (pg/ml) of PBMC and whole blood (20% v/v) from human donors to aqueous TGN1412. PBMC (left) or whole blood (right) were incubated with TGN1412 at the doses shown for 16 - 18 h and with endotoxin (positive control) at doses ranging 0.025 IU (2.5 pg)/ml to 0.15 IU (150 pg)/ml for PBMC and 0.05 IU (5 pg)/ml to 0.8 IU (80 pg)/ml for whole blood and TGN1412 formulation buffer. Values for TNFα, IL-6 and IL-8 are means ± S.E.M of 4 donors.

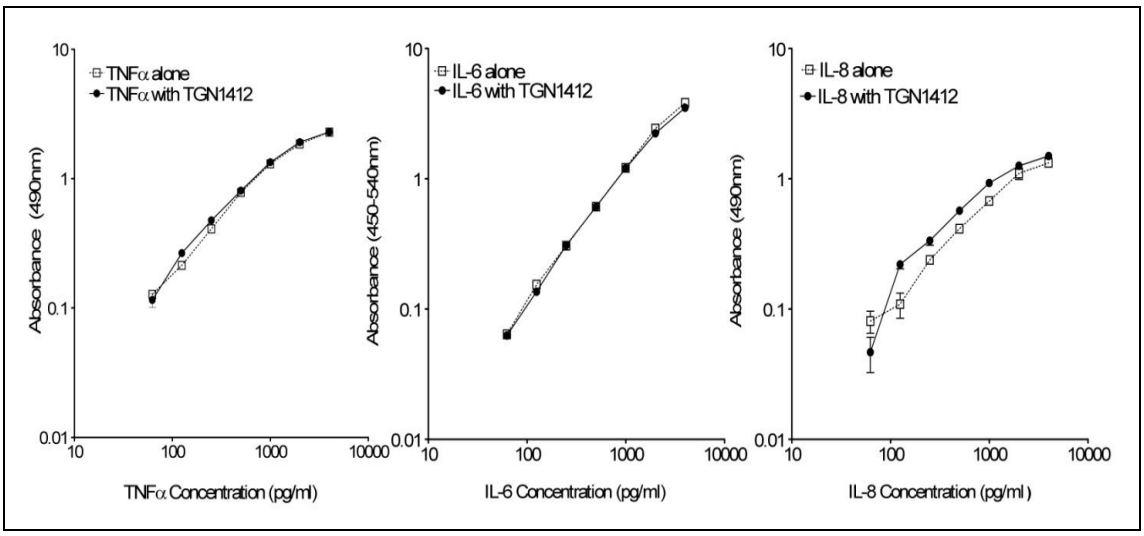


Figure 12. Effect of TGN1412 in the detection of cytokines by cytokine-specific ELISAs. Dilution series of TNFα (left), IL-6 (middle) or IL-8 (right) recombinant cytokine standard were prepared in the presence and absence of 1000 µg/ml TGN1412. Cytokine specific ELISAs were carried out in each of the dilution series prepared. Values are means ± S.E.M of 4 replicates for each concentration of cytokine tested with or without TGN1412, in one assay.

3.4 Discussion

The pro-inflammatory cytokines TNFα, IL-6 and IL-8 were elevated in the serum of all six volunteers in the TGN1412 clinical trial within 4 h of infusion of a clinical grade batch of the drug (Suntharalingam et al., 2006). As noted above, it was conceivable that the "cytokine storm" suffered by the volunteers was caused by a non-endotoxin pyrogen (not detected in the bacterial endotoxins test) to which rabbits were insensitive at the dose that caused such profound effects in man. However, the absence of TNFα, IL-6 and IL-8 cytokine responses of human PBMC to aqueous phase TGN1412 after a 16 – 18 h culture (in a test system optimised for the detection of pyrogens), suggests that this was not the case. The absence of cytokine responses of whole blood to aqueous TGN1412 confirms that the absence of cytokine responses to PBMC was not due to the removal of essential blood components such as neutrophils, eosinophils and basophils, excluded during the PBMC isolation process. It was therefore concluded that the pro-inflammatory effects of the mAb were likely to be due to the intrinsic activity of the molecule itself. Only negligible amounts of TNFα, IL-6 and IL-8 were released *in vitro* in response to 2 µg/ml TGN1412 (i.e. a dose approximately equivalent to the serum

concentration of TGN1412 in the volunteers) and to concentrations up to 500x this dose, i.e. 1000 µg/ml. These negligible amounts in no way mimic the magnitude of TNFα, IL-6 and IL-8 responses to TGN1412 in the trial volunteers (Suntharalingam et al., 2006). Therefore, these conventional cell-based assays using PBMC or whole blood, in which the product is incubated as an aqueous solution, despite being suitable for the detection of pyrogenic contamination, were unable to predict the intrinsic activity of TGN1412. This was surprising, especially as TGN1412 was administered to the trial volunteers in aqueous phase and because CD28 is expressed by T cells, which are present in PBMC.

Following the clinical trial of TGN1412, the Secretary of State for Health set up an Expert Scientific Group which was chaired by Professor Gordon Duff (now Professor Sir Gordon Duff). The Group's aim was to identify what could be learned from the TGN1412 trial and to make recommendations to improve the safety of future clinical trials in man. The data in Figure 11 above showing that TGN1412 incubated with PBMC/whole blood did not stimulate cytokine responses was reported in the Expert Scientific Group Final Report otherwise known as the Duff Report (Expert Group on Phase One Clinical Trials, 2006). It was difficult to compare this data with that generated in pre-clinical tests using PBMC since such data was not reported in the TGN1412 Investigational Medicinal Product Dossier (TeGenero AG, 2005a) or in the Investigators Brochure (TeGenero AG, 2005b). In fact, during the investigation of the clinical trial, TeGenero were criticised for not reporting the results of these assays in the Investigator's Brochure (Kenter and Cohen, 2006). It was confirmed in a report (Hanke, 2006) written by the Chief Scientific Officer of TeGenero that such assays had been conducted and the results were in accordance with those reported by the Expert Scientific Group (Expert Group on Phase One Clinical Trials, 2006), i.e. the data presented here.

It was conceivable that if the dose-response curve for TGN1412 was bell-shaped, the dose of TGN1412 given to Cynomolgus macaques during the pre-clinical tests, i.e. at 50x and 500x the dose subsequently given in man, was too large to elicit a cytokine storm, whereas the dose given in man was closer to the maximum immuno-stimulatory dose. If this were the case, it could have explained why the human volunteers showed adverse reactions that were absent in the Cynomolgus macaques and this was why, first of all, a broadly equivalent dose range was

tested using aqueous TGN1412 *in vitro*. However, the inability of TGN1412 to evoke any responses by PBMC or whole blood meant that this hypothesis could not be tested in this study. Subsequently it was shown that Cynomolgus macaques infused with a wide range of doses of TGN1412, including the 0.1 mg/kg dose that proved to be so harmful in man, suffered no adverse reactions, thus Cynomolgus macaques were, with hindsight, a poor animal model for predicting the clinical safety of TGN1412 (Stebbings et al., 2007).

The inability of the PBMC or whole blood-based assays to predict the intrinsic activity of TGN1412 in aqueous phase (but in tests/assays optimised for the detection of pyrogenic contaminants) was the driver to develop novel *in vitro* methods that were capable of doing so. This is addressed in the following chapters.

Chapter 4: Development of antibody immobilisation techniques to predict the clinical effects of TGN1412 and other monoclonal antibodies

CHAPTER 4: Development of antibody immobilisation techniques to predict the clinical effects of TGN1412 and other therapeutic mAbs

4.1 Introduction

As discussed previously, the failure of animal models and conventional cell-based methods to predict the pro-inflammatory intrinsic nature of TGN1412 signalled the need for better predictive assays. Antibody immobilisation (i.e. the binding of antibody to a solid support) has been reported to be a requirement for full T cell activation using mAbs which target the TCR-CD3 complex (Luhder et al., 2003). Immobilisation of anti-CD3 mAbs by a solid support is thought to mimic the binding of these mAbs to Fc receptors expressed by monocytes (van Lier et al., 1989). The possibility that immobilised TGN1412 could stimulate cytokine responses from PBMC was first proposed by Dr Richard Stebbings. Indeed this was found to be the case and is described in Stebbings et al. (2007). Immobilisation of an antibody is likely to change the manner in which it is presented to cells relative to its addition as an aqueous solution. This is illustrated in Figure 13 below.

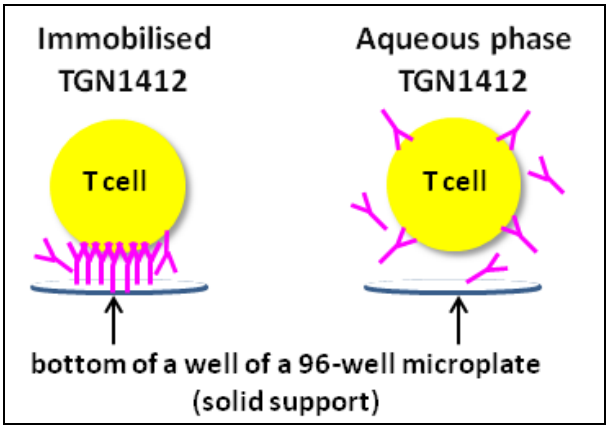


Figure 13. Interaction of immobilised and aqueous phase (soluble) TGN1412 with T cells.

The exact mechanism by which CD28 superagonists interact with their target to stimulate T cell responses without prior engagement of the TCR is not known although a number of models are discussed in section 1.6. What is known is that superagonists bind to an epitope on

the extracellular domain of CD28 which is proximal to the cell membrane, unlike conventional agonistic CD28 antibodies which bind to a membrane-distal epitope as shown in Figure 7 above. Antibody immobilisation may present TGN1412 to CD28 in a manner that facilitates binding of the membrane-proximal epitope and formation of a linear complex as illustrated in Figure 7 above. This may achieve a level of CD28 cross-linking above a threshold required for T cell activation. The main objective of the experiments described in this chapter was to further develop the *in vitro* antibody immobilisation test proposed by Dr Richard Stebbings.

Other studies have suggested that the type of microtitre plate on which the cells are incubated can influence cellular responses to certain stimuli (Poole and Patel, 2010). The surface properties of different microtitre plates (usually polypropylene or polystyrene plates) may affect the manner in which TGN1412 is immobilised and presented to cells. Several antibody immobilisation techniques on different types of microtitre plates are evaluated in this chapter for their value in predicting the clinical effects of TGN1412 and other therapeutic mAbs. These other mAbs include Herceptin and Campath-1H which are associated with a significant incidence of pro-inflammatory clinical infusion reactions and Tysabri and Avastin, which are not associated with a significant incidence of clinical infusion reactions (Dillman, 1999; Chung, 2008; Hellwig et al., 2008; Hansel et al., 2010).

In addition to developing the antibody immobilisation method in this chapter, the reason why aqueous TGN1412 did not stimulate cytokine responses from PBMC is also investigated. Furthermore, the kinetics of cytokine responses to immobilised TGN1412 are compared with the kinetics of TGN1412-stimulated cytokine responses by the trial volunteers. Reverse transcription (RT)-PCR is used to investigate whether or not TGN1412 could have stimulated *de novo* cytokine synthesis in the trial volunteers within one hour post-infusion. This is because the rapid cytokine responses to TGN1412 by the trial volunteers suggested that the cytokines may have been pre-formed and released from intracellular stores following TGN1412 infusion (Suntharalingam et al., 2006). The release of pre-formed intracellular stores of pro-inflammatory cytokines resulting in a cytokine storm, indeed has been reported elsewhere (McILwain et al., 2010).

4.2 Methods

In the experiments described in this chapter, culture medium was RPMI 1640 (Sigma R0883) supplemented with 2mM L-glutamine (Sigma G7513), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma P0781) and non-essential amino acids (Gibco 11140).

4.2.1 Comparison of cytokine responses of PBMC and diluted whole blood to TGN1412 added as an aqueous solution or immobilised by air-drying or wet-coating onto polypropylene and polystyrene culture plates

TGN1412 and an isotype-matched negative control, with an irrelevant specificity, were immobilised by coating onto the walls of wells of untreated 96-well, U-bottomed polypropylene microtitre plates (Corning 3790) and tissue culture treated 96-well, U-bottomed polystyrene microtitre plates (Falcon 353077) by different methods: air-drying and wet-coating. To coat by air-drying, 50 µl of the antibody solution, diluted to the relevant concentration in PBS B (sterile), was added to each well. Plates were left overnight in a class II laminar flow cabinet with the lids removed to allow the solutions to slowly evaporate. To coat using the wet-coating method, 50 µl of the diluted antibody solutions was added to each well and plates were then covered (to prevent evaporation of the solutions) and left overnight in the class II laminar flow cabinet. Antibody solutions were removed from the wet-coated wells with a multi-channel pipette and the wells of all coated plates were washed twice with 200 µl PBS B using a multi-channel pipette to remove the salt crystals (from the air-dried plates) and unbound antibody. PBMC (125,000 cells/well) were incubated for 16 - 24 h at 37°C, 5% CO_2, either in the coated wells of the polypropylene and polystyrene plates containing 250 µl culture medium and 2% donor's own plasma or in wells containing 250 µl aqueous antibody (TGN1412 or the isotype-matched negative control diluted to the required concentration with culture medium) and 2% donors own plasma.

Human whole blood was heparinised as described in section 2.2 and was incubated for 16 - 24 h at 37°C, 5% CO_2, at a final concentration of 20% v/v (diluted with PBS B), with

TGN1412 and the isotype-matched negative control coated at the relevant concentrations by air-drying onto the walls of wells of either polypropylene or polystyrene plates. Antibodies were air-dried using the same procedure as described for PBMC.

After incubation, cell-conditioned medium was removed from the PBMC or whole blood (for both cell sources the cells had settled to the bottom of wells by the end of the incubations) and assayed in cytokine-specific ELISAs as described in section 2.4. Standard curves for each cytokine (TNFα, IL-6 and IL-8) were prepared in culture medium containing 2% (final) pooled donor plasma (i.e. plasma from the four donors of PBMC, used for any one assay) for the PBMC assays and PBS B containing 1% BSA for whole blood assays.

4.2.2 Comparison of cytokine responses of PBMC to TGN1412 immobilised by air-drying and warm-air drying

TGN1412 and an isotype-matched negative control were immobilised by coating onto the walls of wells of polypropylene plates (Corning 3790) by two different methods: air-drying and warm air-drying. The air-drying method was carried out as described in section 4.2.1. The method for warm air-drying was similar except that a hair-dryer was used to blow warm air (40 - 50°C) across the wells of the plates inside a laminar flow cabinet, to evaporate the antibody solutions from the wells (approximately 15 – 20 min/plate). The wells of all coated plates were washed twice with 200 µl PBS B to remove the salt crystals and unbound antibody. PBMC (125,000 cells/well) were incubated for 16 - 24 h at 37°C, 5% CO_2, in the coated wells of the plates containing 250 µl culture medium and 2% donor's own plasma. After incubation, cell-conditioned medium was removed and assayed for cytokines by ELISA as described in section 4.2.1.

4.2.3 Effect of different coating methods on the total amount of TGN1412 immobilised onto plates

TGN1412 or an isotype-matched negative control and the blank (PBS B) were coated by either air-drying, warm air-drying or wet-coating onto polypropylene (Corning 3790) or polystyrene (Falcon 353077) plates as described in sections 4.2.1 and 4.2.2. The solutions were removed from the wet-coated plates and all coated plates were washed twice with 200 µl PBS B, using a multi-channel pipette. (The same PBS was used here as for the experiments where cell responses were measured.) The wells of all plates were incubated with 50 µl PBS B containing 1% ovalbumin for 1 h at 37°C to diminish non-specific binding of the detecting antibodies. The wells of all plates were washed three times with PBS B as before. The wells of all plates were incubated with 50 µl rabbit anti-human IgG (gamma chain specific) antibody conjugated to horseradish peroxidase (Dako P0214) diluted 1 in 3000 with PBS B containing 0.5% BSA, for 2.5 h at room temperature. The wells of all plates were washed four times with PBS B as before. The wells of all plates were incubated with 50 µl substrate solution (OPD tablets, Biotstat 5204530, dissolved in water containing hydrogen peroxide, as described in the manufacturer's instructions). The colour was left to develop for 5 – 10 min at room temperature and the reaction was stopped with 50 µl Stop Solution. After mixing with a multi-channel pipette, the solutions in each of the wells from all plates were transferred to flat bottomed, polystyrene, 96-well plates (Falcon 3530720) and the absorbance was read at 490nm. All absorbance values were baseline corrected by subtracting the mean absorbance for the blank for the corresponding coating method.

4.2.4 Effect of different coating methods on the quantity of TGN1412 immobilised onto plates and (correctly) orientated such that it was able to bind a CD28 fusion protein

TGN1412 and an isotype-matched negative control were coated by either air-drying, warm air-drying or wet-coating onto polypropylene (Corning 3790) or polystyrene (Falcon 353077) plates as described in sections 4.2.1 and 4.2.2. The solutions were removed from the

wet-coated plates and all coated plates were washed twice with 200 µl PBS B using a multi-channel pipette. The walls of wells of all plates were incubated with 50 µl PBS B containing 1% ovalbumin for 1 h at 37°C to diminish non-specific binding of the detecting antibodies. The wells of all plates were washed three times with PBS B as before prior to incubation with 50 µl of CD28 fusion protein from R and D Systems, 342-CD (human recombinant CD28 conjugated to human IgG1 Fc to form a homodimeric molecule) diluted to 1 µg/ml with PBS B containing 0.1% BSA, for 90 min at room temperature. The wells were washed three times with PBS B as before prior to incubation with 50 µl biotinylated mouse anti-human IgG1 Fc antibody (Stratech Scientific Ltd, 6069B) diluted 1 in 2000 with PBS B containing 1% BSA for 2 h at room temperature. The wells were washed four times with tris-buffered saline (TBS) as before prior to incubation with 50 µl extravidin-alkaline phosphatase (Sigma E2636) diluted 1 in 2000 with TBS containing 1% BSA for 30 min at room temperature. Wells were washed four times with TBS as before. The wells of all plates were incubated with 50 µl substrate solution (p-nitrophenyl phosphate tablets, Sigma N2770, dissolved in water as described in the manufacturer's instructions). The colour was left to develop for 5 – 10 min at room temperature and the reaction was stopped with 50 µl 3M sodium hydroxide. After mixing with a multi-channel pipette, the solution in each of the wells from all plates was transferred to flat-bottomed, polystyrene, 96-well plates (Falcon 3530720) and the absorbance was read at 405nm. The wells for the blanks in this method contained all reagents as for the experimental wells (including coated TGN1412 or the isotype-matched negative control) but the CD28 fusion protein was omitted. All values for absorbance from the experimental wells were baseline corrected by subtracting the mean absorbance for the blank for the corresponding TGN1412 or isotype-matched negative control concentration for the corresponding coating method.

4.2.5 Interference by aqueous TGN1412 of cytokine responses of PBMC to immobilised TGN1412

TGN1412 (50 µl/well) was immobilised by air-drying onto polypropylene plates (Corning 3790) using the method described in section 4.2.1. PBMC (125,000 cells/well) were

incubated with immobilised TGN1412 in the absence and presence of either aqueous TGN1412 at a range of concentrations or the isotype-matched negative control at a range of concentrations. PBMC were incubated in 250 µl culture medium and 2% final donor's own plasma, for 16 - 24 h at 37°C, 5% CO_2. After incubation, cell-conditioned medium was removed and assayed for cytokines as described in section 4.2.1.

4.2.6 Detection of early cytokine responses to immobilised TGN1412 using quantitative reverse-transcription polymerase chain reaction (RT-PCR) and by ELISA

Cytokine gene expression in PBMC stimulated with immobilised TGN1412 (for 30, 60 and 90 min) was measured using quantitative RT-PCR analysis. Cytokine release by TGN1412-stimulated cells (for 30, 60 and 90 min) was also measured by ELISA.

4.2.6.1 Stimulation of cells with air-dried TGN1412

TGN1412 (50 µl/well) and an isotype-matched negative control were immobilised by air-drying onto wells of polypropylene plates (Corning 3790) using the method described in section 4.2.1. After the washing step, 125 µl culture medium was added to wells. PBMC (125,000 cells/well) were incubated with immobilised antibody in a final volume of 250 µl culture medium containing 2% donor's own plasma, with 125 µl cells added to wells at different time points using a multichannel pipette. For example, for 90 minute incubations with antibody, PBMC were added to the relevant wells of the first plate at T (time) = 0 min, after which the timer was started. At T = 30 min, PBMC were added to the relevant wells of a second plate (for 60 min incubation with antibody). At T = 60 min, PBMC were added to the relevant wells of a third plate (for 30 min incubation with antibody). At T = 90 min, all plates were centrifuged at 340 x g for 10 min at room temperature. Cell conditioned medium was aspirated from the cell pellets and stored in 96-well plates at -20°C.

4.2.6.2 Measurement of early cytokine responses by ELISA

Cell conditioned medium aspirated from TGN1412-stimulated cells was thawed at room temperature prior to assay of TNFα, IL-6 and IL-8 in cytokine-specific ELISAs as described in section 4.2.1.

4.2.6.3 Measurement of early gene expression by quantitative RT-PCR.

Activation of gene transcription leads to the production of mRNA which is subsequently translated into protein. Therefore, gene expression can be measured using methods that quantify the amount of mRNA produced by cells. The method described here was as described previously (Burns et al., 2008). In the first instance, total RNA is extracted from cells using an RNeasy spin column and stable cDNA is produced from single-stranded mRNA. The cDNA is then used as a template in quantitative RT-PCR involving SYBR Green I dye to quantify the amount of mRNA produced by transcription of the gene of interest by TGN1412-stimulated cells.

4.2.6.3.1 RNA extraction

In order to extract total RNA from stimulated cells, 200 µl PBS B was added to 2 of the 4 replicate wells for each sample (i.e. TGN1412 or isotype control), cells were resuspended and duplicates pooled into Eppendorf tubes prior to microfuging at 4000 x g. Supernatants were removed and from this point forward, total RNA was extracted from cells using the RNeasy Kit (Qiagen 74106) following the manufacturer's instructions. Briefly, 350 µl of a denaturing lysis buffer was added to lyse the cells and denature RNases such that stable intact RNA was isolated. An equal volume of 70% ethanol was added to optimise binding of RNA to the silica-gel membrane in the spin columns. Lysates were loaded into spin columns placed in collection tubes prior to centrifugation of the spin columns at 8000 x g for 1 min at room temperature. Flow-through in the collection tubes was discarded and 350 µl Buffer RW1 was added to each

column prior to centrifuging at 8000 x g for 1 min. Flow-through was discarded. In order to digest any contaminating genomic DNA in the samples, DNase digestion was performed using the RNase-free DNase Set (Qiagen 79254). A working solution of DNase I was prepared as described in the kit instructions and 80 µl was added directly to each spin column membrane prior to 15 min incubation at room temperature. Buffer RW1 (350 µl) was added to each column prior to centrifuging at 8000 x g for 1 min and discarding the flow-through. The bound RNA was further washed to remove contaminants to allow elution of pure RNA. Columns were transferred to new collection tubes and 500 µl Buffer RPE was added to each column prior to centrifuging at 8000 x g for 1 min and discarding the flow-through. A further 500 µl Buffer RPE was added to the columns, prior to centrifuging again at 8000 x g for 2 min and discarding the flow-through. The columns were centrifuged dry at 8000 x g for 1 min and then placed in an RNase- and DNase-free 1.5 ml tube. RNA was eluted from the membranes using a low salt concentration, i.e. RNase- and DNase-free water (30 µl), which was added directly to each membrane. Columns were incubated for 1 min at room temperature prior to centrifuging at 8000 x g for 1 min to elute the RNA. The eluate was re-applied to the column membrane prior to centrifuging again at 8000 x g for 1 min to maximise RNA concentration. Isolated RNA was stored at -80°C.

4.2.6.3.2 Production of cDNA

To carry out quantitative RT-PCR, isolated RNA from PBMC must be reverse transcribed into stable cDNA as the enzyme used in quantitative RT-PCR is a DNA polymerase. cDNA synthesis is carried out using the enzyme Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT).

Following elution from the spin column, 9.5 µl isolated total RNA was incubated with 1 µl Oligo $(dT)_{18}$ primers (Promega C1101) and 1 µl random primers (Promega C1181) at 70ºC in 0.5 ml PCR tubes for 5 min to melt secondary structure creating a linear RNA template. The tube was immediately cooled on ice for 5 min to avoid denaturing the MMLV-RT added at the next step. Random primers bind to random sequences in the RNA and also help prevent

secondary structure formation, while oligonucleotide $(dT)_{15}$ primers bind to the polyA tail of the mRNA within the total RNA. A master mix was prepared as described in Table 1 and 8.5 µl was added to the PCR tube prior to incubation in a PCR machine (Eppendorf Mastercycler) for 50 min at 42°C followed by 15 min at 70°C. The former was to activate the MMLV-RT enzyme and allow the synthesis of cDNA and the latter to subsequently inactivate the enzyme. The 20 µl cDNA samples were stored at -20°C.

Table 1. Reverse transcription master mix

Reagent	**Reaction volume (1x)**	**Supplier**
5x Reaction buffer	4 µl	Promega M170B
10 mM dNTPs	1 µl	Promega U151B
40 U/µl RNAsin	2 µl	Promega N2511
400 mM DTT	0.5 µl	Promega V3151
MMLV-RT	1 µl	Promega M170B

dNTPs; deoxyribonucleotidetriphosphates, RNAsin; RNase inhibitor, DTT; dithiothreitol, MMLV-RT; Moloney Murine Leukemia Virus Reverse Transcriptase.

4.2.6.3.3 Quantitative RT-PCR – method overview

PCR amplifies DNA sequences and permits the detection of sequences when present in low amounts. cDNA samples used as templates for PCR were generated from mRNAs expressed by the TGN1412- or control-stimulated cells. Primers (oligonucleotides) complementary to the nucleic acid sequences of the cytokine of interest were used in PCR reactions to provide specificity to the response measured. PCR was initiated by a heating step to activate the DNA polymerase, prior to cooling to a temperature to allow specific primers to anneal to their complementary DNA sequences. Primer extension by DNA Polymerase was carried out using cDNA as a template. Repeated cycles of DNA denaturation, primer annealing and primer extension led to amplification of specific nucleic acid sequences.

The use of a fluorescent dye during the amplification process allows quantification of the amount of cytokine cDNA present in a sample. SYBR (Synergy Brands) Green I is a dye that

only fluoresces when intercalated within the minor groove of double-stranded DNA. Unbound dye exhibits very little fluorescence. During PCR, DNA is single-stranded after the initial denaturing step, and so SYBR Green I does not bind and low levels of fluorescence are detected. During the elongation step, new double-stranded DNA is synthesised leading to an increase in the amount of fluorescent dye that is bound. To monitor the increasing amount of amplified DNA, fluorescence was measured at the end of each elongation step in every PCR cycle.

Samples containing more cDNA for the cytokine of interest require fewer PCR cycles to reach an assigned threshold amount of fluorescence, compared with samples containing less cDNA. A dilution series of a purified PCR product, amplified by PCR specific for the target cytokine, is subject to quantitative PCR simultaneously with unknown cDNA samples. The amplification of these "standards" allows the generation of a standard curve showing the relationship between the assigned (arbitrary) value of the standards and the number of cycles to reach threshold fluorescence. Using the number of PCR cycles to reach threshold fluorescence (Ct) for each unknown sample, a value can be assigned to these unknown samples using the standard curve. The relative expression of mRNA for the cytokine of interest in unknown samples is normalised by dividing by the value for mRNA for a "house-keeping" gene, i.e. a gene constitutively and stably expressed at high levels (in most tissues) which can be used as an endogenous control. The "house-keeping" gene used in this study was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme involved in cell metabolism, more specifically; glycolysis. GAPDH expression in unknown samples is determined from a GAPDH standard curve as described above. Data are presented as normalised gene of interest expression for TGN1412-stimulated cells relative to that of the isotype-matched negative control-stimulated cells.

4.2.6.3.4 Confirmation of PCR specificity using melt curve analysis

SYBR Green I binds to double-stranded DNA non-specifically and so confirmation that the measured fluorescence is from a PCR product specific for the target cytokine is achieved by DNA melting curve analysis. PCR products are slowly heated above the elongation temperature (typically 72°C) to 95°C after the final cycle of PCR and fluorescence is measured at 1°C increments. The fluorescence of SYBR Green I dye suddenly plummets as PCR product is denatured or "melted" by the increased temperature. When the rate of change of fluorescence is plotted against temperature (x-axis), the observed "melting peak" allows confirmation that a single specific nucleic acid sequence has been amplified. The melting temperature (Tm) of the amplified product is the temperature at which 50% of the DNA becomes single-stranded.

4.2.6.3.5 Quantitative RT-PCR – procedure

Primers specific for each gene of interest were available "in house". The sequence and annealing temperature for each primer used and the size of the target cytokine PCR product are described in Table 2. Lyophilised primers were reconstituted to a 100 μM stock concentration and were subsequently diluted to a 10 μM working concentration in PCR grade water (Sigma W4502).

Table 2. RT-PCR primer sequences, product sizes and annealing temperatures

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)	Annealing temp. (°C)
TNFα	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	123	56
IL-6	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT	196	55
IL-8	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCCTT	180	55
GAPDH	GTCAGTGGTGGACCTGACCT	CCCTGTTGCTGTAGCCAAAT	251	55 - 56

Standards for each gene of interest (TNFα, IL-6 and IL-8) and GAPDH were available "in house", produced as previously described (Burns et al., 2008). A working solution of each standard was prepared in PCR grade water. Dilutions were 1/10 for TNFα, IL-6 and GAPDH and 1/20 for IL-8. These working solutions were used as the largest concentration in a 10-fold dilution series giving 7 different concentrations. A master mix was prepared as described in Table 3. SensiMix listed in this table contained reaction buffer, heat-activated DNA Polymerase, dNTPs, 6 mM $MgCl_2$ and stabilisers. cDNA samples prepared from isolated RNA were diluted 1/3 with PCR grade water and 18 µl master mix was added to 2 µl diluted cDNA to produce a 20 µl final reaction volume. PCR grade water was used as a substitute for cDNA in a negative control. Preparation of the standard dilution series for each gene of interest and for GAPDH and preparation of the master mix and its addition to DNA samples/standard were carried out by a CAS-1200 automated PCR robot.

Table 3. Master mix for quantitative RT-PCR

Reagent	**Volume/Final Concentration**	**Source**
2x SensiMix dT	10 µl (1x)	SensiMix dT kit, Quantace QT6T3
PCR grade water	5.6 µl	Sigma W4502
Forward primer	1 µl (0.5 µM)	-
Reverse primer	1 µl (0.5 µM)	-
SYBR Green I	0.4 µl (1x)	SensiMix dT kit, Quantace QT6T3

Final reaction volume (including 2 µl cDNA template) was 20 µl.

A Rotor-Gene™ 6000 was used to carry out the PCR reactions. The reaction conditions were as follows: 10 min at 95°C to activate the polymerase prior to 40 cycles of 5 sec at 95°C to denature the DNA, 15 sec at primer annealing temperature (see Table 2) and 15 sec at 72°C for elongation. Fluorescence was measured at the end of the elongation step (at 72°C) in every cycle for TNFα and IL-6. However, for IL-8, fluorescence was measured in every cycle at an elevated step (77°C) at the end of the elongation phase. This was to "melt" non-specific

amplified DNA so that fluorescence measurements were from specific amplification of the gene of interest. These conditions were optimised in previous studies (Burns et al., 2008). Data acquisition and analyses were carried out using Rotor-Gene™ 6000 software version 1.7.

4.2.7 Comparison of cytokine responses of PBMC to mAbs other than TGN1412 added as aqueous solutions or immobilised by air-drying or wet-coating onto polypropylene and polystyrene culture plates

Clinical grade mAbs Tysabri, Avastin, Herceptin, Campath-1H and an IgG4κ negative control (described in section 2.6) were compared for their capabilities to stimulate the release of TNFα using protocols described in section 4.2.1 for TGN1412.

4.3 Results

4.3.1 TGN1412 immobilised by air-drying stimulates the largest cytokine responses from PBMC

Figure 14 shows a comparison of 6 different methods of applying TGN1412 on its capability to stimulate TNFα, IL-6 and IL-8 responses from PBMC when cultured with these cells for 16 - 24 h. The methods were: immobilising TGN1412 by air-drying onto polypropylene, untreated 96-well U-bottomed microtitre plates and polystyrene, tissue culture treated, 96-well U-bottomed microtitre plates, immobilising TGN1412 by wet-coating onto polypropylene and polystyrene plates and adding TGN1412 as an aqueous solution to polypropylene and polystyrene plates.

TNFα, IL-6 and IL-8 responses of PBMC to TGN1412 added in aqueous phase to polypropylene and polystyrene plates were very small, with little difference from responses to the isotype-matched negative control added in the same manner. As a consequence of transferring cell-conditioned medium to the ELISA plates for assay of cytokines, aqueous TGN1412 (unlike immobilised TGN1412) would also have been transferred. However, there

was no detectable difference between the values for absorbance for TNFα, IL-6 and IL-8 cytokine standard curves prepared in the absence and presence of aqueous TGN1412 (1000 µg/ml, data not shown). This showed that aqueous TGN1412 was not inhibiting the detection of cytokines by ELISA.

TGN1412 immobilised by air-drying onto the walls of wells of both polypropylene and polystyrene microtitre plates stimulated the release of TNFα, IL-6 and IL-8 to concentrations significantly above those evoked by the isotype-matched negative control ($p<0.01$ for all three cytokines when comparing responses to TGN1412 with responses to the isotype-matched negative control on the same plate, Wilcoxon's paired signed rank test). Absolute values for the three cytokines were substantial for TGN1412 air-dried onto polypropylene and polystyrene plates with the exception of small IL-6 responses to TGN1412 immobilised by air-drying onto polystyrene plates. Cytokine responses to air-dried TGN1412 showed a bell-shaped dose-response curve with 10 µg/well TGN1412 giving the largest response: 4067 ± 722 pg/ml TNFα, 1338 ± 449 pg/ml IL-6 and 63,650 ± 10,561 pg/ml IL-8 for TGN1412 air-dried onto polypropylene plates and 1671 ± 343 pg/ml TNFα, 109 ± 22 pg/ml IL-6 and 30,040 ± 7178 pg/ml IL-8 when TGN1412 was air-dried onto polystyrene plates, values are means ± S.E.M of 10 donors, from 3 independent assays.

Absolute TNFα, IL-6 and IL-8 responses were much smaller to TGN1412 wet-coated onto polypropylene plates compared with responses to TGN1412 air-dried onto polypropylene plates ($p<0.05$ when comparing responses for TNFα, IL-6 and IL-8, where TGN1412 = 10 µg/well, paired t test). Responses were also smaller to TGN1412 wet-coated onto polystyrene plates compared with responses to TGN1412 air-dried onto polystyrene plates ($p<0.05$ when comparing responses for TNFα, IL-6 and IL-8, where TGN1412 = 10 µg/well, paired t test). The values were as follows: 1576 ± 349 pg/ml TNFα, 286 ± 121 pg/ml IL-6 and 29,346 ± 4452 pg/ml IL-8 for TGN1412 wet-coated onto polypropylene plates at 10 µg/well and 343 ± 160 pg/ml TNFα, 38 ± 16 pg/ml IL-6 and 6254 ± 2322 pg/ml IL-8 for TGN1412 wet-coated onto polystyrene plates at 10 µg/well. Values are means ± S.E.M of 10 donors, from 3 independent assays. Absolute values for all three cytokines were smallest when TGN1412 was immobilised by wet-coating onto polystyrene plates.

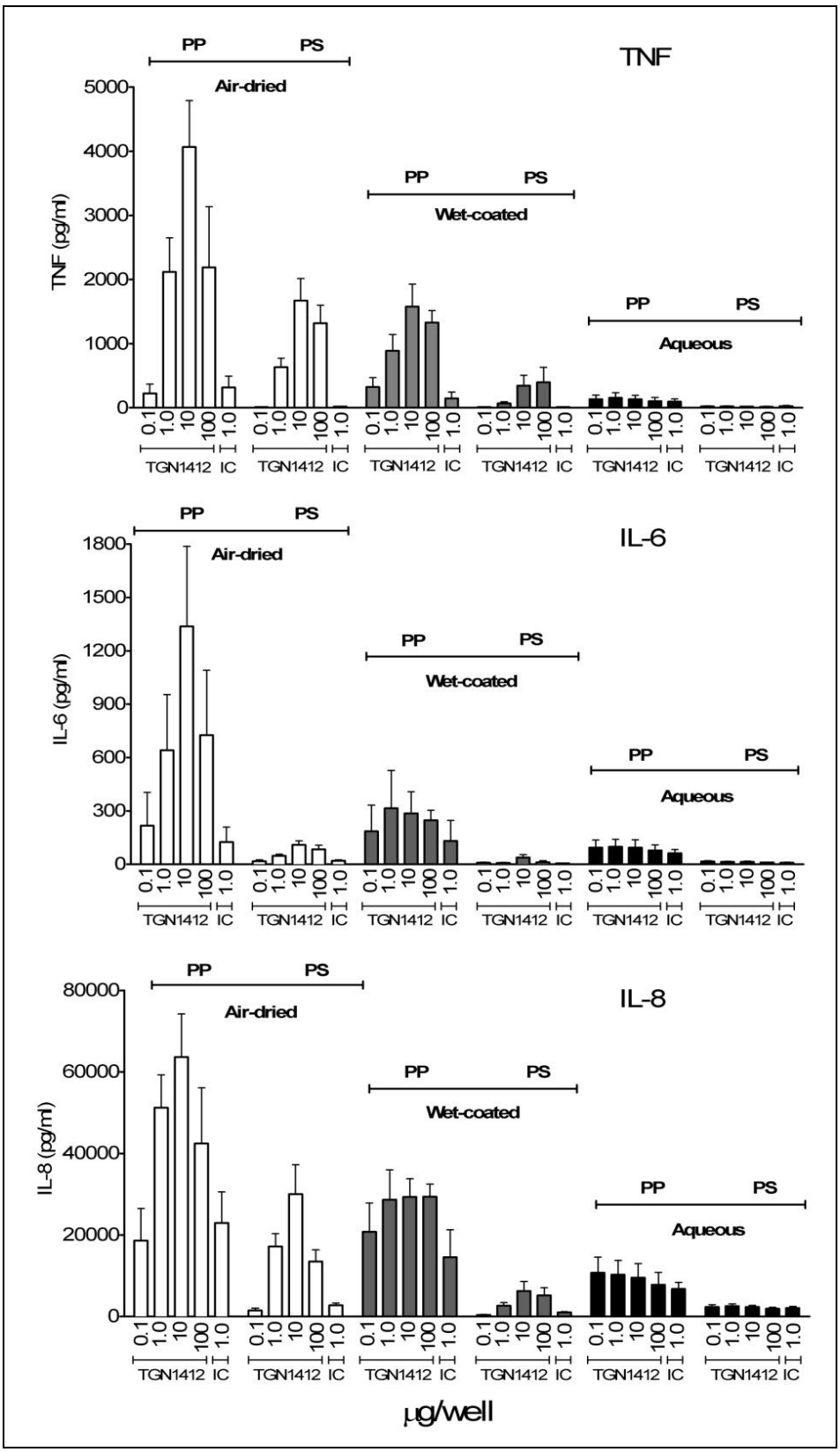


Figure 14. Cytokine responses of PBMC to TGN1412 either immobilised onto wells of microtitre plates or added as an aqueous solution. PBMC were cultured for 16 – 24 h with TGN1412 or the isotype-matched negative control (IC), which had either been immobilised by air-drying or wet-coating, at the doses shown, onto wells of polystyrene (PS) or polypropylene (PP) 96-well microtitre plates or that had been added as an aqueous solution. The values (pg/ml) for TNFα, IL-6 and IL-8 are means ± S.E.M of 6 - 10 donors of PBMC from 3 independent assays.

Although caution must be exercised when comparing *in vivo* and *in vitro* cytokine concentrations, Table 4 shows that the concentrations of TNFα and IL-8 stimulated by TGN1412 immobilised onto polypropylene plates (i.e. the method that stimulated the largest cytokine responses) were broadly similar to the concentrations measured in the blood of the TGN1412 trial volunteers post-infusion. In contrast, levels of IL-6 stimulated by immobilised TGN1412 were less than half of the concentrations measured in the blood of the trial volunteers.

Table 4. Comparison of peak cytokine responses (pg/ml) to TGN1412 *in vivo* (Suntharalingam et al., 2006) with those of PBMC to 10 μg/well TGN1412 immobilised by air-drying onto polypropylene.

Cytokine	***In vivo* peak responses* (*medians for* 6 patients)**	**Largest PBMC responses to immobilised TGN1412*[1]**
TNFα	4676	4067 ± 722
IL-6	3400	1338 ± 449
IL-8	≥5000	63,650 ± 10,561

* Peak responses were at 4 h post infusion of TGN1412. *[1] Values are means and S.E.M from Figure 14.

4.3.2 TGN1412 immobilised by air-drying stimulates cytokine responses from whole blood

Figure 15 shows that TGN1412 immobilised by air-drying onto the walls of wells of both polypropylene and polystyrene microtitre plates incubated with 20% (v/v) whole blood for 16 - 24 h stimulated TNFα, IL-6 and IL-8 responses above those of the isotype-matched negative control ($p<0.05$ for all cytokines when comparing responses to TGN1412 with responses to the isotype-matched negative control for the same plate, Wilcoxon's paired signed rank test). These responses to TGN1412 were dose-dependent. The values for TGN1412 at 100 μg/well were as follows: 45 ± 9 pg/ml TNFα, 60 ± 26 pg/ml IL-6 and 1147 ± 387 pg/ml IL-8 for TGN1412 air-dried onto polypropylene plates and 34 ± 8 pg/ml TNFα, 20 ± 9 pg/ml IL-6 and 376 ± 108 pg/ml IL-8 for TGN1412 air-dried onto polystyrene plates (values are means ± S.E.M of 8 donors, from 2 independent assays). However, absolute cytokine responses of whole blood to TGN1412 were, in general, much smaller than those of PBMC.

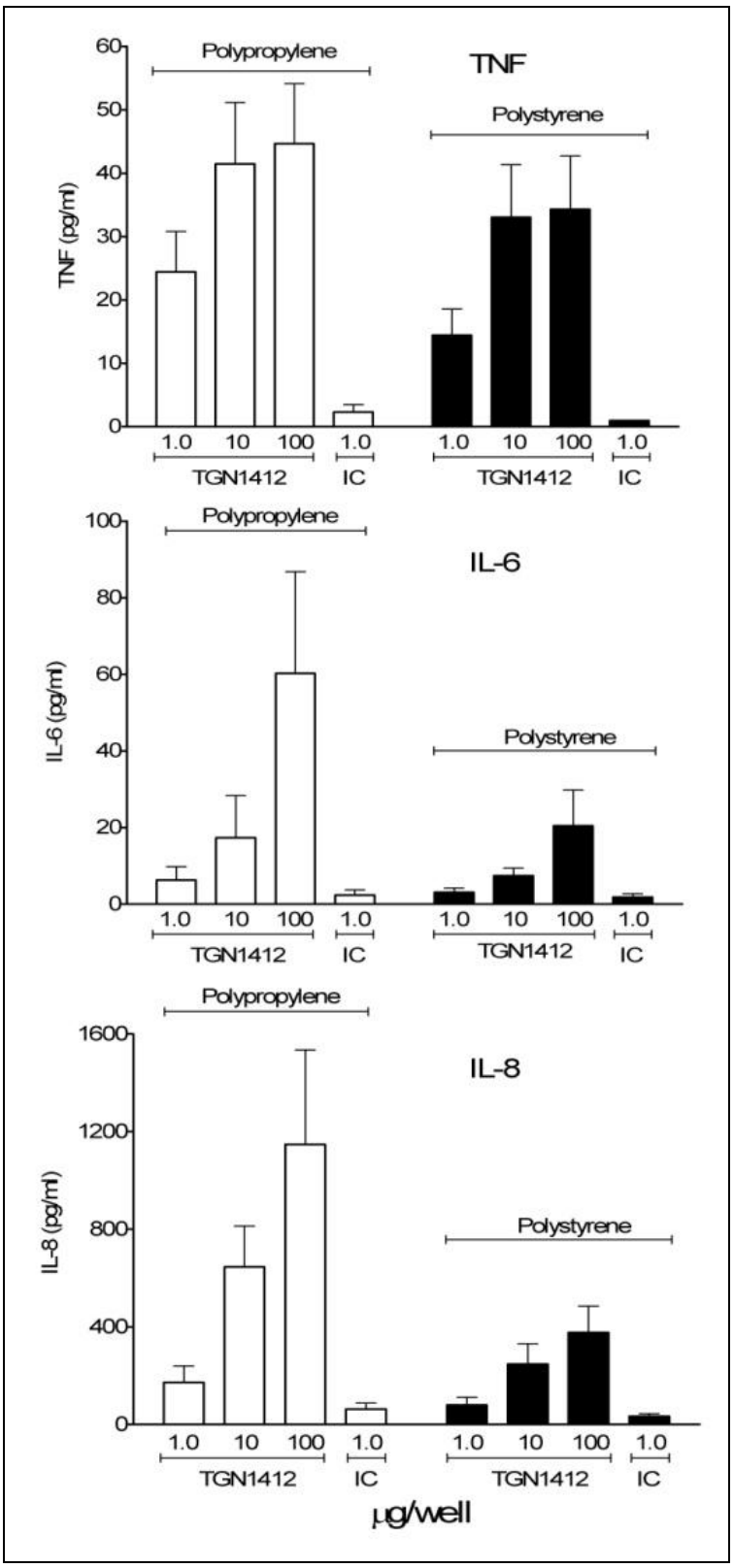


Figure 15. Cytokine responses of 20% (v/v) human whole blood to TGN1412 immobilised by coating using the air-drying method onto either polypropylene or polystyrene microtitre plates. Diluted whole blood was incubated for 16 – 24 h with TGN1412 or the isotype-matched negative control (IC) that had been coated by air-drying onto wells of either polystyrene or polypropylene 96-well microtitre plates. The values (pg/ml) for TNFα, IL-6 and IL-8 are means ± S.E.M of 8 donors of blood from 2 independent assays.

4.3.3 Two different methods for air-drying TGN1412 onto polypropylene microtitre plates give very similar cytokine responses

Two methods of air-drying TGN1412 and isotype-matched negative control onto the walls of wells of polypropylene plates were compared. The first was to leave the TGN1412 solution in the wells of microtitre plates overnight in a laminar flow cabinet to allow the solution of antibody to evaporate slowly. The second was to use a hair-dryer to rapidly evaporate the solution. TGN1412 immobilised by either of these methods at 1, 10 and 100 µg/well stimulated both TNFα and IL-8 cytokine responses from PBMC above those to the isotype-matched negative control when cells were cultured with TGN1412 for 24 h a shown in Figure 16. Very little difference was found between the cytokine responses for the two methods (4360 ± 565 pg/ml TNFα and 44,531 ± 5756 pg/ml IL-8 for TGN1412 air-dried at 10 µg/well, overnight in a laminar flow cabinet versus 4427 ± 466 pg/ml TNFα and 45,113 ± 2193 pg/ml IL-8 for TGN1412 air-dried at 10 µg/well with the use of a hair-dryer, values are means and S.E.M of 4 donors, one experiment).

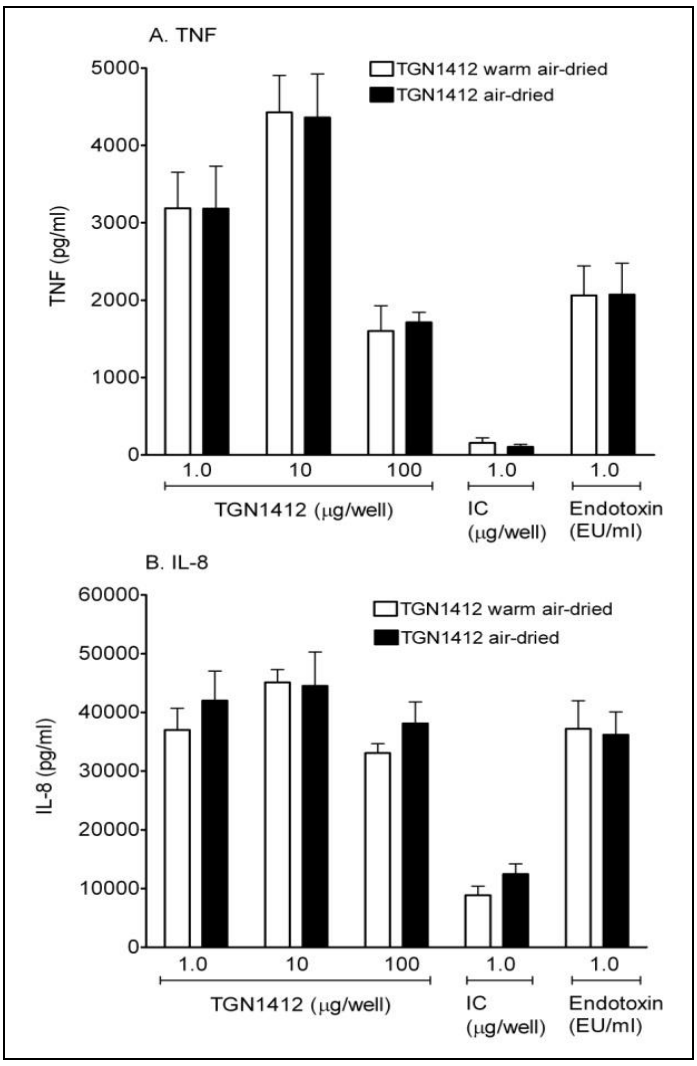


Figure 16. Cytokine responses of PBMC to TGN1412 air-dried onto walls of wells of microtitre plates by two different methods. TGN1412 and the IC were immobilised by coating onto the walls of wells of polypropylene 96-well microtitre plates either by blowing warm air across the wells (warm air-dried) or by leaving the solution to evaporate at room temperature (air-dried). PBMC were cultured for 16 – 24 h with the immobilised TGN1412 or the isotype-matched negative control (IC) at the doses shown. Endotoxin was used as a positive control and was added as an aqueous solution. The values (pg/ml) for TNFα (A) and IL-8 (B) are means ± S.E.M of 4 donors of PBMC, from 1 assay.

4.3.4 Different coating methods, using different types of microtitre plate, affect the quantity and quality of TGN1412 that remains immobilised

The total amount of TGN1412 immobilised onto polypropylene and polystyrene plates after coating using the wet-coating or air-drying methods (as described above) was measured by ELISA using an anti-human IgG antibody (gamma chain specific) conjugated to horseradish peroxidase. Figure 17A shows small differences between the resulting values for absorbance

when 10 µg/well TGN1412 (chosen because this was the optimum dose to stimulate the largest cytokine release from PBMC for a majority of the methods) was coated by air-drying or warm air-drying onto polypropylene and polystyrene plates and by wet-coating onto polypropylene plates. However, Figure 17A also shows much smaller resulting values for absorbance when TGN1412 was immobilised by wet-coating onto polystyrene plates compared with the other methods. These data suggest a 60% reduction in the total amount of immobilised TGN1412 when wet-coated onto polystyrene plates compared with the total amount immobilised when wet-coated onto polypropylene plates ($p<0.01$, t test).

To determine the effect of different coating methods on the quantity of TGN1412 immobilised and orientated so as to bind to CD28 via the variable region, i.e. "usefully bound", TGN1412 (10 µg/well) was coated onto the walls of wells of polypropylene and polystyrene plates using the various coating methods described in Figure 17. After washing, the bound TGN1412 was incubated with a CD28/IgG1 fusion protein. The quantity of plate-bound TGN1412 that was in turn able to bind CD28 was measured using a biotinylated anti-human IgG1 Fc specific antibody. Figure 17B shows little difference between the resulting values for absorbance when TGN1412 was coated by air-drying or warm air-drying onto polypropylene and polystyrene plates and by wet-coating onto polypropylene plates. In contrast, Figure 17B shows a much smaller resulting value for absorbance when TGN1412 was immobilised by wet-coating onto polystyrene plates. These data suggest a 90% reduction in the amount of "usefully bound" TGN1412 when wet-coated onto the wells of polystyrene plates compared with the amount "usefully bound" when wet-coated onto polypropylene plates ($p<0.01$, t test).

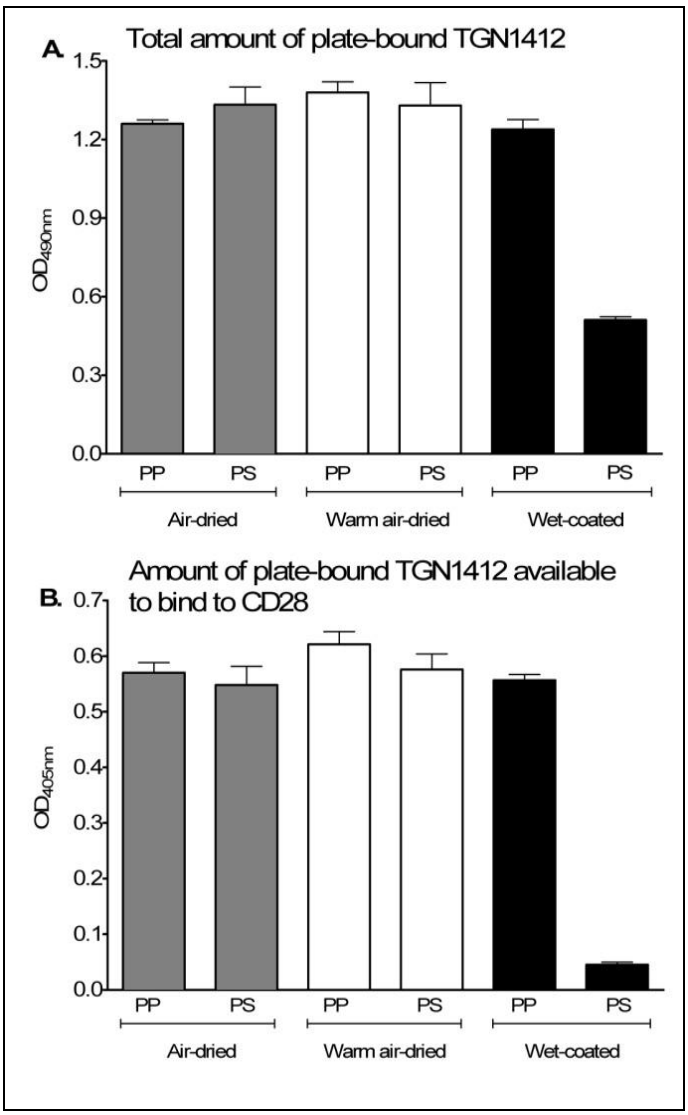


Figure 17. The effects of different coating methods on the amount and orientation of TGN1412 bound to wells of 96-well microtitre plates. TGN1412 was immobilised by coating at 10 µg/well either by air-drying, warm air-drying or wet-coating onto wells of polypropylene (PP) or polystyrene (PS) plates. Panel A shows the total amount of plate-bound TGN1412 for the different methods after washing, quantified using a horseradish peroxidase labelled anti-human IgG antibody (gamma chain specific). Panel B shows the amount of plate-bound TGN1412 (after washing), orientated such that CD28 (conjugated to IgG1 Fc) was able to bind. The amount of plate-bound TGN1412, which was able to bind the CD28 fusion protein was determined using a biotinylated anti-human IgG1 Fc antibody. Values are means ± S.E.M of 4 replicates from 1 assay and are representative of 2 independent assays. All values were baseline corrected as described in the Methods section.

The increase in the percentage reduction for wet-coating onto polystyrene from 60% for total TGN1412 bound to 90% for the amount "usefully bound" suggests that the reduction found in the "usefully bound" TGN1412 was not entirely the result of less total TGN1412 being bound but because wet-coating onto polystyrene did not favour the binding of TGN1412 in a

"useful" orientation. (The above percentages were calculated from the means of 4 replicates, shown in Figure 17, the values being representative of two independent experiments).

Experiments were carried out to determine the optimum concentrations of reagents used in the binding experiments described above. Using these optimised concentrations, it was possible to detect ten-fold dilutions of immobilised TGN1412 (air-dried onto polypropylene plates) ranging from 0.1 – 10 µg/well for both types of ELISA, suggesting that the lack of difference in the values for absorbance for the different coating methods was not due to the reagents being a limiting factor. The isotype-matched (to TGN1412) negative control was also coated onto plates at 1 µg/well, using the methods employed to coat TGN1412. Values for absorbance for the isotype-matched negative control were comparable to those for TGN1412 when using an anti-human IgG antibody specific for the gamma chain to quantify the total amount of antibody bound to the plates, suggesting that the control antibody had been immobilised. Therefore, the lack of responses of PBMC and whole blood to the isotype-matched negative control, as shown in Figure 14 and Figure 15 were not a consequence of this control antibody not binding to the plates. However, when the coated isotype-matched negative control was incubated with the CD28 fusion protein, the values for absorbance were not above baseline values for all coating methods, suggesting that the CD28 fusion protein bound only to the variable region of TGN1412 and not to the regions constant of all IgG4 antibodies. Consequently, only plate-bound TGN1412 orientated such that the CD28 fusion protein was able to bind to it, i.e. "usefully bound", was detected in such ELISAs.

4.3.5 Aqueous TGN1412 inhibits cytokine responses of PBMC to immobilised TGN1412

The TNFα response of PBMC incubated with TGN1412 immobilised by air-drying onto polypropylene plates at 10 µg/well for 24 h was reduced upon the addition of TGN1412 in aqueous phase as shown in Figure 18. The extent of inhibition of the response increased in a dose-dependent manner as the concentration of aqueous TGN1412 added increased (values ranged from 8070 ± 867 pg/ml TNFα where no aqueous TGN1412 was added, to 3542 ± 281 pg/ml where 100 µg/well aqueous TGN1412 was added, values are means and S.E.M of 8

donors of PBMC, from 2 independent experiments). All responses to immobilised TGN1412 were larger (including those where aqueous TGN1412 had been added) than responses to the isotype-matched negative control ($p<0.01$, Wilcoxon's signed rank test). The TNFα responses of PBMC incubated with TGN1412 immobilised by air-drying onto polypropylene plates at 10 µg/well for 24 h was not reduced upon the addition of an isotype-matched negative control in aqueous phase at the same concentrations used for the aqueous TGN1412 (values ranged from 2977 ± 613 pg/ml TNFα where no aqueous isotype-matched control was added to 3311 ± 1093 pg/ml TNFα where 100 µg/well aqueous isotype-matched control was added, values are means and S.E.M of 7 donors from 2 independent experiments).

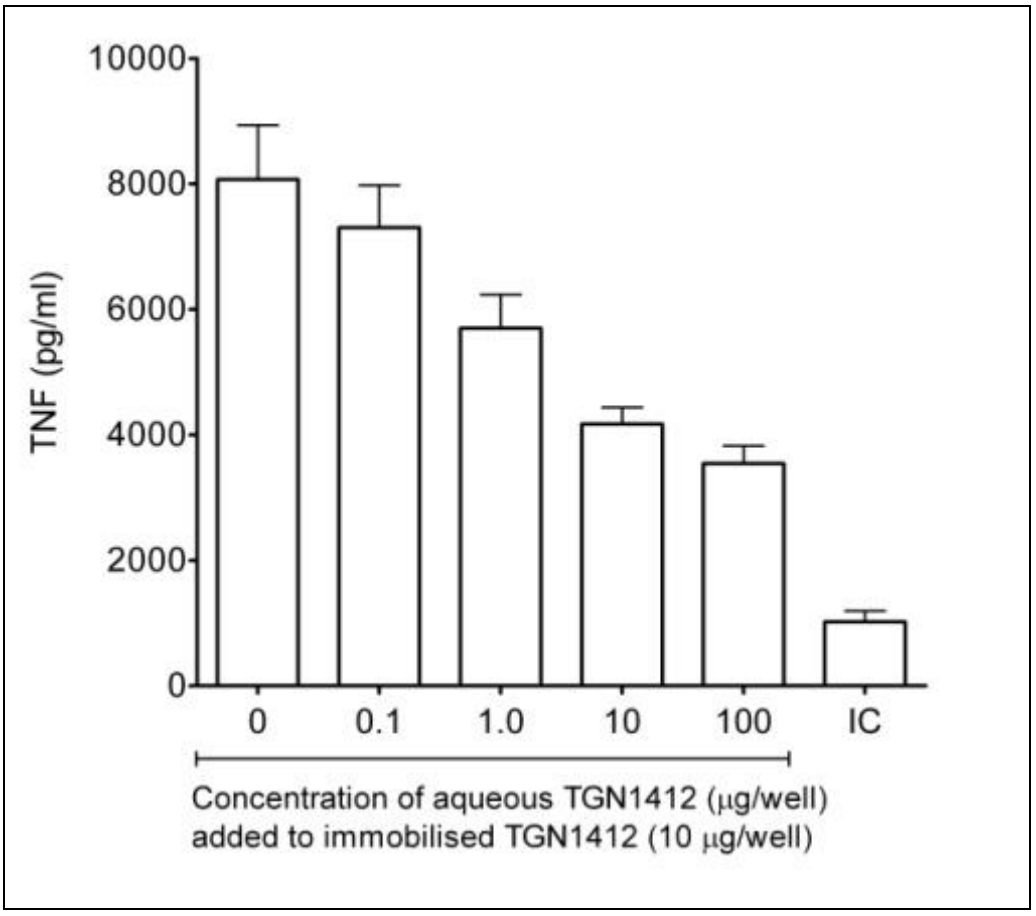


Figure 18. Inhibition of cytokine responses of PBMC to immobilised TGN1412, caused by aqueous TGN1412. PBMC were cultured for 16 – 24 h with TGN1412 (10 µg/well), immobilised by coating (using the air-drying method), onto wells of polypropylene plates on its own or in the presence of a range of concentrations (as shown) of aqueous TGN1412. PBMC were also cultured with an isotype-matched negative control (IC) immobilised as for TGN1412 but at 1 µg/well, in the absence of aqueous TGN1412. The values (pg/ml) for TNFα are means ± S.E.M of 8 donors of PBMC, from 2 independent assays.

4.3.6 Early TNFα, IL-6 and IL-8 gene expression and cytokine responses of PBMC to immobilised TGN1412 measured by quantitative RT-PCR and by ELISA respectively

The purpose for measuring early cytokine responses to immobilised TGN1412 was to investigate the kinetics of cytokine release stimulated by immobilised TGN1412 and to determine whether or not TGN1412 could have stimulated *de novo* cytokine synthesis within one hour post-infusion. PBMC were incubated with immobilised (using the air-drying method) TGN1412 or an isotype-matched negative control antibody at 1 μg/well in wells of polypropylene plates for either 30, 60 or 90 min.

4.3.6.1 Early TNFα, IL-6 and IL-8 gene expression

TNFα, IL-6 and IL-8 gene expression in PBMC was measured by quantitative RT-PCR analysis, the results of which are shown in Figure 19A. Gene expression was measured in terms of the ratio between normalised mRNA levels synthesised in response to TGN1412 and those to the isotype-matched negative control and values are means ± S.E.M of 6 donors of PBMC from 3 independent experiments. TNFα gene expression by PBMC was elevated after just 30 min incubation with immobilised TGN1412. Levels were approximately 2.5x higher than those measured in response to the isotype-matched negative control. After 60 min, TNFα gene expression stimulated by TGN1412 further increased and was approximately 5.5x higher than that stimulated by the isotype-matched control. The elevated level of mRNA production was sustained at 90 min but no further increase was detected. In contrast to TNFα, TGN1412-stimulated IL-6 and IL-8 gene expression by PBMC did not increase above that stimulated by the isotype control at 30, 60 and 90 min incubations.

Cytokine-specific primers were used in PCR reactions, however melt curve analyses were carried out on all quantitative PCR amplified products to confirm amplification of a single gene product within a reaction. Figure 20 shows typical melting profiles for TNFα- IL-6-, IL-8- or

GAPDH specific primers. The PCR product amplified using TNFα-specific primers in samples from TGN1412-stimulated cells and from the isotype-matched negative control-stimulated cells had the same melting profiles as the product amplified by the same primers in a sample containing TNFα standard suggesting that the PCR products amplified were all identical and that the product was TNFα. This was also the case in PCR reactions using IL-6-, IL-8- and GAPDH-specific primers and standards. A "no template" (H_2O) control was included for each primer pair (i.e. specific for TNFα, IL-6-, IL-8- or GAPDH) to demonstrate the absence of contamination in the samples. Figure 20 shows peaks in the melting profile for the "no template" control, in particular for TNFα and IL-6. However these profiles are different from those of the test samples suggesting that the amplified product (which amplified at very high cycle numbers) in the negative control was not the same as that in test samples and is likely to have been the product resulting from primer sets binding to each other in the absence of template.

4.3.6.2 Early TNFα, IL-6 and IL-8 cytokine production

Cell-conditioned medium from the PBMC used for quantitative RT-PCR (described above) was assayed for TNFα, IL-6 and IL-8 in cytokine-specific ELISAs to determine cytokine concentrations produced by PBMCs in response to TGN1412, the results of which are shown in Figure 19B. Cytokine responses are expressed in terms of the ratio between cytokine concentrations produced in response to TGN1412 and those to the isotype-matched negative control and values are means ± S.E.M of 6 donors of PBMC from 3 independent experiments. The pattern of cytokine responses of PBMC stimulated with TGN1412 was broadly similar to the pattern of gene expression by PBMC stimulated with TGN1412. However, in contrast to gene expression, elevated concentrations of TNFα could not be detected in the cell-conditioned medium after a 30 min incubation of PBMC with TGN1412. Although mRNA synthesis precedes protein production, the TNFα ELISA may not have been sensitive enough to detect any small amounts of TNFα protein. Elevated concentrations of this cytokine, however, were

detected by ELISA after 60 min incubation with TGN1412, and TGN1412-stimulated responses were approximately 3x larger than those to the isotype-matched negative control. In contrast to TNFα gene expression, TNFα protein concentrations further increased after 90 min incubation and were approximately 5 times larger than responses to the isotype-control. IL-6 and IL-8 cytokine responses of PBMC stimulated by TGN1412, as with IL-6 and IL-8 gene expression, were not elevated above responses to the isotype control at 30, 60 or 90 minutes.

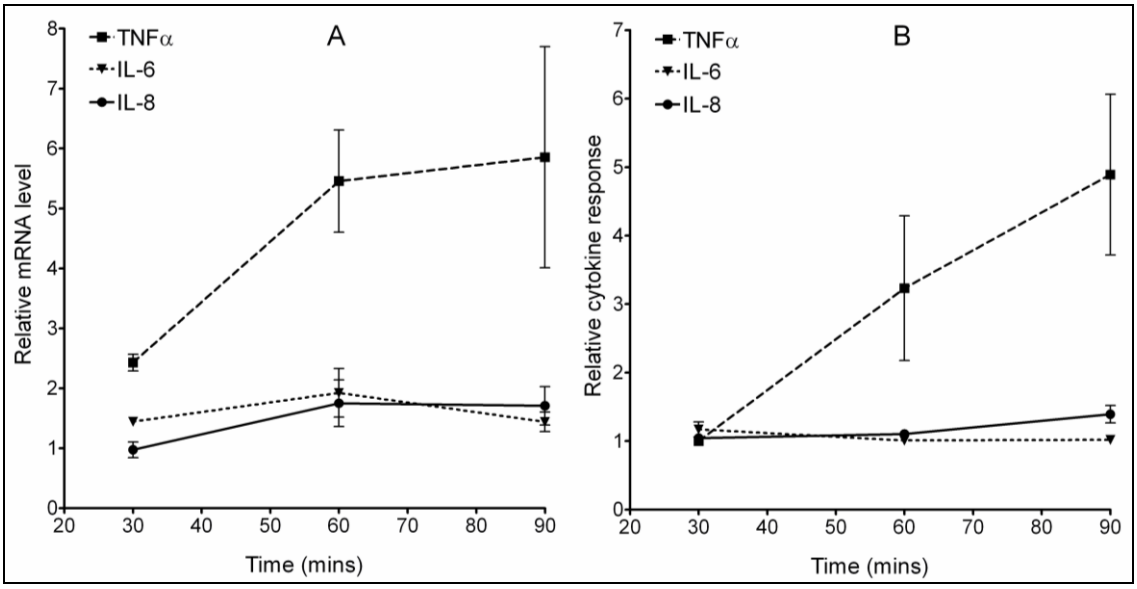


Figure 19. Early TNFα, IL-6 and IL-8 gene expression by PBMC (panel A) and cytokine responses (panel B) to immobilised TGN1412. PBMC were cultured for 30, 60 and 90 min with TGN1412 or an isotype-matched negative control at 1 µg/well, immobilised by coating (using the air-drying method), onto wells of polypropylene plates. Gene expression by PBMC was quantified by RT-PCR analysis and cytokine release into cell-conditioned media was measured by ELISA. Gene expression (panel A) is represented as the ratio of normalised mRNA levels synthesised in response to TGN1412 to those in response to the isotype-control. Cytokine responses are expressed in terms of the ratio of cytokine concentrations stimulated by TGN1412 to those stimulated by the isotype-matched negative control. Values plotted are means ± S.E.M of 6 donors from 3 independent experiments.

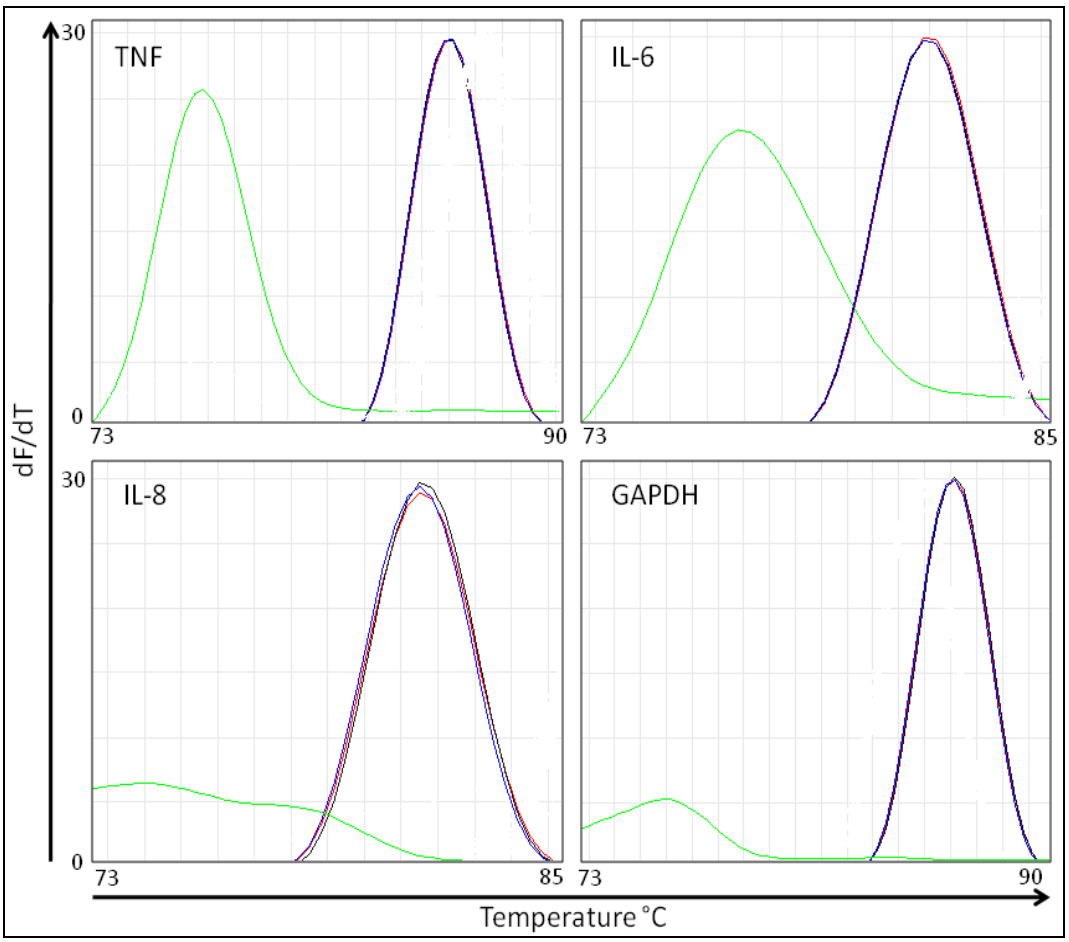


Figure 20. Typical TNFα, IL-6, IL-8 and GAPDH PCR product melting profiles from melt curve analyses. Green line: no template (negative) control; Red line: TNFα, IL-6, IL-8 and GAPDH standard; Black line: TGN1412-stimulated cells; Blue line: isotype-matched negative control-stimulated cells.

4.3.7 TNFα responses of PBMC to immobilised mAbs in addition to TGN1412

Figure 21 shows TNFα responses of PBMC when cultured for 24 h with therapeutic mAbs Tysabri, Herceptin, Campath-1H, TGN1412 and a negative control immobilised by either wet-coating or air-drying onto wells of polypropylene and polystyrene 96-well U-bottomed plates or added as an aqueous solution to wells of polypropylene and polystyrene 96-well U-bottomed plates. TNFα responses of PBMC to all mAbs added in aqueous phase to polypropylene and polystyrene plates were very small. As discussed in section 2.6 (see also Appendix II), none of the therapeutic mAbs tested inhibited the detection of TNFα by ELISA. As with TGN1412, TNFα responses of PBMC to Herceptin and Campath-1H immobilised by air-drying or wet-coating onto wells of polypropylene and polystyrene plates were larger when

compared with responses to these antibodies when they were added in aqueous phase. For the methods air-drying onto polypropylene and polystyrene and wet-coating onto polypropylene, TNFα responses of PBMC were largest for TGN1412 and smallest for Tysabri. The TNFα responses to Herceptin and Campath-1H, using these methods, were similar and were of a magnitude intermediate between responses to Tysabri and TGN1412.

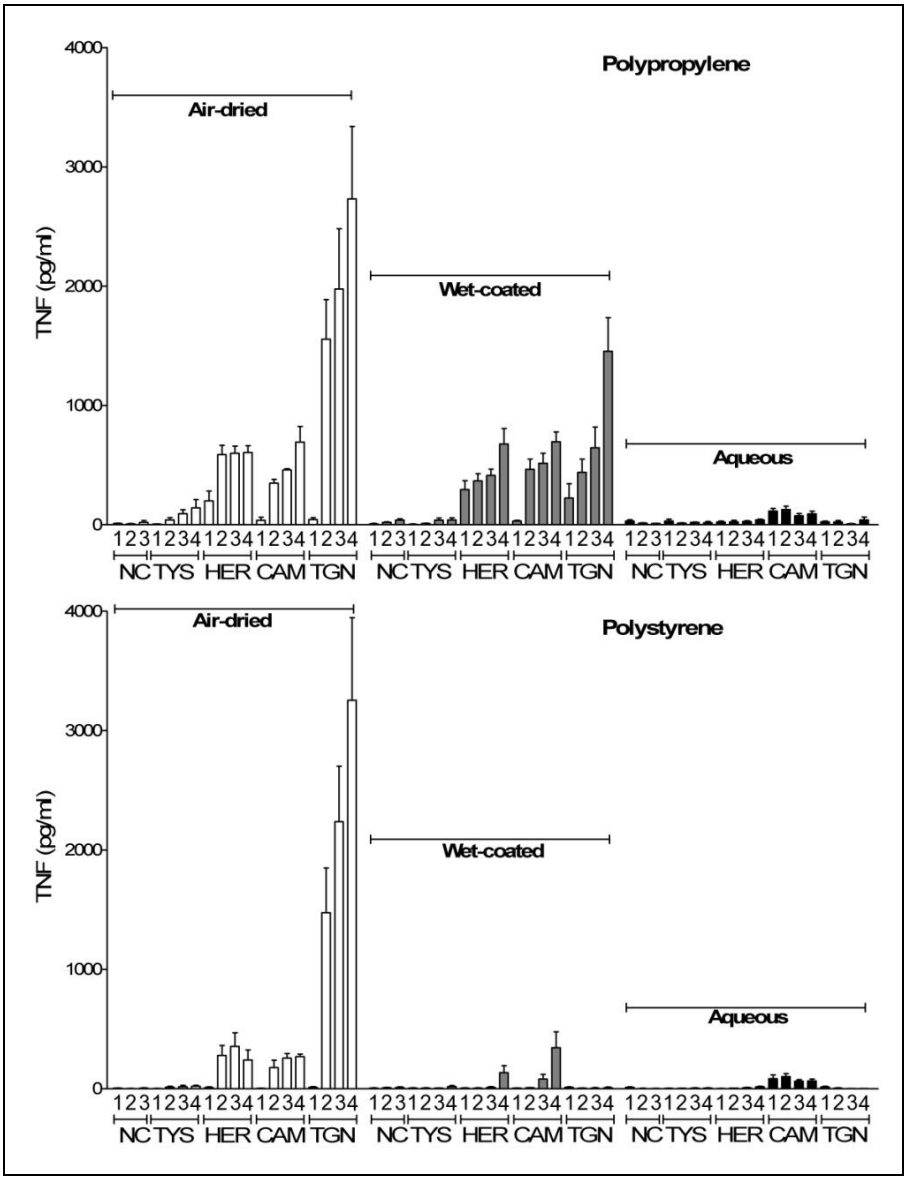


Figure 21. TNFα responses of PBMC to mAbs either immobilised onto wells of microtitre plates or added as an aqueous solution. PBMC were cultured for 24 h with mAbs Tysabri (TYS), Herceptin (HER), Campath-1H (CAM), TGN1412 (TGN) and a negative control (NC) that had either been immobilised by air-drying or wet-coating, at three or four different doses (1=0.1, 2=1.0, 3=10 and 4=100 µg/well) onto wells of polystyrene or polypropylene 96-well microtitre plates or that had been added as an aqueous solution. The values (pg/ml) for TNFα are means ± S.E.M of 8 donors of PBMC from 2 independent experiments.

Figure 22 shows TNFα responses of PBMC when cultured for 24 h with therapeutic mAbs Avastin, Herceptin, Campath-1H, TGN1412 and the negative control either immobilised by air-drying onto wells of polypropylene 96-well U-bottomed plates or added as an aqueous solution to wells of polypropylene 96-well U-bottomed plates. Responses were smallest for all four therapeutic mAbs when they were added in aqueous phase. Responses were increased when the therapeutic mAbs were air-dried onto plates. TNFα responses were the smallest to Avastin (114 ± 37 pg/ml) and the largest to TGN1412 (2929 ± 667 pg/ml). The TNFα responses to Herceptin and Campath-1H were similar (663 ± 301 pg/ml for Herceptin and 594 ± 176 pg/ml for Campath-1H) and were of a magnitude intermediate between responses to TGN1412 and Avastin. The values are means ± S.E.M of 4 donors of PBMC, values used to calculate the mean responses were from the doses of antibodies that gave the largest responses for each donor (10 – 100 µg/well).

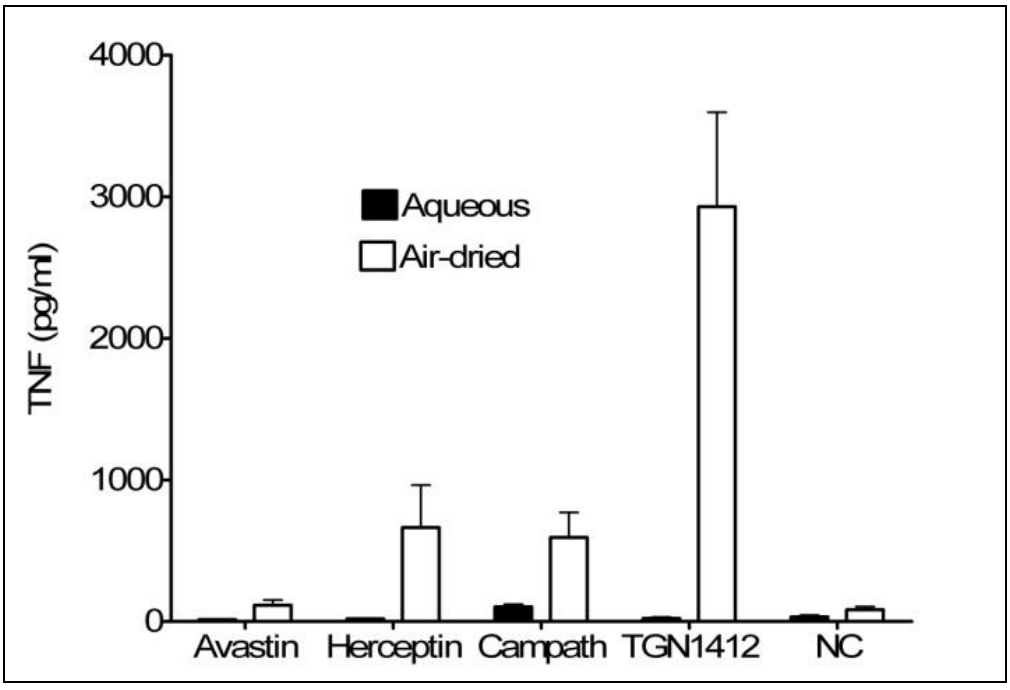


Figure 22. TNFα responses of PBMC to mAbs either immobilised onto wells of microtitre plates or added as an aqueous solution. PBMC were cultured for 24 h with mAbs Avastin, Herceptin, Campath-1H, TGN1412 and a negative control (NC) that had either been immobilised by air-drying onto wells of polypropylene 96-well microtitre plates or that had been added as an aqueous solution. The TNFα values used (pg/ml) to calculate the means in the figure were from the dose of antibody which gave the largest response for each donor of PBMC (10 – 100 µg/well). Values shown are means ± S.E.M of 4 donors of PBMC.

4.4 Discussion

The failure of pre-clinical testing to predict the adverse pro-inflammatory effects of TGN1412 emphasised the need for methods to better predict the clinical safety of such therapeutic antibodies. In this chapter, six different methods of applying TGN1412 to 96-well microtitre plates were investigated to determine the capability of these *in vitro* methods to predict the observed *in vivo* cytokine-releasing activity of TGN1412.

Cytokine responses of PBMC to TGN1412 added in aqueous phase to polypropylene and polystyrene plates were very small, being much the same as responses to the isotype-matched negative control added in the same manner (results consistent with published data from (Stebbings et al., 2007). In contrast, immobilised TGN1412 stimulated cytokine responses above those induced by the isotype-matched negative control. Cytokine responses stimulated by TGN1412 immobilised by air-drying onto polypropylene and polystyrene plates were much larger than responses to TGN1412 wet-coated onto polypropylene and polystyrene plates, respectively. The immobilisation method which gave the smallest absolute cytokine values in response to TGN1412 was when TGN1412 was immobilised by wet-coating onto polystyrene plates. PBMC were found to be more sensitive to TGN1412 than whole blood and elicited larger cytokine responses. This may have been because the isolation procedure somehow primed the PBMC or removed a negative influence or influences from the whole blood, or both. Alternatively, it may simply be because the immobilised TGN1412 is rapidly covered with the large excess of red blood cells in diluted whole blood rather than with the lymphocytes that express the CD28 receptor.

It is not known why, *in vitro*, aqueous TGN1412 failed to stimulate cytokine responses from PBMC but was able to do so when immobilised by coating onto the walls of wells of microtitre plates. What is clear, however, is that the immobilisation allowed TGN1412 to stimulate cytokine responses from PBMC similar to those observed *in vivo* in man (Suntharalingam et al., 2006). Through binding of the Fc portions of mAbs, Fc receptors expressed by, for example, monocytes can promote the clustering of target-bound mAbs leading to the activation of the target cell (Bugelski et al., 2009). It is possible that, *in vivo*, TGN1412

bound to CD28 receptors on T cells via its variable region and was then cross-linked in some manner, for example by Fc receptors. Such cross-linking of TGN1412 may have "presented" this antibody in a specific conformation that allowed linear complex formation with CD28 (as described in Figure 7) and subsequent superagonistic activation of T cells. It is possible that, *in vitro*, immobilising TGN1412, potentially with its Fc region bound to a plastic surface, cross-linked TGN1412 itself, allowing it to be presented to CD28 receptors in a manner that was, perhaps, analogous to the *in vivo* situation. However, one could ask why aqueous TGN1412 did not stimulate cytokine release by PBMC as Fc receptors are expressed by cells such as monocytes in the PBMC fraction. One explanation could be that immobilisation of TGN1412 onto microtitre plates may have presented this antibody to T cells such that it was able to bind to CD28 receptors within a concentrated area of the cell surface (i.e. polarised) and so cross-link CD28 above threshold levels required for T cell activation. Further support for this theory of polarised CD28 cross-linking came from the finding that the TNFα responses of PBMC to TGN1412 immobilised by air-drying onto polypropylene plates was inhibited in a dose-dependent manner by the addition of aqueous TGN1412. It is possible that the aqueous TGN1412 bound at random to CD28 receptors distributed over the entire T cell surface and so was effectively an antagonist of the plate-bound TGN1412. In such a situation the disruption of (polarised) binding and cross-linking of CD28 receptors by TGN1412 would have prevented the subsequent interaction of the relevant downstream signalling molecules in specific regions of the cell surface. Further evidence for the notion of polarised CD28 cross-linking came from the finding that when anti-human IgG4 Fc specific antibodies were incubated in solution with aqueous TGN1412 and PBMC, T cell activation did not occur but when the anti-human IgG4 Fc-specific antibodies were immobilised by air-drying and used to capture (and hence immobilise) aqueous TGN1412, profound T cell activation was evident, measured in terms of IL-2 production (Stebbings et al., 2007). What cannot be explained is exactly how this polarised cross-linking of CD28 receptors would have been achieved *in vivo*. It is possible that TGN1412 was immobilised in some manner by molecules on the surface of other cells such as endothelial cells (i.e. cells not found within a PBMC fraction).

It is not obvious why air-dried TGN1412 stimulated larger cytokine responses than wet-coated TGN1412 or why responses to TGN1412 immobilised by wet-coating onto polystyrene were smaller. A reasonable hypothesis is that the different methods affected either the quality or quantity, or both, of immobilised TGN1412. In this context, quality means orientated with the CD28-binding domain more readily available to bind CD28 expressed on T cells. No differences were found in the total amount of TGN1412 bound to the plates when coated by air-drying or warm air-drying onto polypropylene and polystyrene plates or by wet-coating onto polypropylene plates; nor was it found that these different methods affected the orientation of TGN1412 bound to the plates. What was found, however, was that less TGN1412 remained bound to polystyrene plates when immobilised by wet-coating, compared with the other methods. In addition, wet-coating to polystyrene favoured TGN1412 being immobilised with an orientation such that the CD28-specific variable region was less available to bind to the CD28 fusion protein used in these experiments. This finding may be responsible for the smaller cytokine responses of PBMC to TGN1412 immobilised by wet-coating onto polystyrene plates.

During the TGN1412 clinical trial, volunteers presented with pain approximately 1 h post-infusion. TNFα was the first pro-inflammatory cytokine to be elevated in the serum approximately 1 h post-infusion and this was followed by IL-8 and IL-6 within 4 h post-infusion (Suntharalingam et al., 2006). To compare the kinetics of cytokine responses of PBMC to immobilised TGN1412 with the kinetics of responses observed in the TGN1412 trial volunteers, RT-PCR was used to measure gene expression by PBMC incubated with TGN1412 for 30, 60 or 90 minutes. Elevated levels of TNFα gene expression could be measured after a 30 min stimulation, and these increased further after 60 min. This was consistent with the *in vivo* situation although measurements of serum cytokine concentrations were not carried out 30 min post-infusion. Furthermore, TNFα concentrations measured by ELISA in the cell-conditioned medium from TGN1412-stimulated cells were also elevated after a 60 min incubation. These data seem to suggest that the kinetics for TNFα responses were similar *in vivo* and *in vitro*. Elevated IL-8 and IL-6 responses of PBMC to immobilised TGN1412 could not be measured in terms of gene expression (by RT-PCR) or protein production (by ELISA) at 30, 60 or 90 min post stimulation with TGN1412. This is consistent with the *in vivo* situation in that IL-8 and IL-

6 responses followed TNFα responses but the literature states "within 4 h post-infusion", thus longer *in vitro* incubations would have been required to further investigate the *in vitro* model. It is conceivable from these data that cells bearing CD28 receptors and which are directly stimulated by TGN1412 produce TNFα which subsequently stimulates other cells bearing TNFα receptors (CD120-positive cells), such as monocytes, to release IL-6 and IL-8. Measurement of gene expression by RT-PCR was also used to determine whether or not TNFα in the serum of the volunteers was likely to have been pre-formed prior to its (TGN1412-stimulated) release from cells as opposed to TGN1412 activating TNFα gene expression and synthesis, since elevated concentrations of TNFα occurred so soon (within 60 min) after infusion of TGN1412 (Suntharalingam et al., 2006). The finding in this study that TNFα gene expression was elevated in TGN1412-stimulated cells suggests that serum concentrations of this cytokine in the trial volunteers derived, at least in part, from TNFα synthesised *de novo* rather than from pre-formed intracellular stores.

Although *in vitro* and *in vivo* comparisons should be made with caution, the concentrations of TNFα and IL-8 stimulated by TGN1412 immobilised onto polypropylene plates were broadly similar to the concentrations measured in the blood of the trial volunteers. This further validates this assay as an *in vitro* model for predicting *in vivo* responses to TGN1412. However, the concentrations of IL-6 stimulated by immobilised TGN1412 were much lower than concentrations measured in the blood of the trial volunteers. It is conceivable that, *in vivo*, cells not included in the PBMC fraction of blood were responsible, at least in part, for TGN1412-stimulated (directly or indirectly) IL-6 production, for example, endothelial cells.

In view of the success of the new procedures with TGN1412, the next step was to determine whether or not any of the procedures used to immobilise TGN1412 were better than simply adding aqueous mAb at predicting unwanted pro-inflammatory activities of mAbs other than TGN1412. The mAbs Herceptin, an IgG1 anti-Her2, and Campath-1H, an IgG1 anti-CD52, were tested because they are antibodies that are frequently associated with pro-inflammatory clinical infusion reactions (Chung, 2008). Tysabri, an IgG4κ anti-α4 integrin was also tested because it is an antibody only rarely associated with clinical infusion reactions. TNFα responses

of PBMC to these antibodies were very small when the mAbs were added in aqueous phase as was observed with TGN1412. However, larger responses were evoked when these antibodies were immobilised. When air-dried onto polypropylene and polystyrene and wet-coated onto polypropylene, the capability of the mAbs to stimulate TNFα responses of PBMC could be ranked as follows: Tysabri < Herceptin = Campath-1H < TGN1412 (based on absolute values of TNFα released by PBMC). Thus, it was only when the antibodies were air-dried onto polypropylene or polystyrene or wet-coated onto polypropylene (but not when wet-coated onto polystyrene), that all three antibodies associated with clinical infusion reactions, stimulated TNFα responses. The mAb Tysabri, which is only rarely associated with clinical infusion reactions, stimulated small TNFα responses in the above experiments. Like Tysabri, the mAb Avastin (an IgG1 anti-VEGF) is rarely associated with clinical infusion reactions (Chung, 2008). Avastin stimulated smaller TNFα responses when air-dried onto polypropylene than the mAbs more frequently associated with clinical infusion reactions: Herceptin, Campath-1H and TGN1412. The results from the above experiments with air-drying therefore broadly mirrored reported clinical infusion reactions to these mAbs.

The present study suggests that antibody immobilisation by air-drying onto plastic increases the likelihood of predicting whether or not antibodies other than TGN1412 are likely to cause clinical infusion reactions, suggesting that this technique is applicable to antibodies which target receptors other than CD28. It was conceivable that this method may be specific for IgG4 mAbs and that IgG1 molecules may elicit cytokine responses when immobilised regardless of their capability to cause clinical infusion reactions, as a result of their being more biologically active than IgG4κ molecules. This study does not support this notion since the IgG1 mAb Avastin, only rarely associated with clinical infusion reactions, stimulated only small TNFα responses of PBMC. Consequently, the larger responses stimulated by the immobilised IgG1 molecules Herceptin and Campath-1H (which are frequently associated with clinical infusion reactions) are unlikely to have resulted only from these mAbs being of the IgG1 subclass.

Of course, caution must be exercised when drawing conclusions from investigations with just five therapeutic mAbs. Firstly, it may not be necessary to immobilise an antibody in order for it to stimulate cytokine responses. Campath-1H was able to stimulate small TNFα responses

when incubated in aqueous phase in this study. This is consistent with the findings of Wing et al. (1995) who incubated Campath-1H in aqueous phase with ex-vivo human whole blood cultures (Wing et al., 1995). Campath-1H was able to stimulate TNFα responses when incubated in aqueous phase with ex-vivo human whole blood cultures (Wing et al., 1995). Secondly, it should not be assumed that an antibody immobilised by air-drying that stimulates pro-inflammatory cytokine responses of PBMC would automatically do so in man. In contrast, an antibody that fails to stimulate pro-inflammatory cytokine responses of PBMC – even when it is immobilised by air-drying – would appear to be unlikely to do so *in vivo* in man. Nonetheless, all antibodies should continue to be considered on a case by case basis with the testing of immobilised antibody now considered as well as the usual testing in aqueous phase. Caution must also be exercised when drawing conclusions from comparisons of just one type of polystyrene plate from a single vendor versus just one type of polypropylene plate from a single vendor. It is possible that the plate properties may vary from one vendor to another and from one batch to another which again could influence the outcome of an experiment. Indeed the dose of TGN1412 which stimulated the largest cytokine responses in this study shifted from 10 µg/well (Figure 14) to 100 µg/well (Figure 21) which may be a result of the use of different plate batches. Polystyrene plates used in the above experiments, unlike the polypropylene plates, were "tissue culture treated" and so exposed to vacuum gas plasma treatment which leads to the incorporation of anionic functional groups and a hydrophilic surface. These properties promote cell adhesion and for this reason most "cell-culture grade" polystyrene plates are "tissue culture treated". A supplier of non-treated U bottom polystyrene plates certified as sterile and pyrogen-free (which was essential in this study because pyrogens stimulate the release of pro-inflammatory cytokines) could not be identified. It is therefore conceivable that the lack of binding of TGN1412 to polystyrene plates used in this study could have been a result of the tissue culture treatment that they had received and not due to the fact that they were made of polystyrene. This chapter is not intended to be an exhaustive survey of the many different brands of 96-well plates on the market or, indeed, of all immunomodulatory therapeutic mAbs. Rather, it is a study with the objective of developing/improving *in vitro* methods to allow better detection and quantification of unwanted pro-inflammatory activities of mAbs. That said,

immobilisation of mAb onto plastic using the methods described in this chapter bears little resemblance of the *in vivo* situation, i.e. plastic surfaces are not physiological and TGN1412 and other mAbs are infused in aqueous phase. A more physiological method capable of predicting the pro-inflammatory effects of therapeutic antibodies in man may give a better insight to the underlying physiological mechanisms that mediate such responses *in vivo*.

CHAPTER 5:
The development of a method that can predict the clinical effects of TGN1412 in aqueous phase

CHAPTER 5: The development of a method that can predict the clinical effects of TGN1412 in aqueous phase

5.1 Introduction

The aim of the work described in this chapter was to develop a procedure that was able to predict the clinical effects of TGN1412 in aqueous phase, i.e. a procedure that was more physiologically relevant than immobilising the antibody onto plastic. It has been shown so far in this study that aqueous TGN1412 does not stimulate profound cytokine release (as it did *in vivo*) when incubated with PBMC alone. This is not so surprising when one considers that many different cell types are involved in mediating immunological responses *in vivo*. Although it would be impossible to create an *in vitro* environment truly representative of the *in vivo* situation, the inclusion of at least one more relevant cell type (in addition to those of PBMC) into an *in vitro* test to create a co-culture was considered to be logical and practicable.

TGN1412 infused into the blood of the trial volunteers could have activated target T cells in the blood, within the lymph nodes (where large populations of T cells reside) and within tissues. Not only do endothelial cells form the lining of large vascular blood vessels, they also form microvessels such as arterioles and venules, the main site of leukocyte migration from blood into tissues. They also form the high endothelial venules which facilitate the movement of lymphocytes from the blood into lymph nodes. It is therefore almost certain that TGN1412 would have activated T cells in the presence of endothelial cells. Endothelial cells play a pivotal role in mediating inflammatory responses (Pober and Sessa, 2007), and the responses of the clinical trial volunteers to TGN1412 could certainly be described as inflammatory. More specifically, endothelial cells express adhesion molecules on their surface which interact with ligands expressed by migrating leukocytes, resulting in their selective movement across the endothelial barrier into tissue (Muller, 2002). In fact, adhesion molecules expressed by endothelial cells regulate the circulation of lymphocytes through tissues by acting as tissue specific “homing receptors” on endothelial cells to which specific lymphocyte subsets adhere (Hamann et al., 1994). Since lymphopenia in the peripheral blood of the trial volunteers

followed soon after TGN1412 was infused (Suntharalingam et al., 2006), it was assumed that lymphocytes would have migrated across endothelium (although it is not certain whether or not lymphocytes migrated into tissues or to peripheral lymphoid organs). Therefore it was conceivable that lymphocyte/endothelial cell interaction may have contributed to the inflammatory responses to TGN1412. In this chapter, the hypothesis that PBMC co-cultured over a monolayer of endothelial cells (illustrated in Figure 23) in a cytokine release assay provides a means of detecting the toxic effects of TGN1412 in aqueous phase is tested. After establishing "proof of principle", efforts were made to optimise the method. The mechanisms underlying the contribution of endothelial cells to the inflammatory responses to TGN1412 are addressed in the following chapter (Chapter 6).

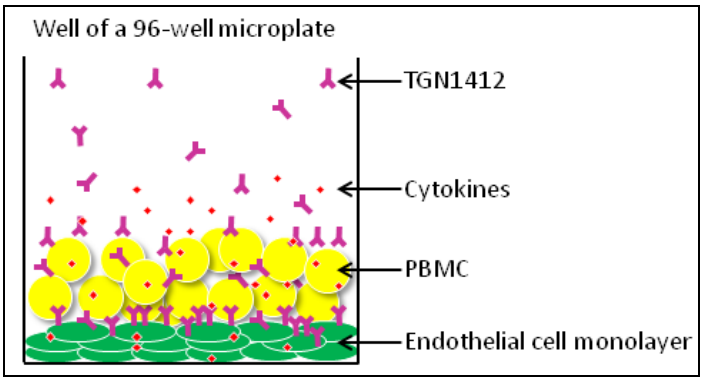


Figure 23. Co-culture: PBMC over a monolayer of endothelial cells.

5.2 Methods

Trypsin-EDTA was from Sigma (T4049) or Clonetics, Lonza (CC-5012). Human AB (hAB) serum was from Biosera (4190-100) and was pooled from a number of donors. Foetal calf serum (FCS) was either from Sera Laboratories International Ltd (EU-000FI) or was used from the endothelial growth medium kit (EGM-2; Clonetics Lonza CC-3162) described below. Heat inactivation of FCS (hi-FCS) or hAB serum (hi-hAB) at NIBSC was carried out by heating stocks to 56°C for 25 min. Heat-inactivation of hAB serum and FCS was to inactivate complement to eliminate any cytotoxic effects caused by these proteins. All cell incubations were in a humidified incubator at 37°C in 5% CO_2.

5.2.1 Isolation and culture of primary endothelial cells from umbilical vein of fresh human cords (C-HUVEC)

Umbilical cords, from which primary C-HUVEC were isolated, were obtained under the approval of the relevant ethics committees from consenting donors at the Queen Elizabeth II (QEII) Hospital, Welwyn Garden City, Herts and at Rochdale Infirmary, Rochdale, Lancs. Cords were collected in sterile jars containing 1x Hank's balanced salt solution (Sigma H1641) supplemented with 20 mM HEPES buffer (Sigma H0887), 0.08 % sodium-bicarbonate (Sigma S8761), 0.05 mg/ml gentamycin (Sigma G1272), 50 U/ml penicillin and 0.05 mg/ml streptomycin (stabilised solution; Sigma P4458). Umbilical cords were processed using sterile technique in a class II laminar flow cabinet on a dissecting board covered with aluminium foil, sterilised with 70% (w/w) ethanol. Scissors, forceps, luer adapters, cable ties and suture thread were pre-soaked in 70% ethanol. Cords were examined and both ends were trimmed with scissors. Those with puncture wounds were discarded. A luer adapter was inserted into each end of the umbilical vein and these were held securely in place with a cable tie and suture thread as shown in Figure 24. Using 10 ml syringes connected to each end of the umbilical vein via luer adapters, the umbilical veins were flushed aseptically several times with sterile PBS A. Collagenase (0.1% solution, Roche 10103586001) was injected into the umbilical vein prior to a 20 min incubation in a humidified incubator at 37°C in 5% CO_2. Cords were wrapped in cling film during this incubation. Endothelial cells were collected by massaging the cord and flushing the vein with warm sterile PBS A. The cell suspension was centrifuged at 400 g for 10 min at room temperature. The supernatant was removed prior to resuspension of the cells in 10 ml EGM-2 containing 10% FCS. Cells were transferred to 25 cm^2 flasks and incubated overnight to allow the cells to adhere to the flask. Following incubation, cells were washed twice with warm sterile PBS A prior to further incubation in 20% of the existing EGM-2 (centrifuged at 800 x g for 5 min) and fresh EGM-2 containing 10% FCS. C-HUVEC from different cords were cultured separately and, after 2 – 3 days, were maintained in EGM-2 containing 2% FCS.

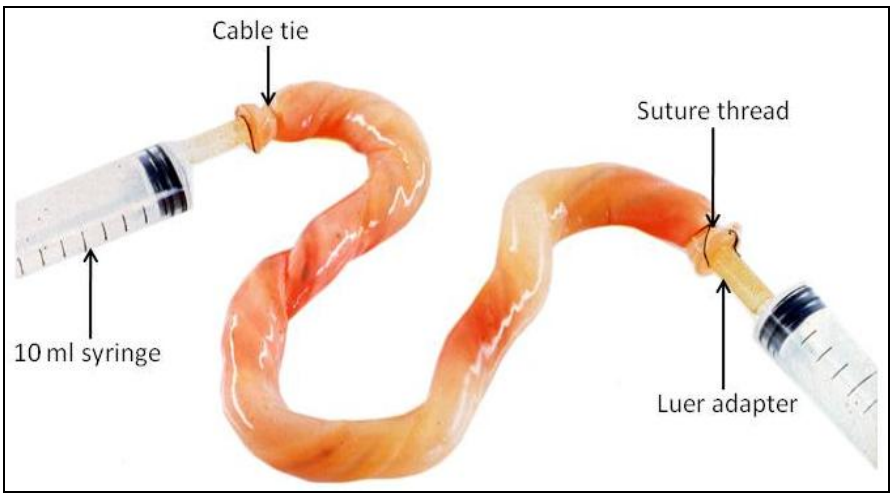


Figure 24. Isolation of endothelial cells from human umbilical vein. Image from NIBSC photo library.

To passage, cells were rinsed with 5 ml HEPES-buffered saline solution (Clonetics CC-5024, Lonza), prior to the addition of 5 ml trypsin-EDTA. Cells were examined under phase contrast at 100x magnification. When cells appeared "rounded" but still remained attached to the flasks, trypsin-EDTA was removed to leave 0.5 ml of fluid remaining and flasks were tapped on a hard surface several times to dislodge cells. Cells were resuspended in 10 ml EGM-2 containing 2% FCS and transferred to a separate tube. Cell counts were carried out and C-HUVEC were seeded at 0.8 x 10^6 cells per 75 cm^2 flask in 25 ml culture medium. C-HUVEC were used between passage 2 and passage 8 in the experiments described below.

5.2.2 Culture of human umbilical vein endothelium derived cells - HUVECjr2 and HUVECyn

HUVECjr2 and HUVECyn (pooled) were from TCS Biologicals (product code ZHC-2101, batch 23203T). These endothelial cells are from human umbilical vein and had not been immortalised. HUVECjr2 were purchased in 1999 and were cultured as described for C-HUVEC in EGM-2 medium containing 2% FCS to produce a single working cell bank expanded from the original stock (by Dr C.J Robinson, NIBSC). HUVECjr2 used in the experiments described in this study were from this single working cell bank and were cultured as for C-HUVEC. HUVECjr2 were used in experiments between passage 2 and passage 13.

HUVECyn were purchased in 2001. A single working cell bank was prepared (by Dr Y. Zhao, NIBSC) using cells expanded from the original source. In this study, HUVECyn from this single working cell bank were used at passages 3 and 4 and were maintained in EGM-2 containing 2% FCS. Prior to passage, new culture flasks (75 cm^2) were first coated with gelatin by adding 10 ml gelatin (Sigma G1393) mixed 1:1 with sterile PBS A prior to a 10 min incubation at room temperature. Gelatin was then removed and flasks were washed 2x with sterile PBS A. Cells were passaged as described for C-HUVEC but were washed with 10 ml sterile PBS A, trypsinised with 5 ml trypsin-EDTA diluted 1 in 5 with sterile PBS A and were seeded at 0.5 x 10^6 cells in each gelatin coated flask.

5.2.3 Culture of human saphenous vein endothelial cells (HSVEC)

HSVEC were a kind gift from Professor A. George (Imperial College, London). Endothelial cells isolated from human saphenous vein from one consenting donor, under a project approved by the relevant ethics committee, were cryogenically frozen at passage 2 at Imperial College London, Department of Medicine. Cells used in these experiments were from this single working cell bank and were used at passages 4 and 5. HSVEC were maintained in medium prepared as described in Table 5 (HSVEC culture medium) and were passaged as described for C-HUVEC; after removing the existing culture medium, cells were washed with 5 ml sterile PBS A and trypsinised with 5 ml trypsin-EDTA diluted 1 in 2 with sterile PBS A. Cells resuspended in fresh culture medium (5 - 10 ml/flask) were pooled, counted and seeded at 0.5 x 10^6 cells/flask in 20 ml HSVEC culture medium.

Table 5. Components of HSVEC culture medium (500 ml total volume)

Component	Final Concentration/Volume	Source
Endothelial Cell Serum Free Media	189 ml stock solution	Gibco 11111-044
Endothelial Cell Basal Medium (EBM)	189 ml stock solution	Lonza cc-3156
Penicillin, Streptomycin, Amphotericin B (PSA)	50 U/ml pen, 50 U/ml strep & 0.25 µg/ml amphotericin B (5 ml of PSA* aliquot)	Lonza DE17-603E (pen & strep solution), Sigma A2942 (amphotericin B)
L-Glutamine	2 mM (5 ml of 200 mM stock solution)	Gibco 25030-032
Endothelial Cell Growth Supplement (ECGS)	0.1 mg/ml (10 ml of 5 mg/ml ECGS* aliquot)	BD Biosciences 356006
Hi-FCS	20% v/v (100 ml stock serum)	NIBSC
Heparin	20 IU/ml (2 ml of 5000 IU/ml stock solution)	CP Pharmaceuticals Multiparin

*PSA: 10 ml amphotericin B stock solution added to 100 ml pen & strep stock solution; 5 ml aliquots stored below -20°C. *ECGS: reconstituted in 20 ml EBM to make 5 mg/ml solution; 10 ml aliquots stored below -20°C.

5.2.4 Co-culture assays (endothelial cells and PBMC)

Culture medium used for assays was RPMI 1640 (Sigma R0883) supplemented with 2mM L-glutamine (Sigma G7513), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Sigma P0781) and 1x non-essential amino acids (100x stock, Gibco 11140). Peripheral blood collection from healthy donors and isolation of PBMC was as described in General Methods. TGN1412 and controls were incubated with 125,000 PBMC/well in 300 µl culture medium containing 2% hi-hAB serum, over monolayers of endothelial cells seeded at 15,000 – 30,000 cells/well the previous day in 100 µl of culture medium containing 10-15% hi-hAB serum (all "spent" medium was aspirated prior to addition of PBMC), in 96-well polystyrene, tissue-

culture treated, flat bottomed microtitre plates (Nunc 167008). Usually, cell-conditioned medium was harvested after 24 h, which was extended to 48 h when IFNγ was to be measured. Cell-conditioned medium was assayed using cytokine-specific ELISAs for some or all of the following: TNFα, IL-2, IL-6, IL-8 and IFNγ, as described in General Methods. Standard curves for each cytokine were prepared in culture medium containing 2% hi-hAB serum. Where relevant, cell proliferative responses of remaining cells were quantified by measuring 3H-thymidine incorporation, as described in General Methods, added to wells in culture medium containing 2% hi-hAB serum.

The previous paragraph describes the procedure for typical co-culture assays. However, any deviations from this procedure in an attempt to optimise the assay are described in the figures presenting the results. These deviations included substitution of hi-hAB serum with hi-FCS in the final culture medium, changes to the percentage hi-hAB serum in the final culture medium (2, 10 or 15%), and changes to endothelial cell and/or PBMC cell density seeded in the wells (30,000 or 60,000 endothelial cells and 125,000 or 200,000 PBMC per well). Where deviations from the typical procedure were made, cytokine standard curves for ELISAs were prepared in the same culture medium used for the co-culture assay, for example, if 10% hi-hAB serum was used in the co-culture assay, cytokine standard curves were prepared in culture medium containing 10% hi-hAB serum. Similarly, when cell proliferative responses were measured, 3H-thymidine was added in the same supplemented culture medium used for the co-culture assay.

Positive controls used include bacterial endotoxin (WHO IS 94/580 from NIBSC) which stimulated IL-6 release from both endothelial cells and PBMC and the mitogenic plant-derived lectin phytohaemagglutinin, PHA (Sigma). PHA in solution can activate polyclonal T cells in the absence of MHC/antigen complexes and co-stimulatory molecules. It cross-links and clusters TCRs resulting in the production a various cytokines including TNFα, IL-2, IL-6, IL-8, and IFNγ. Other controls were an isotype-matched (to TGN1412) negative control, culture medium only, and omitting the cell monolayer or omitting the PBMC.

5.2.5 Co-culture assays (endothelial cells and diluted whole blood)

Human whole blood was collected from consenting donors as described in section 2.2. Co-culture assays using whole blood were conducted as described for PBMC in section 5.2.4 with TGN1412 and controls incubated with 20% whole blood in 300 µl sterile PBS B over a monolayer of HUVECjr2 for 24 h. Cell-conditioned medium was assayed using cytokine-specific ELISAs as described in General Methods.

5.2.6 Statistical analyses

Unless otherwise stated in the results section, significant differences between data sets were established using Wilcoxon's paired signed rank test.

5.3 Results

5.3.1 The effect of co-culturing PBMC over a monolayer of C-HUVEC on TNFα, IL-2, IL-6, IL-8 and cell proliferative responses to TGN1412

Figure 25 shows that TNFα, IL-6, IL-8, IL-2 and cell proliferative responses to TGN1412 incubated in aqueous phase for 16 – 24 h with C-HUVEC or PBMC alone were very small and were not notably different from responses to the isotype-matched negative control. In contrast, TNFα, IL-6, IL-8, IL-2 and cell proliferative responses to TGN1412 incubated in aqueous phase for 16 – 24 h with PBMC cultured over a monolayer of C-HUVEC were significantly larger than responses to the isotype-matched negative control ($p<0.005$ when comparing responses to TGN1412 at 1 µg/well with responses to the isotype-matched negative control at 1 µg/well). Values for responses of cells in co-culture to 1 µg/well TGN1412 were 415 ± 76 pg/ml TNFα, 5510 ± 910 pg/ml IL-6, 30,423 ± 2573 pg/ml IL-8 and 204 ± 26 pg/ml IL-2, values are means ± S.E.M of 10 donors of PBMC and 4 donors of umbilical cords from 4 independent experiments consisting of the following: 1st cord + PBMC from 2 donors (experiment 1), 2nd cord + PBMC

from 4 donors (experiment 2), 3rd cord + PBMC from 2 donors (experiment 3) and 4th cord + PBMC from 2 donors (experiment 4).

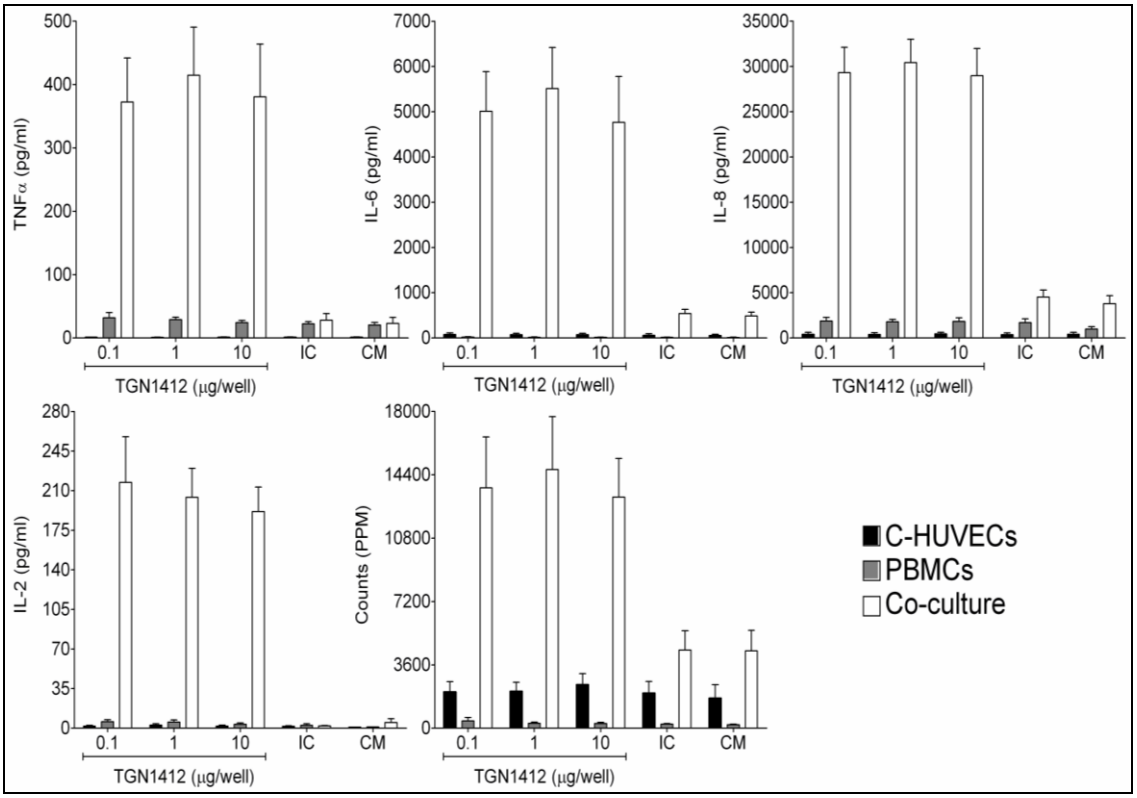


Figure 25. The effect of co-culturing PBMC over a monolayer of C-HUVEC on TNFα, IL-6, IL-8, IL-2 and cell proliferative responses to TGN1412. TGN1412 at doses 0.1, 1 and 10 μg/well, an isotype-matched negative control (IC) at 1 μg/well and culture medium alone (CM) were incubated for 16 - 24 h in aqueous phase with either C-HUVEC i.e. freshly isolated from umbilical cords (black) or with PBMC (grey) or with PBMC co-cultured over a monolayer of C-HUVEC (white). Cell-proliferative responses were measured in wells containing PBMC alone and PBMC co-cultured with C-HUVEC. Values for the cytokine and cell proliferative responses are means ± S.E.M of 10 donors of PBMC and 4 donors of umbilical cords from 4 independent experiments.

5.3.2 The effect of co-culturing PBMC over a monolayer of HUVECyn on TNFα, IL-6 and IL-8 responses to TGN1412

Figure 26 shows that TNFα, IL-6 and IL-8 responses to TGN1412 at doses of 0.1, 1 and 10 μg/well were only marginally larger than responses to the isotype-matched negative control at 1 μg/well when incubated for 24 h with PBMC cultured over a monolayer of HUVECyn endothelial cells suggesting that these endothelial cells were poor at mediating TGN1412-

stimulated responses in co-culture assays. A repeat of the experiment with an additional 4 donors of PBMC gave a similar pattern of results.

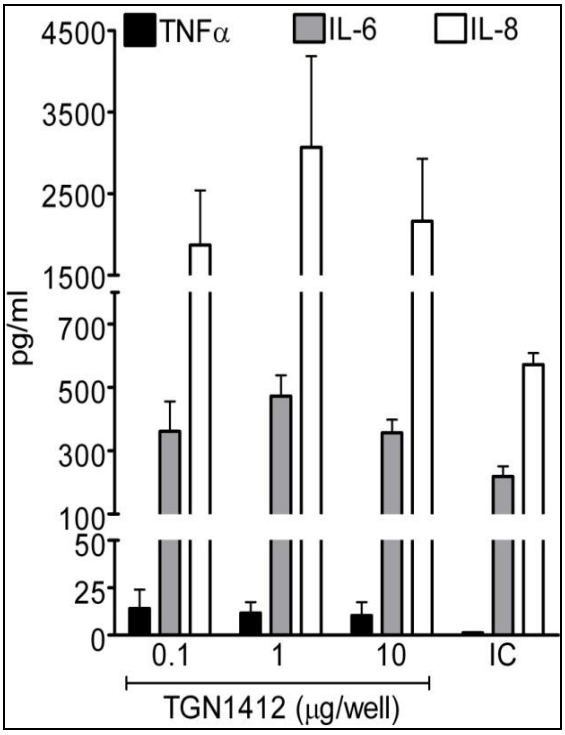


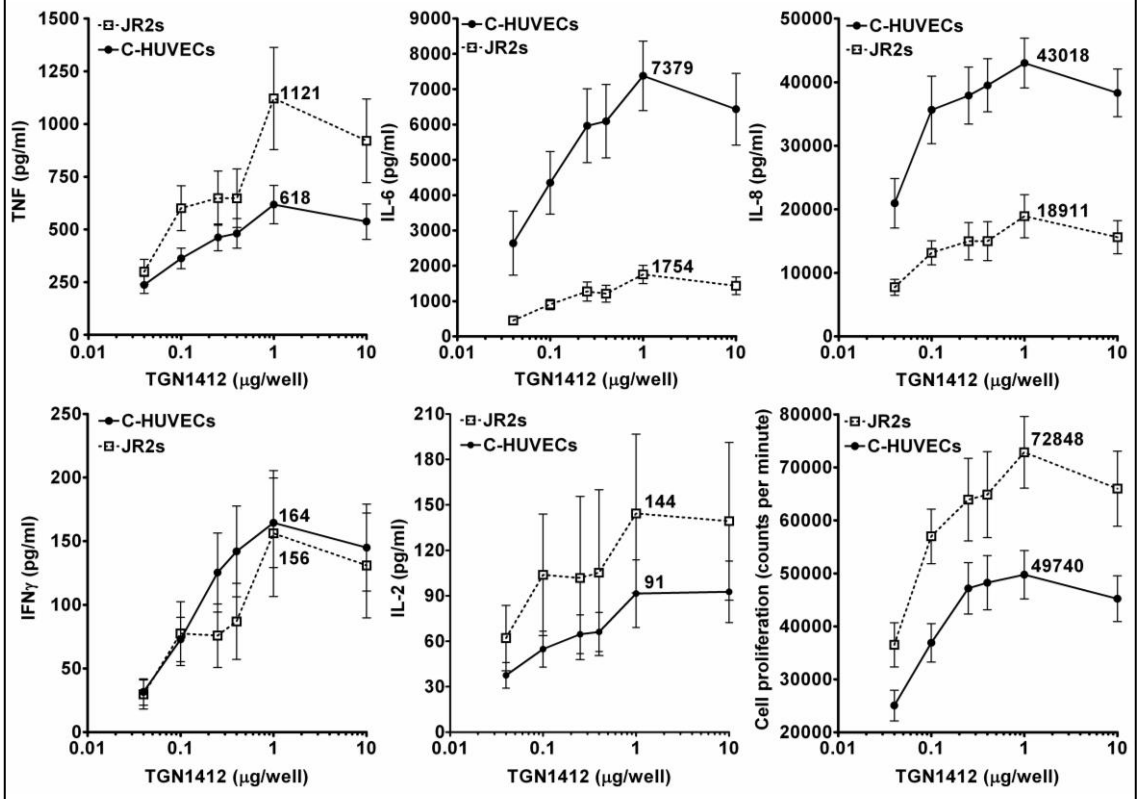
Figure 26. The effect of co-culturing PBMC over a monolayer of human umbilical vein endothelial cells (HUVECyn) on TNFα, IL-6 and IL-8 responses to TGN1412. Cytokine responses of co-cultured cells (PBMC and HUVECyn) to TGN1412 and an isotype-matched negative control (IC) at 1 μg/well after a 24 h incubation period. Values for cytokine release (pg/ml) are means ± S.E.M of 4 donors of PBMC from 1 independent experiment but are representative are results from 2 independent experiments, each with 4 different donors of PBMC.

5.3.3 Comparison of the capability of primary endothelial cells (C-HUVEC) with endothelium derived cells (HUVECjr2) to mediate TGN1412-induced cytokine and cell proliferative responses in co-culture assays with PBMC.

Figure 27A shows that aqueous TGN1412 stimulated dose-related release of TNFα, IL-6, IL-8, IFNγ, IL-2 and cell proliferative responses when incubated for 48 h with PBMC cultured over a monolayer of either C-HUVEC or HUVECjr2. Under the same experimental conditions, cytokine and cell proliferative responses to an isotype-matched negative control, after baseline correction, were smaller than responses to the smallest dose of TGN1412 tested. For both C-

HUVEC and HUVECjr2, the smallest dose of TGN1412 tested (0.04 µg/well, equivalent to 0.13 µg/ml) stimulated cytokine responses for all cytokines measured and cell proliferative responses, and 1 µg/well TGN1412 (equivalent to 3.3 µg/ml) stimulated the largest cytokine responses (for all cytokines measured) and the largest cell proliferative responses. TGN1412 stimulated larger TNFα, IL-2 and cell proliferative responses in co-culture assays using HUVECjr2 as the monolayer. In contrast, IL-6, IL-8 and IFNγ responses to TGN1412 were larger when C-HUVEC were used as the monolayer. Larger concentrations of background IL-6 and IL-8 were measured in co-culture assays where C-HUVEC were used instead of HUVECjr2 (Figure 27B), however this did not contribute to the larger IL-6 and IL-8 responses to TGN1412 in co-cultures using C-HUVEC because data for these assays were baseline corrected.

A.



B.

Cytokine	JR2s (pg/ml)	C-HUVECs (pg/ml)
TNFα	92 ± 38	83 ± 17
IL-6	132 ± 26	522 ± 107
IL-8	2615 ± 743	5429 ± 1095
IFNγ	8 ± 4	13 ± 4
IL-2	3 ± 1	6 ± 3

Figure 27. Comparison of human umbilical vein endothelial cells of different origins on their capability to interact with PBMCs to support cytokine and cell proliferative responses to aqueous TGN1412 when incubated with human PBMC. TNFα, IL-6, IL-8, IFNγ, IL-2 and cell proliferative responses to TGN1412 (panel A) at 0.04 – 10 μg/well and supplemented culture medium alone (panel B) incubated for 48 h with PBMC cultured over a monolayer of endothelial cells from fresh human umbilical vein (C-HUVEC) or endothelium derived cells (HUVECjr2, denoted as JR2). Values for cytokine and cell proliferative responses are means ± S.E.M of 11-16 and 22-28 donations of PBMC (from 15 different donors) for the HUVECjr2 and C-HUVEC data respectively and 5 different umbilical cords for values for C-HUVEC, from 4 independent experiments.

Isolated C-HUVEC and HUVECjr2 were cultured under exactly the same conditions when maintained between assays as described in Methods for this chapter. Figure 28 shows their appearance to be very similar in culture flasks despite C-HUVEC being more confluent.

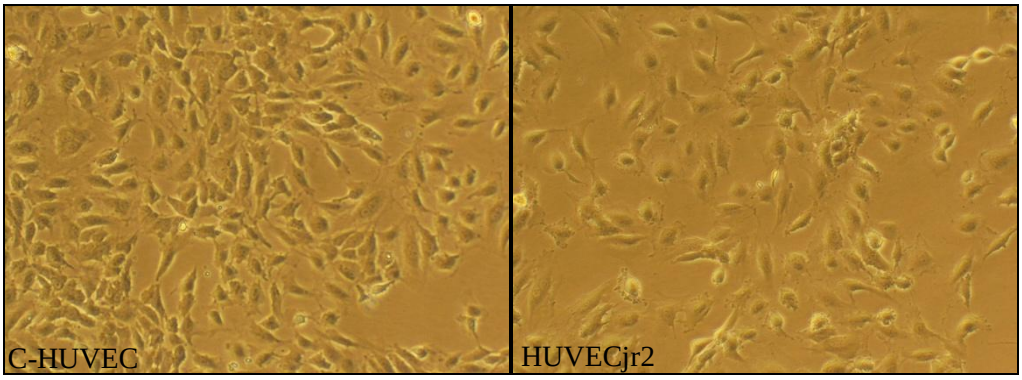


Figure 28. Typical appearance of C-HUVEC and HUVECjr2 cultured in 75 cm^2 flasks in EGM-2 culture medium containing 2% FCS. Viewed under phase contrast microscopy at 100x magnification.

Although caution must be exercised when comparing *in vivo* and *in vitro* cytokine concentrations, Table 6 shows that TGN1412 stimulated smaller concentrations of TNFα, IL-2 and IFNγ release after 24 – 48 h incubations in co-culture assays compared with peak concentrations measured in the blood of the clinical trial volunteers; in contrast, IL-6 and IL-8 concentrations in co-culture assays were broadly comparable with those measured *in vivo*. Of course, responses varied from one trial volunteer to the next but it was not possible from the information given (Suntharalingam et al., 2006) to estimate the individual values for all six recipients in the trial. The data in Table 6 summarises as far as is practicable the magnitude of responses to TGN1412 obtained *in vivo* and *in vitro*.

Table 6. Comparison of peak cytokine responses (pg/ml) to TGN1412 *in vivo* (Suntharalingam et al., 2006) and of cells in co-culture assays (PBMC with either C-HUVEC or HUVECjr2).

Cytokine	***In vivo* peak responses***	**Co-culture**		
	(medians for 6 patients)	**C-HUVEC**		**HUVECjr2**
		24 h*[1]	**48 h*[2]**	**48 h*[2]**
TNFα	4676	415 ± 76	618 ± 91	1121 ± 242
IL-6	3400	5510 ± 910	7379 ± 985	1754 ± 259
IL-8	≥5000	30423 ± 2573	43018 ± 3918	18911 ± 3382
IL-2	4200	204 ± 26	91 ± 22	144 ± 52
IFNγ	≥5000	ND	164 ± 35	156 ± 49

* Peak responses were at 4 h post infusion of TGN1412. *[1] Values are from Figure 25. *[2] Values are from Figure 27. ND = Not Determined.

5.3.4 The effect of co-culturing human whole blood over a monolayer of HUVECjr2 on TNFα, IL-6 and IL-8 responses to TGN1412

TNFα, IL-6 and IL-8 responses to aqueous TGN1412 (0.1, 1 and 10 μg/well) and the isotype-matched negative control (1 μg/well) incubated for 24 h with 20% human whole blood over a monolayer of HUVECjr2 were all below 7 pg/ml. Cytokine responses to 2.5 IU (250 pg)/ml endotoxin (positive control) were: 428 ± 113 pg/ml TNFα, 4210 ± 21 pg/ml IL-6 and 809 ± 192 pg/ml IL-8, values are means ± S.E.M of 2 donors of blood.

5.3.5 The effect of different serum/plasma supplements on cytokine and cell proliferative responses to TGN1412 in co-culture assays

Figure 29 shows that in culture medium supplemented with hi-hAB serum, TGN1412 stimulated TNFα, IL-6, IL-2 and cell proliferative responses which were significantly larger than those stimulated by the isotype-matched negative control when incubated for 48 h with 125,000 PBMC/well cultured over a monolayer of 30,000/well HUVECjr2 ($p<0.05$ when

comparing responses to 1 µg/well TGN1412 with responses to 1 µg/well negative control). In contrast, under the same experimental conditions but using hi-FCS as the culture medium supplement, cytokine and cell proliferative responses to TGN1412 were very small and not much different from responses to the negative control. Despite the lack of cytokine and cell proliferative responses to TGN1412 in media containing hi-FCS, responses to PHA (positive control) were marked in both FCS and hAB serum supplemented medium. Values shown in Figure 29 are means of responses of 7 donors from 2 independent experiments. Different batches of hi-FCS were used for each experiment and so the absence of responses to TGN1412 when hi-FCS was unlikely to have been attributable to a poor batch of serum.

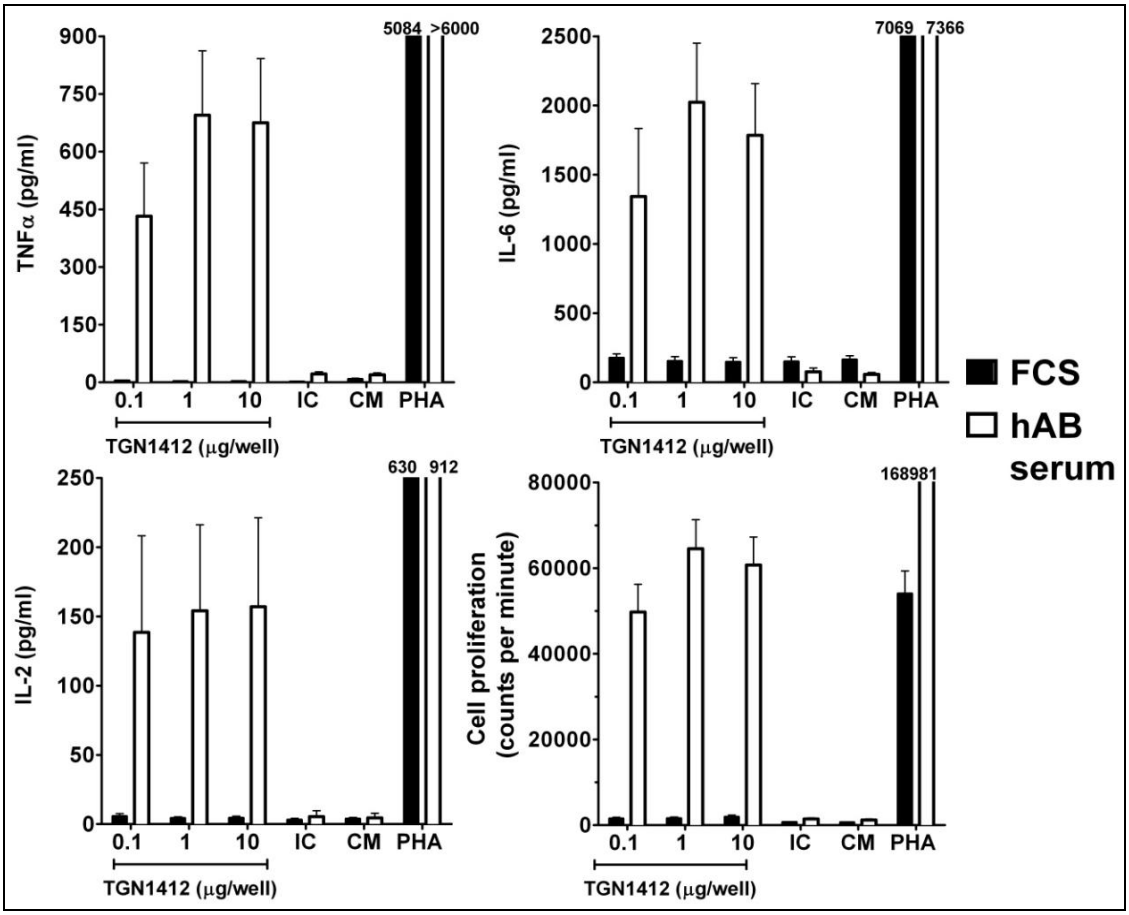


Figure 29. Comparison of cytokine responses to TGN1412 in co-culture assays using either hi-FCS or hi-hAB serum as the supplement. TNFα, IL-6, IL-2 and cell proliferative responses to TGN1412, an isotype-matched negative control (IC) at 1 µg/well, supplemented culture medium alone (CM) and PHA at 10 µg/ml incubated for 48 h with 125,000 PBMC cultured over a monolayer of 30,000 HUVECjr2 in 300 µl culture medium supplemented with either 2% FCS or 2% hAB serum. Values for cytokine and cell proliferative responses are means ± S.E.M of 7 donors of PBMC from 2 independent experiments.

Usually, cytokine values for responses to TGN1412 were read off cytokine ELISA standard curves where the standard was diluted in the same culture medium used for the cell culture and containing the same percentage of plasma/serum. The values in Figure 29 were read off cytokine ELISA standard curves where the standard was diluted in culture medium (as for the cell culture) but containing 2% hAB serum. A comparison of OD values given for cytokine ELISA standard curves prepared in culture medium containing hi-FCS or hi-hAB serum (2%) showed that the type of serum did not affect the concentration of cytokine measured by ELISA (as shown in Figure 30).

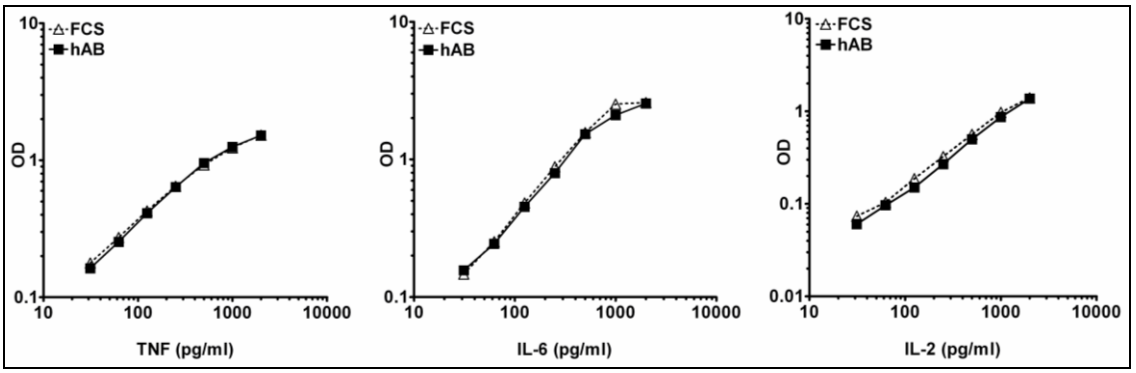


Figure 30. Effects of different types of sera on the detection of TNFα, IL-6 and IL-2 by ELISA. Standard curves of each cytokine were diluted in culture medium containing either hi-FCS or hi-hAB serum (2%) and cytokine concentrations for each curve were measured in terms of optical density (OD) by ELISA.

5.3.6 The effect of cell density on cytokine responses to TGN1412 in co-culture assays

Figure 31 shows that TNFα, IL-6 and IL-2 responses to TGN1412 (0.1, 1 and 10 µg/well) incubated for 24 h with PBMC (at cell densities 125,000 and 200,000 cells/well) cultured over a monolayer of HUVECjr2 (at cell densities 30,000 and 60,000 cells/well) in medium containing either 2 or 10% hi-hAB serum were marked and were larger than responses to the isotype-matched negative control. However, although there were marginal differences between responses for different cell density combinations of PBMC and HUVECjr2 (for cultures in both 2 and 10% hi-hAB serum), none of the combinations gave responses for any of the cytokines which were > 2x responses given by any other combination of cell densities.

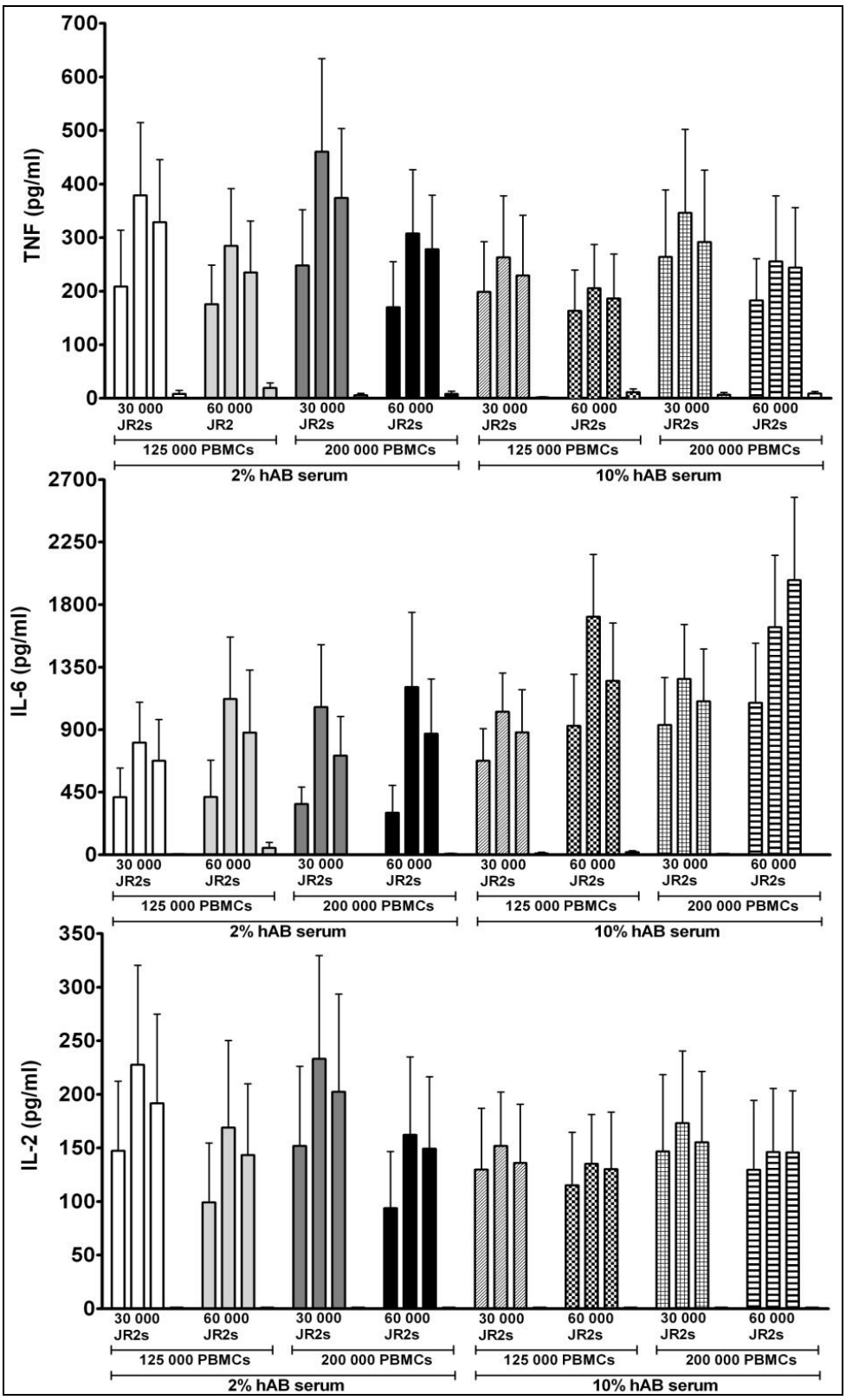


Figure 31. Comparison of TNFα, IL-6 and IL-2 responses to TGN1412 in 24 h co-culture assays using different PBMC and HUVECjr2 (JR2s) cell densities in 2% and 10% hi-hAB serum. From left to right for each experimental condition, responses are to TGN1412 at doses 0.1, 1 and 10 µg/well and an isotype-matched negative control at 1 µg/well. Values are means ± S.E.M of baseline corrected responses from 4 donors of PBMC.

5.3.7 The effect of human AB serum concentration on cytokine responses to TGN1412 in co-culture experiments

Figure 32 shows that TNFα, IL-6 and IL-2 responses to TGN1412 (1 µg/well) incubated for 24 h with 125,000 PBMC cultured over a monolayer of 30,000 HUVECjr2 in medium containing either 2, 10 or 15% hi-hAB serum were marked and were larger than responses to the isotype-matched negative control. Despite there being no significant statistical difference between responses to TGN1412 in 2, 10 and 15% hi-hAB serum for any of the cytokines measured ($p>0.05$, Kruskal-Wallis test with Dunn's Multiple Comparisons procedure), TNFα and IL-2 responses were largest in medium containing 2% hi-hAB serum and smallest in medium containing 15% hi-hAB serum. In contrast, IL-6 responses were largest in medium containing higher concentrations of hi-hAB serum (10-15%) and smallest in medium containing 2% hi-hAB serum. However, for all three cytokines, the largest shift in response caused by changing the serum concentration was no more than 100%. The concentration of hi-hAB serum used had very little effect on background levels of cytokines in co-culture assays, i.e. cells incubated in supplemented culture medium only.

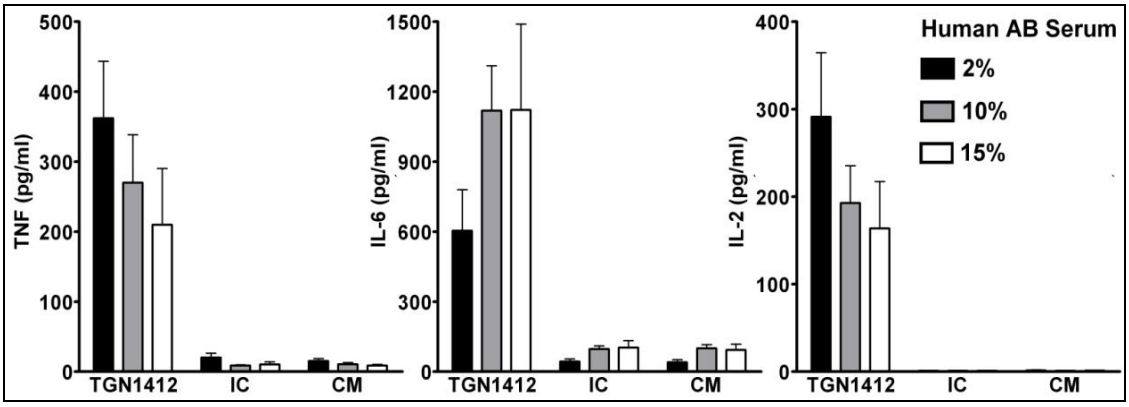


Figure 32. Comparison of TNFα, IL-6 and IL-2 responses to TGN1412 in co-culture assays using different concentrations hi-hAB serum. Cytokine responses to 1 µg/well TGN1412, 1 µg/well isotype-matched negative control (IC) and to culture medium alone (CM) when incubated with 125,000 PBMC and 30,000 HUVECjr2 for 24 h in 2, 10 or 15% hi-hAB serum. Values are means ± S.E.M of 8 donors of PBMC from 2 independent experiments.

The values in Figure 32 were read off standard curves diluted in culture medium (as for the cell culture) but containing only 2% hi-hAB serum. However, a comparison of OD values given for cytokine standard curves prepared in culture medium containing either 2 or 15% hi-hAB serum showed that the concentration of hi-hAB serum used did affect the concentration of cytokine measured by ELISA (as shown in Figure 33)

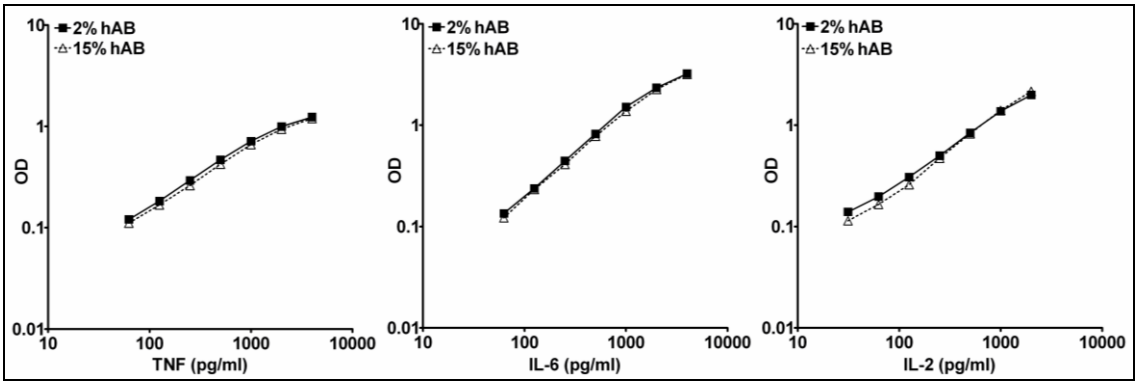
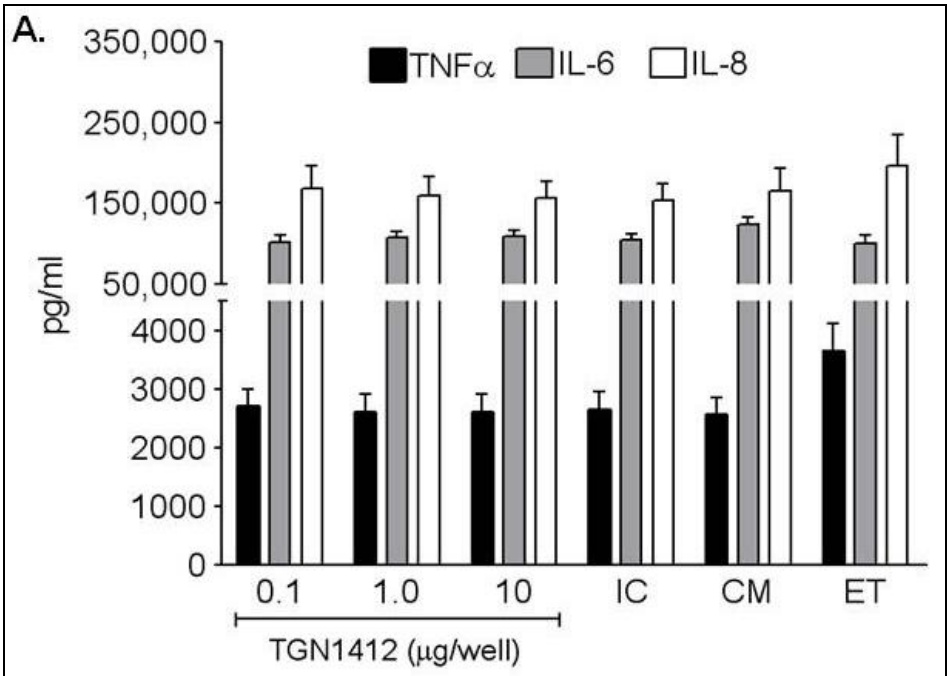


Figure 33. Effects of different human AB serum concentrations on the detection of TNFα, IL-6 and IL-2 by ELISA. Standard curves for each cytokine were diluted in culture medium containing either 2 or 15% hi-hAB serum and cytokine concentrations for each curve were measured in terms of optical density (OD) by ELISA.

5.3.8 The effect of co-culturing PBMC over a monolayer of Human Saphenous Vein Endothelial Cells (HSVECs) on TNFα, IL-6 and IL-8 responses to TGN1412

Figure 34A shows that TNFα, IL-6 and IL-8 responses to TGN1412 incubated in aqueous phase with PBMC cultured over a monolayer of human saphenous vein endothelial cells for 24 h were marked. Cytokine responses to TGN1412 were not notably different from responses to an isotype-matched negative control antibody. Values for responses of cells in co-culture to 1 μg/well TGN1412 were 2601 ± 316 pg/ml TNFα, 108,310 ± 7129 pg/ml IL-6 and 159,123 ± 23,880 pg/ml IL-8. Values are means and S.E.M of 8 donors of PBMC and 1 donor of HSVECs from 2 independent experiments. Figure 34B shows that concentrations of cytokines release by either HSVECs alone or PBMC alone incubated with TGN1412 or supplemented culture medium were small in comparison with the concentrations measured when these cells were

cultured together with TGN1412 or supplemented culture medium which suggests that the large responses of cells in co-culture were not a result of contamination.



B.

	HSVECs		PBMCs
	TGN1412	*Culture medium*	*Culture medium*
TNF	<1	<1	8 ± 3
IL-6	134 ± 25	138 ± 41	<50
IL-8	1185 ± 196	1160 ± 175	675 ± 167

Figure 34. The effect of co-culturing PBMC over a monolayer of human saphenous vein endothelial cells (HSVECs) on TNFα, IL-6 and IL-8 responses to TGN1412. Panel A: cytokine responses of co-cultured cells (PBMC and HSVECs) to TGN1412, an isotype-matched negative control (IC) at 1 µg/well, supplemented culture medium alone (CM) and endotoxin (ET) at 2.5 IU (250 pg)/ml after a 24 h incubation period. Panel B: cytokine release (pg/ml) by HSVECs or PBMC alone incubated with aqueous TGN1412 (1 µg/well) or in supplemented culture medium alone. Values for cytokine release (pg/ml) are means ± S.E.M of 8 donors of PBMC and 1 donor of HSVECs from 2 independent experiments.

5.4 Discussion

Despite the fact that immobilised (to plastic) TGN1412 stimulated profound cytokine release when incubated with human PBMC (Chapter 4), there is no *in vivo* analogue of a plastic surface and so antibody immobilisation methods can justifiably be criticised for not being

physiologically relevant. It is very likely that cells, other than lymphocytes, are involved in responses stimulated by TGN1412, such as other cells known to be involved in immunological responses such as vascular endothelial cells.

An obvious method to try in the first instance was to incubate TGN1412 (in aqueous phase) with PBMC cultured over a monolayer of endothelial cells. Endothelial cells derived from human umbilical vein (HUVEC) were chosen because they are the usual ‘‘fresh’’ cell of choice for studies of human endothelium (Marin et al., 2001). The reason for this is that despite the considerable difficulties that are frequently encountered in obtaining fresh umbilical cords, umbilical veins remain the easiest “fresh” human tissue to source. Initially, freshly isolated endothelial cells from donated human umbilical veins (not subject to cryogenic storage) were used (called C-HUVEC in this study) rather than a commercially available cell line since, for the latter, the expression of functionally relevant molecules can be altered by cryogenic storage and continuous passaging. In this “proof of principle” experiment, TGN1412 in aqueous phase was unable to stimulate significant cytokine and cell proliferative responses when incubated with either PBMC or C-HUVEC alone. Only when C-HUVEC and PBMC were co-cultured could TGN1412 stimulate notable responses and did so for all readouts (TNFα, IL-6, IL-8, IL-2 and cell proliferation) chosen as a representative panel of cytokines stimulated by TGN1412 during its “first time in man” clinical trial (Suntharalingam et al., 2006). Under the same experimental conditions, background cytokine/cell proliferation levels of cells co-cultured in the absence of TGN1412 or to an isotype-matched negative control antibody were negligible.

Given the difficulties in sourcing fresh umbilical cords, their intrinsic variability and the requirement for medicines control testing of readily available endothelial cells, it was important to identify and validate a source of such cells that were able to mediate responses to TGN1412 in a manner similar to C-HUVEC in co-culture experiments with PBMC. Since TNFα, IL-6 and IL-8 responses to TGN1412 incubated with PBMC cultured over a monolayer of HUVECyn cells were only marginally above responses to the isotype-matched negative control, a decision was made not to pursue experiments with these endothelial cells. Repeated passaging, culture in sub-optimal conditions and cryogenic storage of these cells may have altered the expression of molecules relevant to mediating TGN1412 responses. Another source of human umbilical vein

endothelial cells, HUVECjr2, readily available at NIBSC, was investigated. Preliminary experiments carried-out as part of this study showed potential for these endothelial cells for use in co-culture assays to predict the toxicity of TGN1412 as responses to TGN1412 were significantly above those to the isotype-matched negative control (Stebbings et al., 2007). To establish a true "likeness" of HUVECjr2 to non-cryogenically preserved umbilical vein endothelial cells (C-HUVEC), a range of TGN1412-stimulated responses was compared for both cell types: this included TNFα, IL-6, IL-8, IL-2, IFNγ and cell proliferative responses (Suntharalingam et al., 2006). A direct comparison between C-HUVEC and HUVECjr2 when used as monolayers in co-culture experiments with the same donors of PBMC revealed that HUVECjr2 were clearly capable of mediating responses (for all readouts) to TGN1412 above those to the isotype-matched negative control antibody. Furthermore, the pattern of responses mediated by HUVECjr2 and C-HUVEC was very similar. The smallest dose of TGN1412 tested (0.04 µg/well) stimulated responses (for all readouts) above background levels when both C-HUVEC and HUVECjr2 were used. In addition, the same dose of TGN1412 (1 µg/well) stimulated the largest responses (for all readouts) when either C-HUVEC and HUVECjr2 were used. It should be noted that 1 µg/well/300 µl is equal to 3.3 µg/ml which is close to our best estimate of the *in vitro* equivalent (2 µg/ml) of the dose of TGN1412 given to the trial volunteers (0.1 mg/kg) as described in section 3.2.1. Therefore, this co-culture method, regardless of whether HUVECjr2 or C-HUVEC were used, was capable of detecting the toxicity of TGN1412 at physiologically relevant doses. Based upon the *in vitro* data presented here, the trial volunteers were given a dose that was very close to the maximum stimulatory dose based on data from co-culture experiments.

The appearance of HUVECjr2 in culture flasks was very similar to that of C-HUVEC. Confirmation that HUVECjr2 had not lost their endothelial phenotype came from a more comprehensive study conducted with the aim of correlating endothelial phenotype with the capability to mediate TGN1412-stimulated responses in co-culture assays. C-HUVEC isolated from umbilical cords for experiments conducted in this thesis and HUVECjr2 both expressed inducible endothelial markers ICAM-1, VCAM-1 and E-selectin and constitutive endothelial

markers von Willebrand factor (vWF) and CD31 and both C-HUVEC and HUVECjr2 were able to bind Ulex europeus lectin (Findlay et al., 2011).

These findings taken together led to the conclusion that HUVECjr2 were "fit for purpose" as an alternative monolayer to C-HUVEC in co-culture experiments with human PBMC, for predicting the toxic effects of TGN1412. This, however, was a somewhat surprising result since the single working cell bank of HUVECjr2 used for experiments in this study was expanded from the same original batch of cells (i.e. batch ZHC-2101 from TCS Biologicals) as for HUVECyn which, as discussed earlier, were poor mediators of TGN1412-stimulated responses in co-culture assays. Single working cell banks for HUVECjr2 and HUVECyn were produced by two different laboratories using different culture conditions. This emphasises the importance of optimal culture conditions in maintaining endothelial cell phenotype and preventing cell de-differentiation. Other studies have suggested that the tissue-specific phenotype of endothelial cells is dependent on the microenvironment of the tissue and that such cells rapidly undergo de-differentiation *in vitro* (Thum et al., 2000; Lacorre et al., 2004).

There were some obvious differences between responses to TGN1412 when co-cultured with either HUVECjr2 or C-HUVEC. IL-6 and IL-8 responses to TGN1412 were larger when C-HUVEC were used as the monolayer; in contrast, TNFα, IL-2 and cell proliferative responses were larger when HUVECjr2 were used as the monolayer. Some of these differences are investigated further in the next chapter.

TNFα, IL-8, IL-2 and IFNγ (but not IL-6) median cytokine concentrations in the blood of the trial volunteers reduced considerably towards normal concentrations 24 h post infusion of TGN1412 (Suntharalingam et al., 2006). *In vitro* cytokines accumulate in the cell-conditioned medium; therefore *in vitro* one would have expected to find at least similar if not larger concentrations unless, of course, cytokines were metabolised by the cells.

Although caution must be exercised when comparing *in vivo* and *in vitro* cytokine concentrations, Table 6 shows that concentrations of TNFα, IL-2 and IFNγ (but not concentrations of IL-6 and IL-8) stimulated by TGN1412 in 24 - 48 h co-culture experiments were smaller than peak concentrations measured in the blood of the trial volunteers. Furthermore, TNFα responses in co-culture experiments were smaller than those stimulated by

immobilised TGN1412 (Chapter 4). In addition, IL-2, TNFα and IFNγ concentrations stimulated in co-culture assays here were considerably smaller than reported levels stimulated by immobilised TGN1412 (Eastwood et al., 2010). With the aim of optimising the co-culture assay, experiments were conducted to determine the effects of substituting PBMC with whole blood, substituting umbilical vein endothelial cells with saphenous vein endothelial cells, and altering serum type/concentration and cell density on the assay's capability of predicting TGN1412-stimulated cytokine responses. This was to determine whether or not the small cytokine responses in the co-culture, relative to *in vivo* responses, could be enhanced by changes in the experimental conditions to more closely match *in vivo* responses.

Responses to TGN1412 when whole blood was substituted for PBMC in 24 h co-culture experiments using HUVECjr2 as the monolayer were not above background levels suggesting that the presence of a factor or factors in blood that inhibit(s) responses. Since whole blood could be argued to be the most physiologically relevant source of white blood cells, this result was somewhat unexpected. That said, it is likely that due to the lack of flow conditions *in vitro*, the erythrocytes, being the most dense and abundant cells in blood, would have settled under gravity onto the endothelial cells prior to the leukocytes. This would have prevented cell to cell contact between leukocytes and endothelial cells. This supports the notion that cell to cell contact between leukocytes and endothelial cells is necessary for TGN1412-stimulation of cytokine responses in co-culture assays. This notion is investigated further in Chapter 6.

Originally, human serum was chosen rather than bovine serum (FCS) as it was considered to more closely mimic the *in vivo* situation, i.e. that of the trial volunteers. However, FCS is richer in growth factors and, for this reason, is frequently used in cell culture assays. The use of FCS rather than hAB serum in 48 h co-culture assays using 125,000 PBMC and 30,000 HUVECjr2 per well did not increase cytokine responses, in fact, quite the contrary as responses were no different from those stimulated by the isotype-matched control. This was not likely to have been an FCS batch-specific phenomenon since the same result was found in two independent experiments (each with 4 different donors of PBMC), each using a different batch of FCS. It is not clear whether or not FCS lacks something that hAB serum contains or if FCS itself is inhibitory. Endothelial cells were incubated in wells in 10% FCS (or hAB serum) prior

to the day on which TGN1412 and PBMC were added and media was exchanged for that containing 2% FCS (or hAB serum). HUVECjr2 were maintained in flasks in 2% FCS so 10% FCS may have been toxic and could have rendered these cells incapable of eliciting responses to TGN1412. In contrast, human AB serum at 10% was likely to have been less toxic because it was less rich in growth factors, not being of foetal origin. It is unlikely, however, that 10% FCS was toxic to HUVECjr2 since C-HUVEC were maintained in 10% FCS for several days immediately after isolation from umbilical cord and thrived in this concentration of FCS (personal observation). The reason for the absence of TGN1412-stimulated responses in media supplemented with FCS could be related to the finding that bovine transferrin binds human cells with a much lower affinity than human transferrin (Young and Garner, 1990). Usually, iron-bound transferrin binds to transferrin receptor at the surface of the cell where it is internalised. Iron is subsequently separated from transferrin and the transferrin receptor-transferrin complex is recycled back to the membrane. In addition to its role in iron uptake, it has been suggested that membrane bound transferrin receptor redistributes in the immunological synapse, specifically in the pSMAC formed upon T cell stimulation with CD3 and CD28 antibodies (Batista et al., 2004). A deficiency in transferrin (i.e. when FCS is used) may affect the level of membrane-associated transferrin receptor which could interfere with synapse formation and responses to TGN1412. FCS has been used as a media supplement for other experiments in which TGN1412 was able to stimulate T cell responses (Eastwood et al., 2010), however this involved TGN1412 immobilisation and so the mechanism for T cell stimulation is likely to have been different to that in co-culture assays.

Increasing the density of PBMC or endothelial cells in the wells did not notably affect cytokine responses to TGN1412 when 2 or 10% hAB serum was used in 24 h co-culture experiments. It is possible that higher cell densities than those used are required to significantly affect cytokine responses, however if this assay is to be used as a routine assay for testing the safety of medicines, it is important to maintain practical limits and while culturing larger numbers of endothelial cells would not pose a problem, obtaining enough PBMC from blood donors may be more of a challenge. An alternative to fresh PBMC might be a mixture of cell lines.

When TGN1412 was incubated for 24 h in co-culture assays of 125,000 PBMC and 30,000 endothelial cells, the inclusion of 2% hAB serum was associated with larger IL-2 and TNFα responses than 10 – 15% hAB, in contrast, 10 - 15% hAB serum was associated with larger IL-6 responses than 2% hAB serum. The reason for this is unclear, however, it was not due to the presence of IL-6 in the serum since IL-6 responses in culture medium alone (i.e. in the absence of TGN1412) were no different when 10 or 15% hi-hAB serum was used. In addition, optical density values for IL-6 standard curves prepared in culture medium containing either 2% or 15% hi-hAB serum were no different when assayed by ELISA. The maximum effect of increasing or decreasing the concentration of hAB serum was a halving or doubling in the cytokine responses to TGN1412. This was surprising since it was expected that soluble factors such as growth hormones required for the maintenance of cell cultures and other soluble factors that may mediate responses to TGN1412 would be limited in culture medium containing only 2% hAB serum. Although HUVEC are generally cultured in only 2% FCS, FCS is far richer in growth factors than hAB serum. Since a majority of the readouts measured were larger in 2% rather than 10 - 15% hAB serum, 2% hAB serum was chosen for all further experiments as it is not practicable to set up cultures containing different concentrations of hAB serum for each cytokine tested.

Based upon the data presented above, the co-culture assay of TGN1412 appears robust since variations in cell numbers and/or serum concentrations through uncertainty of measurement or operator error would not drastically affect the outcome; however, this cannot be assumed to be the case for other medicines. Although not an exhaustive analysis of experimental parameters that could affect cytokine responses to TGN1412, the above experiments do examine the most obvious assay variables. One other obvious condition was to use donors' own plasma (collected during the isolation of PBMC from blood) in co-culture assays. However, practically, this would be challenging because donor plasma would need to be separated from blood prior to incubating the HUVEC overnight so that this plasma could be used as the supplement in the HUVEC culture medium. Therefore, PBMC isolated from blood at the same time as the plasma would have to be incubated overnight prior to their addition to the HUVEC. An overnight incubation of the PBMC may have implications as cells may stick to

the vessel in which they are incubated. In addition, cytokine concentrations stimulated by TGN1412 in co-culture assays were broadly similar for 24 h and 48 h incubations and so the assay is flexible; a desired characteristic of a method intended for routine use. These optimisation experiments have confirmed that none of the examined conditions give significantly larger cytokine responses than the conditions that were chosen originally, i.e. 125,000 PBMC and 30,000 endothelial cells per well in 2% hAB serum.

Umbilical vein derived endothelial cells were initially selected as the cells to form the monolayer in the co-culture assay for the reasons discussed above. However, endothelial cells involved in responses stimulated by TGN1412 in the clinical trial obviously were not of umbilical origin. Therefore, endothelial cells from saphenous vein (HSVEC) were also evaluated for their use in co-culture assays as these were more physiologically relevant than HUVEC. Although it was not possible to procure fresh saphenous vein tissue, HSVEC cryogenically frozen at low passage (2) following isolation from saphenous vein were available as a gift from the Department of Medicine, Imperial College London. When human saphenous vein endothelial cells (HSVECs) were co-cultured with PBMC for 24 h, TGN1412 did not stimulate TNFα, IL-6 and IL-8 responses significantly above responses to the isotype-matched negative control. However, this may have been because background concentrations of all three cytokines were so large, simply as a result of culturing PBMC and HSVECs together (in the absence of TGN1412).

The fact that none of the conditions described above dramatically increased TNFα and IL-2 cytokine responses to TGN1412 in 24 - 48 h co-culture assays, stimulated further thought as to why cytokine concentrations were small compared with responses to immobilised TGN1412 and with peak concentrations in the blood of the trial volunteers. One possibility was that after a 24 - 48 h incubation of cells in co-culture with TGN1412, the concentration of IL-2 in the cell-conditioned medium was reduced as it was internalized by T cells. It has been reported that interaction of IL-2 with high-affinity IL-2 receptors on the surface of T cells leads to receptor-mediated internalization and activation of T cell proliferation, thus IL-2 is removed from the extracellular environment (Subtil et al., 1994). In TGN1412 immobilisation assays, greater cytokine release could have resulted in their accumulation in the cell-conditioned

medium if saturating levels were achieved. The IL-2 concentrations were reduced by half in cell conditioned medium from 48 h co-culture assays compared with those of 24 h assays (with the caveat that this was not a direct comparison within the same assay and so different donors of PBMC were used). However, it is published that IL-2 concentrations, released by stimulated lymphocytes into cell-conditioned medium, were maximal between 24 – 48 h (Gillis et al., 1978) albeit under different experimental conditions. Another possibility is that IL-2 released into the cell-conditioned medium may at least bind to IL-2 receptors expressed by endothelial cells, if it is not internalised, so preventing its measurement by ELISA. However, only in one report has it been shown that human umbilical vein endothelial cells possess IL-2 receptors (Hicks et al., 1991).

IFNγ responses of PBMC to immobilised TGN1412 (in the absence of endothelial cells) incubated for 24 h were small in comparison with peak IFNγ concentrations in the blood of the TGN1412 trial volunteers. Only after 48 – 72 h stimulation with TGN1412 could higher concentrations of IFNγ be measured. This incubation period was a requirement to allow TGN1412-stimulated naive and central memory T cells (in PBMC) to mature into $CD4^+$ effector memory T cells, the subset responsible for producing TGN1412-stimulated IFNγ release and which generally exist in very low numbers in peripheral blood. *In vivo*, of course, the IFNγ response to TGN1412 was faster because $CD4^+$ effector memory T cells are abundant in tissues such as the lungs and gastrointestinal mucosa (Eastwood et al., 2010). It is for this reason that TGN1412 was incubated for 48 h in co-culture experiments where IFNγ was to be a readout. However, for unknown reasons, cytokine responses were still small in comparison with peak cytokine concentrations in the blood of the trial volunteers. This finding could be indicative of a lack of TGN1412-stimulated T cell maturation into effector memory T cells in co-culture assays. Unpublished work at NIBSC has recently shown that much larger cytokine responses are stimulated by TGN1412 in co-culture assays (involving HUVECjr2) when purified CD4+ T cells were used instead of PBMC. Therefore, TGN1412, whether immobilised or incubated in aqueous phase in co-culture assays, activates CD4+ T cells. However, it is possible that, when immobilised, TGN1412 activated different CD4+ T cell subsets (i.e. naive and memory T cells)

compared with those stimulated in co-culture assays and that this affected the quantities of cytokines released. This is discussed in more detail in Chapters 6 and 8.

It is not yet understood where exactly in the body TGN1412 initiated responses but what is certain is that both TGN1412 and responding leukocytes would have come into contact with endothelial cells. Since endothelial cells are considerably heterogeneic (Garlanda and Dejana, 1997), it is to be expected that endothelial cells from umbilical vein have a different phenotype from endothelial cells located *in vivo* at sites where TGN1412 stimulated responses, and this could be the basis for the smaller 'sub-optimal' concentrations of cytokines (compared with the *in vivo* responses) measured in co-culture assays. In addition, *in vivo*, endothelial cells may act in concert with other cell-types, such as fibroblasts, smooth muscle cells or specialist APCs such as dendritic cells, to mediate TGN1412-stimulated responses.

Despite the gap in our understanding of the mechanisms underlying TGN1412-stimulated responses in the co-culture assay, this co-culture method is one of only two published methods available for predicting cytokine release to TGN1412 and if this assay had been used to test TGN1412 prior to its clinical trial, it is highly unlikely that the trial would have gone ahead and so is a more appropriate test procedure than the pre-clinical safety tests that were conducted. The obvious question is, of course, what do endothelial cells contribute in PBMC assays in which TGN1412 stimulates cytokine release without its prior immobilisation? This is investigated in the next chapter.

CHAPTER 6: Investigation of the physiological mechanisms underlying clinical responses to TGN1412 using co-culture

CHAPTER 6: Investigation of the mechanisms underlying responses to TGN1412 in co-culture assays

6.1 Introduction

It is clear from the findings of the previous chapter that a method involving the co-culture of human PBMC over a monolayer of human endothelial cells is better able to predict clinical responses to aqueous TGN1412 than a method relying upon PBMC alone. Furthermore, the inclusion of endothelial cells negates the requirement for immobilisation of TGN1412 to a plastic surface, a procedure which has little physiological relevance. A number of possibilities of how endothelial cells mediate (aqueous) TGN1412-stimulated responses in co-culture assays are investigated in this chapter since this could give insight to the mechanisms underlying clinical responses to this antibody. These possibilities are described in the following paragraphs.

In the study of transplant rejection, it is well established that endothelial cells play a fundamental role in stimulating rejection of the transplant by the recipient. Since activation of recipient CD4+ T cells (but not CD8+ T cells) is crucial to the rejection process, MHC class II antigens expressed by endothelial cells, i.e. the MHC class recognised by CD4+ T cells, play an important role in organ rejection (Rose, 1998). *In vitro*, IFNγ-induced allogeneic human umbilical vein endothelial cells stimulated CD4+ T cell responses through an MHC class II antigen-dependent mechanism (Page et al., 1994a; Rose, 1998). Allogeneic MHC/antigen stimulation of recipient T cells can be through "direct" stimulation, differing from "indirect" stimulation because it is the foreign MHC molecule itself that is the antigen and is recognised by recipient TCRs. For direct stimulation, the "foreign" MHC molecule does not need to be presented as part of the recipient's MHC molecules expressed by recipient APCs (Rose, 1998). Since CD4+ cells have been reported to be the predominant T cell subset responding to immobilised TGN1412 (Stebbings et al., 2007) and are also stimulated by aqueous TGN1412 in co-culture assays (unpublished data, NIBSC), it was conceivable that, in co-culture assays, the direct stimulation of CD4+ T cell receptors by allogeneic MHC class II antigens expressed by "foreign" endothelial cells played a role in mediating TGN1412-stimulated responses. For

example, TGN1412 may have provided a co-stimulatory signal following a primary signal given through ligation of the TCR by foreign MHC class II expressed by endothelial cells (as illustrated in Figure 35). If this had been the case, the use of co-culture assays for investigating the underlying mechanisms of TGN1412-stimulation *in vivo* could be questioned because the response stimulated could have been dependent on the fact that PBMC and endothelial cells were from different donors. This scenario was clearly not the case when TGN1412 was given in man. There is conflicting evidence in the literature regarding the expression of MHC class II molecules by HUVEC. On the one hand, it has been reported that HUVEC do not constitutively express MHC class II antigens in situ or *in vitro* (Rose, 1998) and, although expression is induced by IFNγ (Collins et al., 1984), HUVEC are 10 – 100 times less sensitive to IFNγ than microvascular endothelial cells regarding MHC class II upregulation (McDouall et al., 1997). Since concentrations of IFNγ in cell-conditioned medium from PBMC/HUVEC co-culture assays with culture medium alone (i.e. no TGN1412) after 48 h were negligible (see Figure 27B), it is unlikely that MHC class II expression was upregulated by IFNγ prior to T cell activation by TGN1412, in which case allogeneic MHC class II expression was unlikely to have been responsible for initial responses to TGN1412. On the other hand, in just one study, non-treated HUVEC (i.e. without IFNγ treatment) incubated with allogeneic T cells induced T cell proliferation in the absence of a mitogen or mAbs directed at the TCR/CD3 complex suggesting that the presentation of alloantigens to T cells occurred (Adams et al., 1992). Furthermore, MHC class II expression by endothelial cells may be induced by other means, for example, allogeneic natural killer lymphocytes were able to upregulate MHC class II expression by endothelial cells through a contact-dependent mechanism (Watson et al., 1995). In light of these findings, the concept that endothelial cells mediate responses to aqueous TGN1412 in co-culture assays because they are allogeneic was investigated.

It was established in Chapter 4 that the manner in which TGN1412 is presented to PBMC is crucial to its capability to induce cytokine responses. It is a possibility that aqueous TGN1412 in co-culture assays is immobilised by endothelial cells, presenting the antibody in a similar manner to that achieved by air-drying or wet-coating the antibody (Chapter 4). This concept is illustrated in Figure 35. One possible mechanism for TGN1412 immobilisation is through

binding of its Fc with Fc receptors expressed on the surface of endothelial cells. Expression of Fc receptors by endothelial cells *in vitro* has been reported (Bjerke et al., 1988; Pan et al., 1998; Pan et al., 1999; Sandilands et al., 2010) and although Fc receptor expression is enhanced by the cytokines TNFα and IFNγ, low level constitutive expression of Fc receptor by endothelial cells cultured *in vitro*, including HUVEC, has also been reported (Pan et al., 1998; Sandilands et al., 2010). Indeed other cells such as monocytes and B lymphocytes within the PBMC fraction express Fc receptors (Gergely et al., 1977; Maeda et al., 1996). Therefore, if immobilisation of TGN1412 by Fc receptor was important, it would be expected that aqueous TGN1412 would have stimulated responses from PBMC in the absence of endothelial cells, which was not found to be the case. However, since endothelial cells in co-culture assays form a monolayer beneath the PBMC, Fc receptors expressed by endothelial cells may have been better positioned than Fc receptor expressed by PBMC to elicit TGN1412-stimulated responses. Consequently, the role of TGN1412 immobilisation by endothelial cells in mediating TGN1412-stimulated responses in co-culture assays was investigated.

Endothelial cells play a fundamental role in mediating inflammatory responses (Pober and Sessa, 2007). The specific interaction of their cell surface adhesion molecules with ligands expressed by immune cells mediates selective migration of immune cells across the endothelial barrier, usually in post-capillary venules, into the infected tissue. A similar process occurs in the high endothelial venules, specialised blood vessels which mediate the migration of lymphocytes from the blood into lymph nodes (Faveeuw et al., 2000; Pittet and Mempel, 2008). Adhesion molecules expressed by endothelial cells also regulate the circulation of lymphocytes through tissues in the absence of infection by acting as tissue-specific "homing receptors" to which lymphocytes adhere (Hamann et al., 1994). Some of the adhesion molecules expressed by endothelial cells that mediate the migration of leukocytes across endothelial barriers are also expressed by specialised APCs. Interaction of adhesion molecules expressed by APCs with their ligands expressed by T cells mediates cell to cell adhesion and the formation of a stable immunological synapse promoting T cell activation by the APC (as described in section 1.4.3). In addition to their role in mediating cell to cell adhesion in the immunological synapse, there

have been numerous reports suggesting that adhesion molecules expressed by APCs also have a co-stimulatory effect during T cell activation.

The first established receptor/ligand interaction mediating cell to cell adhesion was that of LFA-3 with CD2 (Dustin et al., 1987). LFA-3 is a 222 amino-acid surface glycoprotein, comprising extracellular, transmembrane and cytoplasmic domains, is expressed by specialised APCs and endothelial cells (Wallner et al., 1987). Studies involving Jurkat cells (T lymphocyte cell line) expressing CD2 and artificial membrane lipid bilayers expressing LFA-3 showed that LFA-3/CD2 interactions not only promote cell to cell adhesion but also stimulate downstream signalling processes in T cells in the absence of TCR ligation, although the presence of the TCR was required for such events to occur. These signalling processes were the same as those that follow TCR stimulation and which ultimately lead to increases in intracellular calcium levels characteristic of T cell activation (Kaizuka et al., 2009). Similar results were found when anti-CD2 antibodies were used to stimulate T cells (Kanner et al., 1992; Martelli et al., 2000). In addition, the use of artificial lipid bilayers and Jurkat cells revealed that signalling events activated upon LFA-3/CD2 ligation were accompanied by the reorganisation and clustering of CD2 and associated signalling molecules into microdomains and that this was dependent on the actin network. It was proposed that the formation of such microdomains is a requirement for the activation of Lck since they allow Lck activators to cluster and exclude Lck inhibitors. Microdomains of CD2/LFA-3 were spatially segregated from those containing TCRs. CD2/LFA-3 clustered peripherally to TCRs concentrated in the cSMAC. One theory is that CD2/LFA-3 signalling sustains signalling events triggered following TCR activation and spatial segregation of the TCR and CD2 may be important for this process (Kaizuka et al., 2009).

ICAM-1 is also an adhesion molecule expressed by APCs which interacts with LFA-1 (otherwise known as $\alpha_L\beta_2$), the only β_2 integrin expressed by T cells. ICAM-1 is a glycoprotein and has an extracellular domain, a single transmembrane domain and a cytoplasmic domain (Bella et al., 1998). In addition to its role in mediating cell to cell adhesion between APCs and T cells and the formation of a stable immunological synapse, as described in section 1.4.3, as with LFA-3, it is able to provide co-stimulatory signals during T cell activation by APCs. In one study it was suggested that human CD4+ T cell proliferation stimulated by activated monocytes

(i.e. with enhanced APC function) in the presence of CD3 antibody (to provide a signal to T cells through the TCR/CD3 complex) required ICAM-1/LFA-1 interaction (van Seventer et al., 1991b). Other studies using CD4+ T cells from transgenic mice that were CD18 negative (β chain of LFA-1) showed that the presence of LFA-1 was required for the optimal activation of CD4+ T cells stimulated by antigen-TCR (Kandula and Abraham, 2004). Furthermore, T cell proliferation and IL-2 release was induced by stimulator cells that had been transfected with MHC molecules and ICAM-1 but not by those expressing MHC molecules in the absence of ICAM-1 (Zuckerman et al., 1998).

The specific signaling events involved in ICAM-1/LFA-1 co-stimulation have not been fully elucidated (Varga et al., 2010). However, the enhancement of IL-2 production from anti-CD3-stimulated T cells by anti-LFA-1 or ICAM-1 was entirely dependent on the interaction of the adaptor protein SLP-76 with ADAP (adhesion and degranulation-promoting adaptor protein) suggesting that these two adaptor proteins play a role in mediating LFA-1 co-stimulation (Wang et al., 2009). In a different study, beads coated with CD3 antibody alone or with CD3 antibody combined with purified ICAM-1 or with ICAM-1 alone were incubated with human CD4+ T cells. In this system, the interaction of ICAM-1 with LFA-1 prolonged the hydrolysis of PIP_2 in T cells resulting in the production of IP_3 (inositol 1,4,5-triphosphate) and other products of PIP_2. As discussed in section 1.4.2 these products ultimately lead to an increase in intracellular calcium levels during T cell activation. Indeed, in this study using antibody coated beads, a prolonged increase in the level of intracellular calcium in T cells was observed when ICAM-1 was used in conjunction with CD3 antibody (van Seventer et al., 1992).

Given the co-stimulatory role of LFA-3 and ICAM-1 expressed by APCs in T cell activation, it is not unreasonable to assume that these adhesion molecules, when expressed by endothelial cells, may also give co-stimulatory signals in T cell activation. In fact there have been several reports suggesting that endothelial cells have a role *in vivo* as non-specialised APCs, in part owing to the fact that they express adhesion molecules which, when expressed by APCs, have a co-stimulatory effect. Furthermore, endothelial cells are capable of expressing high levels of MHC class I and II molecules *in situ*. Since the only known function of these

molecules is to present antigen to T cells, endothelial cells are clearly well equipped in this regard as discussed above (Westphal et al., 1993a; Choi et al., 2004; Pober and Sessa, 2007).

Also in support of this theory that endothelial cells may function *in vivo* as non-specialised APCs is the fact that endothelial cells (including HUVEC) are capable of co-stimulating the proliferation of T cells incubated with anti-CD3 mAb (Hughes et al., 1990; Westphal et al., 1992) or with mitogen (Shanahan, Jr. et al., 1985; Savage et al., 1991). Endothelial cells also augmented the production of IL-2 from purified CD4+ T cells and PBMC stimulated with the mitogen PHA. The use of transwell plates (i.e. plates containing a membrane insert as shown in Figure 36) revealed that this augmentation of IL-2 production was dependent on direct contact between endothelial cells and PBMC and was inhibited by blocking antibodies to LFA-3 and CD2, but not by blocking antibodies to ICAM-1 and LFA-1 (Hughes et al., 1990). In a different study, augmentation of IL-2 production from PHA-stimulated CD4+ T cells was inhibited by eight different antibodies which target LFA-3. Furthermore, the augmentation effect was delayed when T cells expressing low levels of CD2 were used, and purified LFA-3 was also capable of augmenting IL-2 production. This implies that the release of soluble factors such as cytokines is not responsible for the co-stimulatory effect and is consistent with the notion that endothelial cell to T cell contact is required. Although endothelial cells constitutively express LFA-3 (Smith and Thomas, 1990), they do not usually express CD80 and CD86 (Denton et al., 1999), the natural ligands for CD28 (as described in section 1.4.1). Therefore the co-stimulatory activity of endothelial cells is not likely to involve this classical co-stimulatory pathway.

Consistent with its role on APCs, ICAM-1 is also implicated in the co-stimulatory activity of endothelial cells. The co-stimulatory activity of endothelial cells on anti-CD3 mAb-stimulated or PHA-stimulated T cells was inhibited by blocking antibodies to ICAM-1 (Westphal et al., 1993a; Westphal et al., 1993b; Westphal et al., 1993c). In addition to LFA-3 and ICAM-1, endothelial cells express the cell surface adhesion molecule VCAM-1. VCAM-1 binds to VLA-4; an α4β1 integrin strongly expressed by activated T cells. The interaction of VCAM-1 and VLA-4 plays an important role in mediating the recruitment of effector T cells across endothelial barriers into sites of infection (Murphy et al., 2008) and in mediating the

migration of T cells from the blood across high endothelial venules into lymph nodes (Faveeuw et al., 2000). VCAM-1/VLA-4 interactions have also been reported to provide co-stimulatory signals in T cell activation (Pober and Cotran, 1991; van Seventer et al., 1991a; Udagawa et al., 1996; Nguyen et al., 2008). It has been suggested that the adhesive function of VLA-4 expands the area of the immunological synapse which facilitates antigen recognition by the TCR. Furthermore, VLA-4/VCAM-1 interactions may promote antigen recognition through the appointment of signalling molecules required for the transmission of signals from the TCR/antigen complex. This includes adaptor molecules containing ITAM motifs, tyrosine kinases and the adapter SLP-76. Cytoskeleton movements are responsible for the breakdown of SLP-76 microclusters that are involved in T cell activation. VLA-4 was found to inhibit such cytoskeleton movements leading to sustained signalling (Nguyen et al., 2008). T cells also express the integrin α4β7 which, upon interaction with MAdCAM-1 expressed by endothelial cells, facilitates the entry of T cells into mucosal lymphoid tissues such Peyer's Patches in the gut (Hamann et al., 1994). The interaction of α4β7 with its counterstructures has also been reported to co-simulate anti-CD3 induced T cell proliferation (Lehnert et al., 1998).

It has been shown that a "docking structure" can form at the point of contact between endothelial cells and T cells. In the "docking structure" the endothelial cell membrane forms a "cup-like" shape which engulfs the T cell. It is within this cup that adhesion molecules (including ICAM-1 and VCAM-1) interact with their ligands. In one study, in the presence of a super antigen (i.e. antigens that bind non-specifically to TCRs and so activate polyclonal T cell proliferation), molecules within the cup were organised into a mature synapse involving SMACs including a cSMAC and pSMAC as described in section 1.4.3 for the APC/T cell immunological synapses. Whether or not this docking structure plays a fundamental role in T cell activation by MHC-expressing endothelial cells is yet to be established (Choi et al., 2004).

Co-stimulation of PHA-activated T cells by endothelial cells has been reported to activate transcription factors AP-1, NFAT and NF-κB, i.e. those involved in T cell differentiation, proliferation and T cell effector functions (Mestas and Hughes, 2001). Both the composition and amount of AP-1 is affected by the presence of endothelial cells (Hughes and Pober, 1993) and

the export of NFAT from the nucleus is inhibited by endothelial cells, increasing its activity on gene expression (Murphy and Hughes, 2002).

The evidence to suggest that endothelial cells can co-stimulate T cells and thus act as APCs is very strong. Several theories have been proposed regarding the biological relevance of endothelial cells in antigen presentation and T cell activation. One possibility is that following re-infection with a specific pathogen pathogen-derived peptides are presented locally by endothelial cells in the context of self-MHC molecules. Effector memory T cells may be alerted to the re-appearance of a pathogen in the surrounding tissue following interaction with antigen/MHC complexes presented by endothelial cells resulting in a rapid immunological recall response. In microvessels such as venules effector memory T cells would have intimate contact with endothelial cells as a result of the small lumen (Choi et al., 2004; Pober and Sessa, 2007). In support of this theory, it was revealed that human microvascular endothelial cells presenting antigen were able to stimulate transendothelial migration of CD4+ effector memory T cells (Pober and Sessa, 2007). Interaction of co-stimulatory adhesion molecules expressed by endothelial cells with their ligands on effector memory T cells may also promote T cell activation. Other studies have shown that co-stimulation of T cells by endothelial cells (including HUVEC) is restricted to CD45RO+ memory T cells (Pober and Cotran, 1991; Marelli-Berg et al., 1996; Pober et al., 1997; Ma and Pober, 1998; Murphy et al., 1999). It is thought that naive T cells have more stringent activation requirements than memory T cells and thus can only be activated by specialised APCs. This makes sense as if memory T cells were underactive, the rapid recall responses conferred by memory T cells would be limited. One possibility is that naive T cells may not express the necessary ligands for adhesion molecule-specific interaction with endothelial cells (Choi et al., 2004). For example, it has been reported that naive T cells preferentially express the inactive form of LFA-1 whereas memory T cells express large amounts of the high affinity active form of LFA-1. In the same study, it was found that although memory CD4+ T cells employed LFA-1/ICAM-1, CD2/LFA-3 and CD28/B7 interactions, naive CD4+ T cells employed CD2/LFA-3 or CD28/B7 interactions when transfected CHO cells were used as a model APC (Parra et al., 1993).

The co-stimulatory properties of adhesion molecules expressed by endothelial cells, which are thought to support the non-specialised APC function of endothelial cells *in vivo*, may also provide a means for endothelial cells to promote TGN1412-stimulated responses in co-culture assays and *in vivo* (as shown in Figure 35). The interaction of adhesion molecules expressed by endothelial cells with their ligands expressed by T cells may firstly play a role in optimising the positioning of signalling molecules required for T cell activation upon stimulation with TGN1412. Furthermore, signals generated from the interaction of adhesion molecules with their ligands may themselves promote TGN1412-stimulated responses. The concept that adhesion molecules contribute to TGN1412-stimulated responses in co-culture assays is investigated in this chapter.

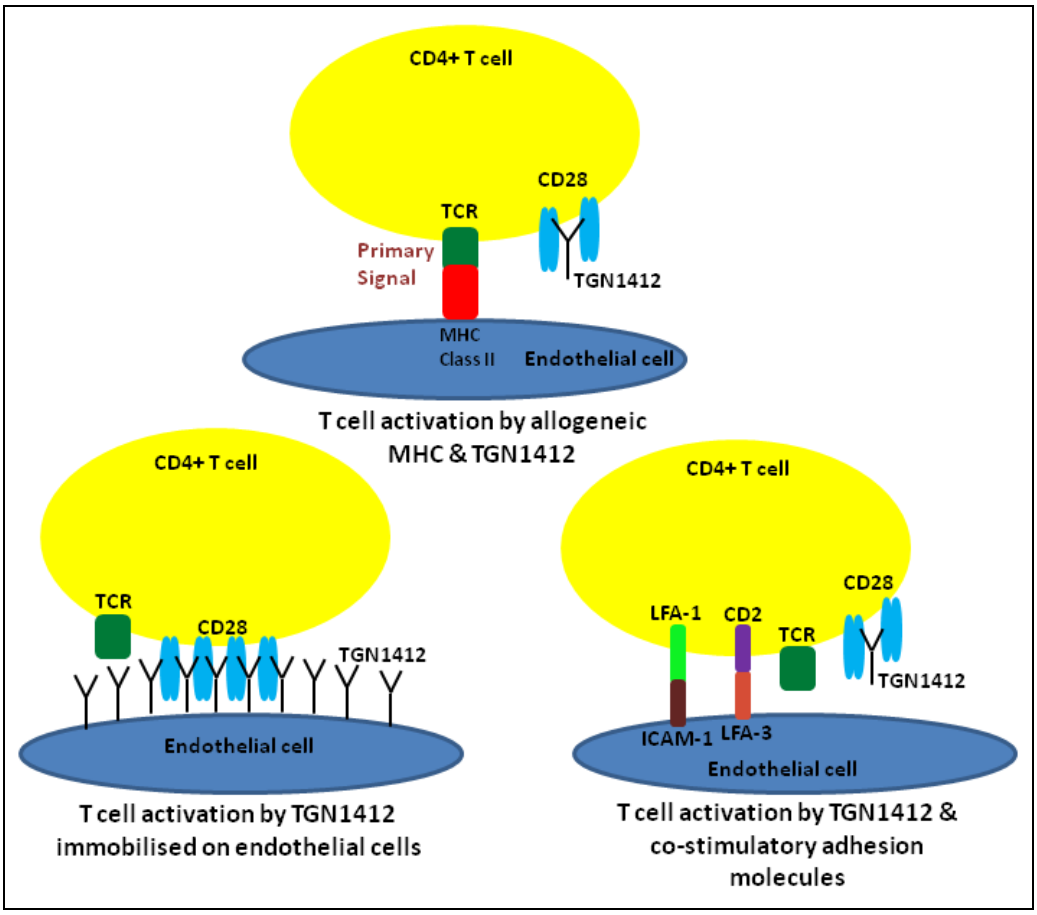


Figure 35. Illustration of three possible roles of endothelial cells in mediating aqueous TGN1412-stimulated T cell responses in co-culture assays. Left: TGN1412 costimulates CD4+ T cells when a primary signal is generated by the interaction of allogeneic MHC class II molecules (expressed by endothelial cells) with the TCR. Middle: Endothelial cells immobilise TGN1412 presenting it to T cells in a manner that is also achieved by immobilising TGN1412 onto plastic. Right: co-stimulatory adhesion molecules (expressed by endothelial cells) promote TGN1412 activation of T cells.

To further evaluate the physiological relevance of the co-culture assay, the biological activity of the IL-2 released in response to TGN1412 was evaluated. In addition, the endothelial cell monolayer was substituted by a monolayer of fibroblasts with the aim of evaluating the importance of the endothelial cell phenotype in this assay.

Finally, the hypothesis that IL-6 responses in co-culture assays and *in vivo* could come, at least in part, from endothelial cells was investigated. Endothelial cells have been reported to release a range of cytokines including TNFα (Meyer, 2000), IFNα, IFNβ, IL-6, IL-11, IL-12 and IL-18 (Choi et al., 2004) and so are involved in immune responses through mechanisms not involving endothelial cell to immune cell contact. The concentrations of IL-6 produced by PBMC (in the absence of endothelial cells) in response to immobilised TGN1412 in Chapter 4 were compared with the concentrations of IL-6 measured in the blood of the TGN1412 trial volunteers. However, this was not the case in co-culture assays. Endothelial cells (including HUVEC) are capable of releasing IL-6 in response to TNFα stimulation (Chi et al., 2001; Huang et al., 2010). TNFα binds to the extracellular region of TNF receptor 1 expressed on the surface of endothelial cells. This induces the formation of a complex (signalosome) consisting of various signalling molecules, which stimulate several kinase cascades resulting in IL-6 expression (Pober and Sessa, 2007). Since TNFα is produced in response to TGN1412 in co-culture assays, IL-6 responses *in vivo* and in co-culture assays could come, at least in part, from endothelial cells.

6.2 Methods

Culture medium used for assays was RPMI 1640 (Sigma R0883) supplemented with 2 mM L-glutamine (Sigma G7513), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Sigma P0781) and non-essential amino acids (Gibco 11140) unless otherwise stated. Human AB serum was from Biosera (4190-100) and was pooled from mixed donors. Heat inactivation of hAB serum at NIBSC is carried out by heating stocks to 56°C for 25 min. Heat-inactivation was to inactivate complement to eliminate any cytotoxic effects caused by these proteins. All cell incubations were in a humidified incubator at 37°C in 5% CO_2.

6.2.1 Transwell experiments

Transwell plates were from Corning Life Sciences (3391) and are illustrated in Figure 36. Transwell plates were used in experiments to investigate the role of endothelial cell to PBMC contact in TGN1412-stimulated responses.

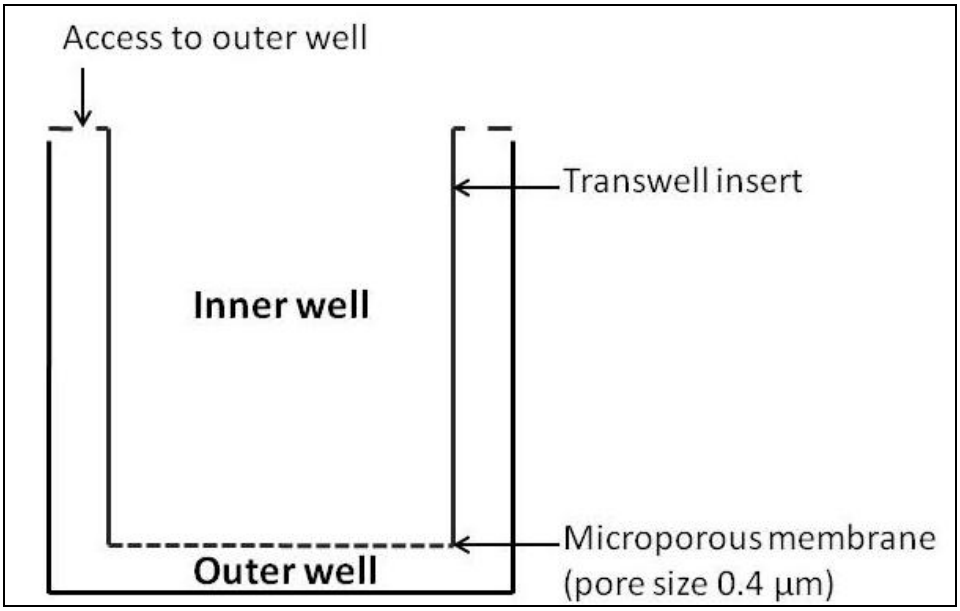


Figure 36. Simplified illustration of a Corning Transwell system.

Maintenance of HUVECjr2 cultures was as described in section 5.2.2. PBMC from healthy donors were isolated from fresh human blood as described in section 2.2. HUVECjr2 were seeded in outer wells (see Figure 36) of transwell plates at 45,000 cells/well in 150 µl culture medium containing 10% hi-hAB serum and incubated overnight to allow the cells to adhere. Spent culture media was replaced with 150 µl culture medium containing 2% hi-hAB serum. Where HUVECjr2 to PBMC contact was to be prevented, pre-soaked (in culture medium containing 2% hi-hAB serum for 1 h) transwell membranes were inserted to create the inner wells (see Figure 36) and 55,000 PBMC in 40 µl culture medium containing 2% hi-hAB serum was added to each inner well. Membranes of the inner wells of transwell plates have a much smaller surface area than the bottoms of wells of NUNC plates used in conventional co-culture assays. For this reason, PBMC were added to each inner well to give a density equivalent to that of PBMC in wells of NUNC plates (385,000 PBMC/cm^2).

TGN1412, an isotype-matched negative control antibody or endotoxin (WHO IS 94/580, NIBSC) as a positive control were added in 40 µl and 150 µl (in culture medium containing 2% hi-hAB serum), to inner and outer wells respectively to give final concentrations 0.3, 3.3 and 33.3 µg/ml TGN1412, 3.3 µg/ml isotype control concentration and 2.5 IU (250 pg)/ml endotoxin in both the inner and outer wells. TGN1412 concentrations are equivalent to 0.1, 1 and 10 µg/well in conventional co-culture assays using NUNC plates where the final volume in the well was 300 µl; a range of concentrations which invariably included the dose which stimulated the largest cytokine responses.

The same experimental conditions were used in wells where HUVECjr2 and PBMC contact was permitted in outer wells but where transwell membranes (inner wells) were not inserted. However, in addition to adding 55,000 PBMC/well, other outer wells received 280,000 PBMC so that in the presence of HUVECjr2 to PBMC contact, both the equivalent cell number (55,000 PBMC/well) and equivalent cell density (280,000 PBMC/well = 385,000 PBMC/cm^2) to PBMC over membranes in inner wells (where cell to cell contact was prevented) was used. Plates were incubated for 24 h prior to the removal of cell-conditioned medium (from the outer and inner wells, when present; medium was not pooled) which was assayed using an IL-6-specific ELISA as described in section 2.4, in which the IL-6 standard was diluted in culture medium containing 2% hi-hAB serum.

In a different experiment, outer wells in which HUVECjr2 to PBMC contact was permitted (using 280 000 PBMC/well) were set up using the experimental conditions described in the paragraph above; however, in contrast, the membranes (inner wells) were inserted above both the HUVECjr2 and PBMC to determine whether or not the presence of membranes in the assay system interfered with IL-6 responses. IL-6 was measured in cell-conditioned medium taken from the outer wells. In addition, PBMC were incubated with endotoxin (2.5 IU [250 pg]/ml) in inner wells, but without HUVECjr2 below the membranes. IL-6 was measured in cell-conditioned medium taken from the inner wells.

The presence of 55,000 PBMC could have potentially "clogged" the transwell membranes (inserted to prevent endothelial cell to PBMC contact). It was therefore important to check that soluble factors were able to move freely across the membrane because, if this was not

the case, the prevention of cell to cell contact could also have prevented "cross-talk" across the membrane mediated by soluble factors. Consequently, PBMC above the membrane (in the inner well) and HUVECjr2 below the membrane (in the outer well) were incubated with endotoxin (2.5 IU [250 pg]/ml) added either only to the inner well or only to the outer well. IL-6 was quantified in cell-conditioned medium taken from the inner well and from the outer well; medium taken from the inner and outer wells was not pooled prior to IL-6 quantification. In addition, in the absence of HUVECjr2 to PBMC cell contact, i.e. with PBMC in the inner well and HUVECjr2 in the outer well, IL-6 (WHO IS 89/548, NIBSC) at concentrations of 62 – 1000 pg/ml were added either to the inner well or to the outer well. TGN1412 was also added to wells (3.3 µg/ml) but, in contrast to IL-6, it was added to both sides of the membrane (i.e. to the inner and outer wells). The transwell plates were incubated for 24 h and IL-6 was quantified by ELISA in cell-conditioned medium taken from the inner and outer wells (this medium was not pooled).

It was thought that mixing the medium in the inner and outer wells would re-suspend the PBMC and establish a more homogenous environment within the entire system by promoting the movement of soluble factors across the membrane. Therefore, cultures of PBMC and HUVECjr2 in transwell plates with 3.3 µg/ml TGN1412 or an isotype-matched (to TGN1412) control or 2.5 IU (250 pg)/ml endotoxin, in the absence and presence of transwell membranes preventing HUVECjr2 to PBMC contact, were mixed (using a pipette) 3 times over the 24 h incubation period. Where membranes were present, cultures within the inner and outer wells were individually mixed. IL-6 was measured in cell-conditioned medium taken from inner and outer wells; medium from the inner and outer wells was not pooled.

To investigate the effects of HUVECjr2 to PBMC contact on TNFα and IL-2 responses to TGN1412 in co-culture assays, cell-conditioned medium remaining from transwell cultures in which IL-6 responses were measured (stored below -20°C) were thawed and assayed in TNFα and IL-2 specific ELISAs. Cytokine standards were prepared in culture medium containing 2% hi-hAB serum; cell-conditioned medium taken from the inner well was pooled with that taken from the outer well as there was not enough medium remaining from the inner well to assay this individually.

6.2.2 Co-culture assays involving conventional CD28 agonistic antibody

Co-culture assays were conducted as described in section 5.2.4 however, in addition to TGN1412 and an isotype control antibody, cells were incubated with a mouse anti-human CD28 mAb (eBioscience 16-0289, clone CD28.2) at 0.1, 1 and 10 µg/well for 48 h. Cell-conditioned medium was assayed for TNFα, IL-6 and IL-2 in specific ELISAs in which cytokine standards were diluted in culture medium containing 2% hi-hAB serum. Cell proliferative responses were measured by 3H-thymidine incorporation as described in section 2.5 in which the 3H-thymidine was added in culture medium containing 2% hi-hAB serum.

6.2.3 Co-culture assays to investigate TGN1412-immobilisation by HUVECjr2

Co-culture assays were conducted as described in section 5.2.4. After removing 100 µl spent medium from the HUVECjr2 monolayer after the overnight incubation following seeding, HUVECjr2 were pre-incubated with 100 µl TGN1412 or an isotype-matched negative control at 1 µg/well in culture medium containing 2% hi-hAB serum or with this supplemented culture medium alone for 5 h. Monolayers were washed gently twice with supplemented culture medium. PBMC were added together with either TGN1412 (for wells not pre-incubated with TGN1412) or supplemented culture medium alone (for wells pre-incubated with TGN1412). Cultures were incubated for 48 h prior to removal of the cell-conditioned medium which was assayed in cytokine-specific ELISAs. Cytokine standard curves were prepared in culture medium containing 2% hi-hAB serum. Cell proliferative responses were measured by 3H-thymidine incorporation as described in section 2.5 in which 3H-thymidine was added to wells in culture medium containing 2% hi-hAB serum.

6.2.4 Flow cytometric analysis of LFA-3 expression by HUVECjr2 and PBMC

HUVECjr2 were cultured as described in section 5.2.2. After trypsinisation, cells from flasks were resuspended and pooled in 20 ml sterile PBS B containing 1% BSA in centrifuge

tubes. Pooled cells were centrifuged twice at 365 x g for 5 min at room temperature. Supernatants were removed after the first centrifugation step prior to re-suspension of the cell pellet in 20 ml PBS B containing 1% BSA. After the second centrifugation step, cells were resuspended in 2 ml PBS B containing 10% rabbit serum (Sigma R9133) prior to counting and adjusting the cell concentration to 2 x 10^6 cells/ml.

PBMC were isolated from fresh blood donated by two healthy individuals at NIBSC and washed as described in section 2.2. Cells from each donor were resuspended in 20 ml PBS B containing 1% BSA and centrifuged at 365 x g for 5 min at room temperature. Supernatants were removed prior to re-suspension of the cell pellets in 2 ml PBS B containing 10% rabbit serum, counting and adjusting to 2 x 10^6 cells/ml.

Tubes containing HUVECjr2 and PBMC were incubated on ice for 10 min, prior to the addition of 100 µl HUVECjr2 or PBMC to wells of a U bottom polystyrene 96-well microtitre plate (Falcon 353077) to give 2 x 10^5 cells/well. Microtitre plates were centrifuged at 80 x g for 3 min at room temperature. Supernatants were removed using a multichannel pipette prior to the addition of 50 µl of either goat anti-human LFA-3 or control antibody (goat IgG with an irrelevant specificity; antibodies used are described in Table 7) diluted to 15 µg/ml in PBS B containing 1% BSA. Microtitre plates were incubated on ice for 30 minutes. Antibody was removed prior to the addition of 200 µl PBS B containing 1% BSA and centrifugation at 80 x g for 3 min. This washing procedure was repeated prior to the addition of 50 µl fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat antibody (Abcam ab6737) diluted 1 in 25 with PBS B containing 0.1% BSA. A smaller concentration of BSA was used here to avoid reducing the activity of formaldehyde used at a later stage. Microtitre plates were incubated on ice for 20 min prior to aspiration of the FITC-conjugated antibody from cells which were subsequently washed twice by adding 200 µl PBS B (without BSA), centrifuging at 80 x g for 3 min at room temperature and aspiration of the supernatant. Cells in each well were resuspended in 200 µl FACs Fix solution and microtitre plates were wrapped in aluminium foil and stored at 4°C overnight. Flow cytometric analysis of the cells was conducted using a FACSCanto II flow cytometer (Becton Dickenson) and FACS Diva software (Becton Dickenson) was used for data acquisition. Data was analysed using Flow Jo software (TreeStar Inc.).

6.2.5 Co-cultures involving blocking antibodies

Blocking and control antibodies used are described in Table 7. Co-culture assays were conducted as described in section 5.2.4. After removing 100 µl spent medium from the HUVECjr2 monolayer after the overnight incubation following seeding, HUVECjr2 were pre-incubated with either 100 µl relevant blocking antibody or a control antibody (class or subclass-matched to the blocking antibody) to give (final) doses shown in the Results section or in culture medium alone containing 2% hi-hAB serum. All antibodies were diluted in culture medium containing 2% hi-hAB serum. After an incubation of approximately 5 h (for blocking antibodies with an intended target expressed by HUVECjr2), 125,000 PBMC in 150 µl culture medium containing 2% hi-hAB serum were added prior to the addition of 50 µl TGN1412 at 1 µg/well as this dose invariably stimulated the largest responses in conventional co-culture assays as described in section 5.3. Other controls were an isotype-matched (to TGN1412) antibody in the absence of blocking antibody, blocking antibody alone (i.e. in the absence of TGN1412) at the largest concentration tested in wells with TGN1412, the isotype-matched (to the blocking antibody) control antibody alone at the same concentration as the blocking antibody tested alone, and culture medium containing 2% hi-hAB serum alone (no antibody). Plates were incubated for 24 or 48 h. Cell conditioned medium was assayed in cytokine-specific ELISAs and cell proliferative responses were measured by 3H-thymidine incorporation as described in Chapter 2. Culture medium containing 2% hi-hAB serum was used to dilute cytokine standards for ELISAs and 3H-thymidine.

In co-culture assays where Tysabri (an IgG4 anti-α4 integrin subunit antibody) was used as a blocking antibody, a TGN1412 analogue (NIB1412-S228P) was used instead of TGN1412 itself. The purpose of using the analogue rather than TGN1412 itself was to prevent the possible formation of half molecules and Fab arm exchange between TGN1412 and Tysabri when they were incubated together since this would have confounded the objective of the experiment by introducing a novel molecule. The analogue of TGN1412 differed from TGN1412 in that the analogue had been rendered incapable of forming half molecules and undergoing Fab arm exchange with other IgG4 molecules (Angal et al., 1993) unlike TGN1412 itself and other IgG4

antibodies including Tysabri (Labrijn et al., 2009). The analogue (NIB1412-S228P) was generated at NIBSC through a serine to proline mutation in the hinge region at amino acid position 228 (Edelman et al., 1969). Cytokine and cell proliferative responses stimulated by this analogue in co-culture assays and when immobilised had been shown to be comparable to those stimulated by TGN1412 itself. In addition, the mutated mAb had been shown to specifically bind CD28 and to be free from detectable pyrogenic contaminants (manuscript in preparation).

Co-culture assays where Tysabri was used as a blocking antibody were conducted as described above for other blocking antibodies used. Tysabri and an isotype-matched (to Tysabri) control antibody were added in 10 µl culture medium containing 2% hi-hAB serum (to give the final doses shown in the Results section) to wells containing attached HUVECjr2 after spent medium was removed. Immediately after the addition of Tysabri/isotype-matched control antibody, 150 µl PBMC were added followed by 150 µl TGN1412 analogue (NIB1412-S228P). An extended incubation of Tysabri with HUVECjr2 prior to the addition of PBMC as described above for other blocking antibodies was deemed to be unnecessary as the intended target for this antibody is expressed by PBMC.

Co-culture assays where anti-human IL-2 antibody was used to block the activity of IL-2 released by cells in response to TGN1412 were conducted as described above for other blocking antibodies. PBMC were added in 150 µl culture medium containing 2% hi-hAB serum to wells containing attached HUVECjr2 after spent medium was removed. Following this, TGN1412 and isotype-matched (to TGN1412) control antibody were added (100 µl/well) prior to the addition of 50 µl anti-IL-2 antibody or control antibody to give the (final) doses shown in the Results section or culture medium containing 2% hi-hAB serum alone.

Table 7. Blocking and control antibodies used. Blocking antibodies were free of toxic preservatives.

Blocking/control antibody	**Source**	**Description**
Anti-LFA-3	R & D Systems; AF1689	Goat IgG specific for recombinant human LFA-3
Control antibody: class-matched to anti-LFA-3	R & D systems; AB-108-C	Goat IgG with an irrelevant specificity (normal IgG from naive [non-immunised] goats)
Anti-ICAM-1	Leinco Technologies; C372, clone 15.2	Mouse IgG1 specific for human ICAM-1
Control antibody: isotype-matched to anti-ICAM-1	R & D Systems; MAB002, clone 11711	Mouse IgG1 with an irrelevant specificity (Keyhole Limpet Hemocyanin; KLH).
Anti-α4 integrin subunit	Tysabri, purchased as clinical grade	Humanised IgG4 specific for human α4 integrin subunit
Control antibody: isotype-matched to anti-α4 integrin subunit (Tysabri)	Clinical grade antibody (see section 2.6)	Humanised IgG4 with an irrelevant specificity
Anti-IL-2	R & D Systems; MAB 602, clone 5355	Mouse IgG2a specific for human IL-2
Control antibody: isotype-matched to anti-IL-2	R & D Systems; MAB003, clone 20102	Mouse IgG2a with an irrelevant specificity (KLH)

6.2.6 Antibody immobilisation assays involving blocking antibodies

The blocking and control antibodies used are described in Table 7. Wells of 96-well polypropylene U bottom plates (Corning 3790) were coated with 50 µl TGN1412 (10 µg/well) or an isotype-matched (to TGN1412) negative control (10 µg/well) in 50 µl sterile PBS B or PBS B alone and left to dry overnight in a class II laminar flow cabinet. Wells were washed 2x with 200 µl PBS B prior to the addition of 100 µl of the relevant blocking antibody or control antibody (class or subclass-matched to the blocking antibody) to give (final) doses shown in the Results section or culture medium containing 2% hi-hAB serum alone. All antibodies were diluted in culture medium containing 2% hi-hAB serum. Other controls were blocking antibody

alone (i.e. in the absence of immobilised TGN1412) at the largest concentration previously tested in wells with TGN1412, the isotype-matched (to the blocking antibody) control antibody alone at the same concentration as the blocking antibody tested alone and culture medium containing 2% hi-hAB serum alone (no antibody). PBMC were added (125,000 cells/well in 150 µl culture medium containing 2% hi-hAB serum) and wells were incubated for 24 h. Cell-conditioned medium was assayed in cytokine-specific ELISAs and cell proliferative responses were measured by 3H-thymidine incorporation as described under General Methods. Culture medium containing 2% hi-hAB serum was used to dilute cytokine standards for ELISAs and 3H-thymidine.

6.2.7 Fibroblast culture

Primary human gingival fibroblasts (HGF) were a kind gift from the Eastman Dental Institute. Cells were maintained in culture medium containing 10% hi-FCS but excluding non-essential amino acids. Cells were seeded at 0.8 x 10^6 cells in 75cm^2 flasks (Falcon, BD) after trypsinisation with trypsin-EDTA (Sigma T4049). Cells used in this study were from a single working cell bank and were used between passage 5 and passage 7.

Human foreskin fibroblasts (HFF) Hs-27 were an expanded stock, originally from European Collection of Cell Culture (94041901). Cells were maintained as for gingival fibroblasts. Cells used in this study were from a single working cell bank and were used at passage 5.

6.2.8 Co-culture assays involving PBMC and fibroblasts as the monolayer

Co-culture assays were conducted as described in section 5.2.4. Fibroblasts were seeded in wells of flat-bottomed 96-well microtitre plates at 15,000 – 20,000 cells per well in 100 µl. HGF were in maintenance medium (described in 6.2.7 above). HFF were cultured in maintenance medium containing 10% hi-hAB serum rather than hi-FCS. Following an overnight incubation at 37°C with 5% CO_2, spent medium was removed from the fibroblast

monolayer. The monolayer was incubated with 125,000 PBMC (per well) and test sample (see below) in culture medium (as described in section 6.2) containing 2% hi-FCS and 2% hi-hAB serum for HGF and 2% hi-hAB serum only for HFF (final volume in well = 300 µl) for 16 - 24 h.

Test samples included TGN1412, an isotype-matched (to TGN1412) negative control, no sample (i.e. culture medium alone) and positive controls IL-1α (International Standard 86/678, NIBSC) and IL-17 (WHO IS 01/420, NIBSC). IL-1α has previously been shown at NIBSC to stimulate pro-inflammatory cytokine release from HGF. IL-17 has also been reported to stimulate pro-inflammatory cytokine release from fibroblasts (Yao et al., 1997).

6.2.9 Stimulation by TNFα of IL-6 release from HUVEC

C-HUVEC and HUVECjr2 were seeded in wells of 96-well microtitre plates (NUNC) at 30,000 cells/well in 100 µl culture medium containing 10-15% hi-hAB serum and incubated overnight. Spent medium was removed prior to the addition of 300 µl TNFα (WHO IS 88/786, NIBSC) at concentrations of 25 – 1000 pg/ml diluted in culture medium containing 2% hi-hAB serum or supplemented culture medium alone. Cell-conditioned medium was removed after 48 h incubation and assayed in an IL-6 specific ELISA as described under General Methods in which IL-6 standard was also diluted in culture medium containing 2% hi-hAB serum.

6.2.10 Statistical analyses

Unless otherwise stated in the results section, significant differences between data sets were established using Wilcoxon's paired signed rank test.

6.3 Results

6.3.1 Effects of endothelial cell to PBMC contact on IL-6 responses stimulated by TGN1412

HUVECjr2 at 45,000 cells/well and PBMC at 55,000 cells/well were incubated for 24 h in transwell plates but were separated by a porous membrane preventing endothelial cell to PBMC contact. PBMC were incubated in inner wells above the HUVECjr2, which were in outer wells in this transwell system (see Figure 36). TGN1412 or an isotype-matched negative control was added above and below the membrane (i.e. to inner and outer wells) to achieve a final mAb concentration of 3.3 µg/ml. Figure 37 shows that under these experimental conditions, TGN1412 was unable to stimulate an IL-6 response significantly above responses to an isotype-matched negative control when IL-6 responses were measured in cell-conditioned medium sampled from the inner and outer wells, i.e. either side of the porous membrane ($p>0.05$). In contrast, IL-6 responses to a positive control, endotoxin, were marked. Under the same experimental conditions, but where PBMC were cultured in outer wells with HUVECjr2, allowing PBMC to endothelial cell contact, TGN1412 (3.3 µg/ml) stimulated IL-6 responses significantly above those to the isotype-matched negative control (3.3 µg/ml). This was found to be the case when PBMC were seeded in the outer wells (over HUVECjr2) either at an equivalent density (385,000 PBMC/cm^2 = 280,000 PBMC/well) to PBMC when seeded on transwell membranes in inner wells or at an equivalent cell number to PBMC when seeded over transwell membranes in inner wells (55 000 PBMC/well); $p<0.001$. Therefore, the lack of IL-6 responses to TGN1412 when PBMC and HUVECjr2 were separated by a membrane was not due to a small number of PBMC over the membrane. The number of PBMC added over the membrane, i.e. in inner wells, was that which gave an equivalent cell density to that of conventional co-culture assays in NUNC plates as described in Chapter 5.

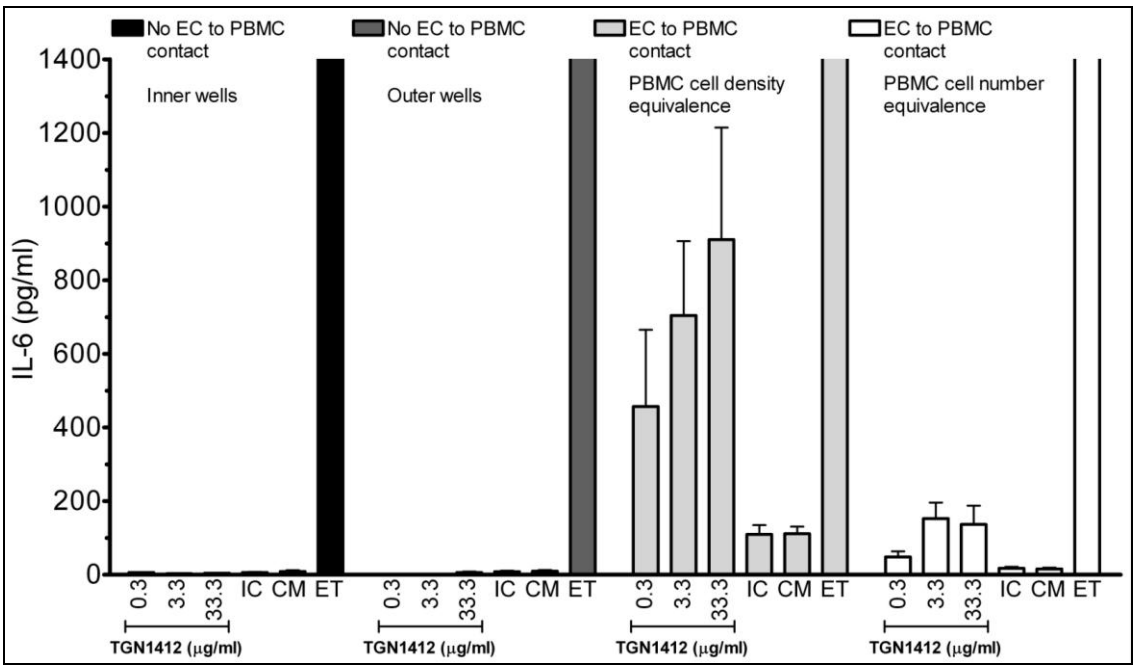


Figure 37. Effect of endothelial cell (EC) to PBMC contact on IL-6 responses to TGN1412 in 24 h co-culture assays. HUVECjr2 (ECs) and PBMC were incubated in transwell plates in which the PBMC seeded in the inner wells above the HUVECjr2 which were in the outer wells were separated by a porous membrane to prevent EC to PBMC contact (see Figure 36). Alternatively, PBMC and HUVECjr2 were incubated together in just the outer wells to allow endothelial cell to PBMC contact. In both situations, cells were incubated with TGN1412 at the doses shown, an isotype-matched negative control (IC) at 3.3 μg/ml, culture medium only or with 2.5 IU (250 pg)/ml endotoxin (ET; positive control). In wells where PBMC to endothelial cell contact was prevented, IL-6 was measured in cell-conditioned medium sampled from both the inner wells and the outer wells. In wells where EC to PBMC contact was permitted (i.e. where the membrane/inner well was omitted), PBMC were seeded either at an equivalent density (385,000 PBMC/cm^2 = 280,000 PBMC/well) or cell number (55,000 PBMC/well) in outer wells with HUVECjr2 to PBMC seeded in inner wells (i.e. over transwell membranes) when they were inserted. HUVECjr2 were seeded at 45,000 cells/well in outer wells. Values are means and S.E.M of 8-12 donors of PBMC in 2-3 independent experiments.

Other experiments showed that the lack of IL-6 responses to TGN1412 when endothelial cell to PBMC contact was prevented was not a result of inhibition caused by the physical presence of the membrane. In these experiments, IL-6 responses stimulated by TGN1412 (3.3 μg/ml) incubated with PBMC and HUVECjr2 in outer wells (i.e. in the presence of endothelial cell to PBMC contact) but with the membrane inserted (inner well) above both cell types, were not notably smaller than IL-6 responses without the membrane inserted. IL-6 was measured in cell-conditioned medium taken from the outer cells. Responses were 467 ± 167 pg/ml IL-6 in the presence of the membrane and 563 ± 156 pg/ml IL-6 in the absence of the membrane, values

are means (pg/ml) and S.E.M of 4 donors of PBMC. Also, PBMC cultured over a membrane (i.e. in inner wells) in a transwell plate with endotoxin but in the absence of endothelial cells in the outer well, released large concentrations of IL-6 in cell-conditioned medium taken from the inner wells. Responses were: 1977, 1975, 3106 and 10,455 pg/ml IL-6, values are means of duplicates for 4 donors of PBMC.

It was important to determine whether or not the 55,000 PBMC in the inner wells, that would have settled on the transwell membranes, hampered the flow of soluble factors through the membrane in wells of a transwell system. In the first experiment, endotoxin, a potent stimulant of monocytes within the PBMC fraction and of endothelial cells, was added either to the inner wells containing PBMC (i.e. above the membrane) or to the outer wells containing HUVECjr2 (i.e. below the membrane) in a transwell plate. Endotoxin was added to give a final concentration in the well (on the assumption that endotoxin was able to pass through the membrane), of 2.5 IU (250 pg)/ml. The data in Table 8 shows that IL-6 release above background concentrations could be measured in cell-conditioned medium taken from the outer and inner wells (i.e. from above and below the membrane), when endotoxin was added as the stimulus to the outer wells only or to the inner wells only. This suggests that IL-6 and/or endotoxin were able to pass in both directions across the membrane in the presence of PBMC.

Table 8. IL-6 responses measured in cell-conditioned medium from above or below a membrane separating PBMC and endothelial cells (HUVECjr2) in wells of a transwell system, to 2.5 IU (250 pg)/ml endotoxin added either above or below the membrane.

Endotoxin added:	**Cell-conditioned medium from:**	
	Above the membrane (inner wells)	**Below the membrane (outer wells)**
Above the membrane (to inner wells)	> 4000	3033 ± 646
Below the membrane (to outer wells)	> 4000	2220 ± 325

Values are means (pg/ml) and S.E.M of 4 donors of PBMC. Background IL-6 concentrations, i.e. in the absence of endotoxin, were below 12 pg/ml in cell-conditioned medium taken from either above or below the membrane.

In a different experiment, IL-6 was added either above or below the membrane (i.e. it was added either to the inner or the outer wells) separating the PBMC from the endothelial cells. TGN1412 (3.3 µg/ml) was added to both the inner and outer wells to mimic the conditions of the experiments in Figure 37. The target final IL-6 concentration in the wells (on the assumption that IL-6 would pass through the membrane after being added either above or below the membrane) was 62.5 – 1000 pg/ml. Figure 38 shows that when IL-6 was added above the membrane (to inner wells), for all 4 donors of PBMC, IL-6 could be measured at a range of concentrations (i.e. small and large) in cell-conditioned medium from both the inner and outer wells. This was also the case when IL-6 was added below the membrane (to outer wells). However, when IL-6 was added above the membrane, slightly less IL-6 was measured in cell-conditioned medium from below the membrane compared with that from above the membrane, suggesting that IL-6 concentrations were not completely homogenous. This was not the case when IL-6 was added below the membrane, i.e. for all 4 donors the concentration measured from cell-conditioned medium above and below the membrane was similar.

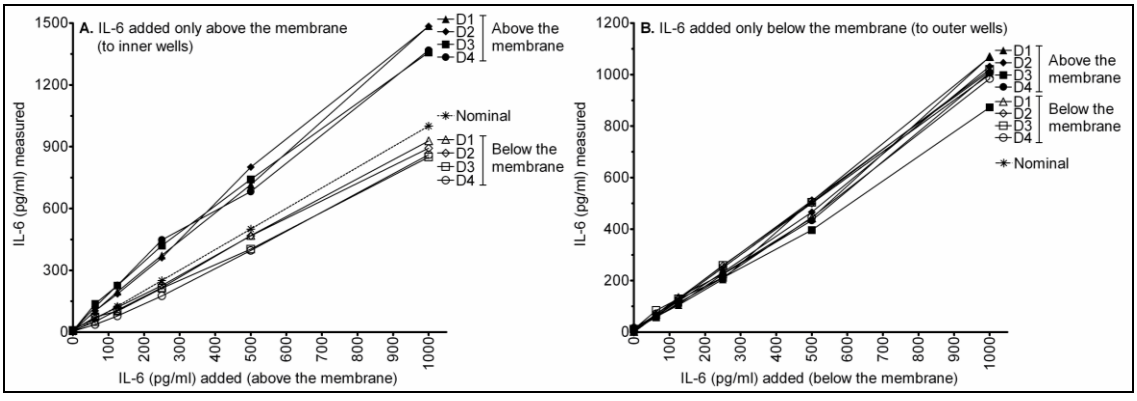


Figure 38. Capability of IL-6 to pass through the porous membrane in a transwell plate in the presence of PBMC and endothelial cells above and below the membrane respectively. IL-6 was added either above (panel A) or below (panel B) a membrane separating PBMC in inner wells (55,000 PBMC/well) from HUVECjr2 in outer wells (see Figure 36) and was incubated for 24 h with 3.3 µg/ml TGN1412. The target final IL-6 concentrations, should IL-6 have passed through the membrane and become homogenous, was 62.5 – 1000 pg/ml. IL-6 was measured in cell-conditioned medium taken from either above or below the membrane (i.e. from inner and outer wells). Values are means of duplicates for 4 different donors of PBMC (D1 – D4) from one experiment. The expected (nominal) concentration of IL-6 to be detected, i.e. the amount added, is also indicated in each panel.

It was thought that mixing the well contents in the inner and outer wells would resuspend PBMC above the membrane and would encourage the free-flow of soluble factors across the membrane, creating a homogenous medium throughout the well. Figure 39 shows that there was no difference between the IL-6 responses to TGN1412 in mixed and non-mixed wells. This was the case for cell-conditioned medium taken from the inner and outer wells. In fact, IL-6 responses to TGN1412 were not greater than those to the isotype-matched negative control in mixed and non-mixed wells, consistent with other experiments in which endothelial cell to PBMC contact was prevented (statistical analysis not possible because IL-6 concentrations were so small that actual values could not be estimated). However, endotoxin was able to stimulate marked IL-6 responses under the same experimental conditions. When endothelial cell to PBMC contact was permitted (i.e. in the absence of the membrane) IL-6 responses to TGN1412 were significantly larger than those to the isotype-matched negative control ($p<0.05$, paired t test) whether or not wells were mixed. There was little difference between IL-6 responses to TGN1412 in mixed and not mixed wells.

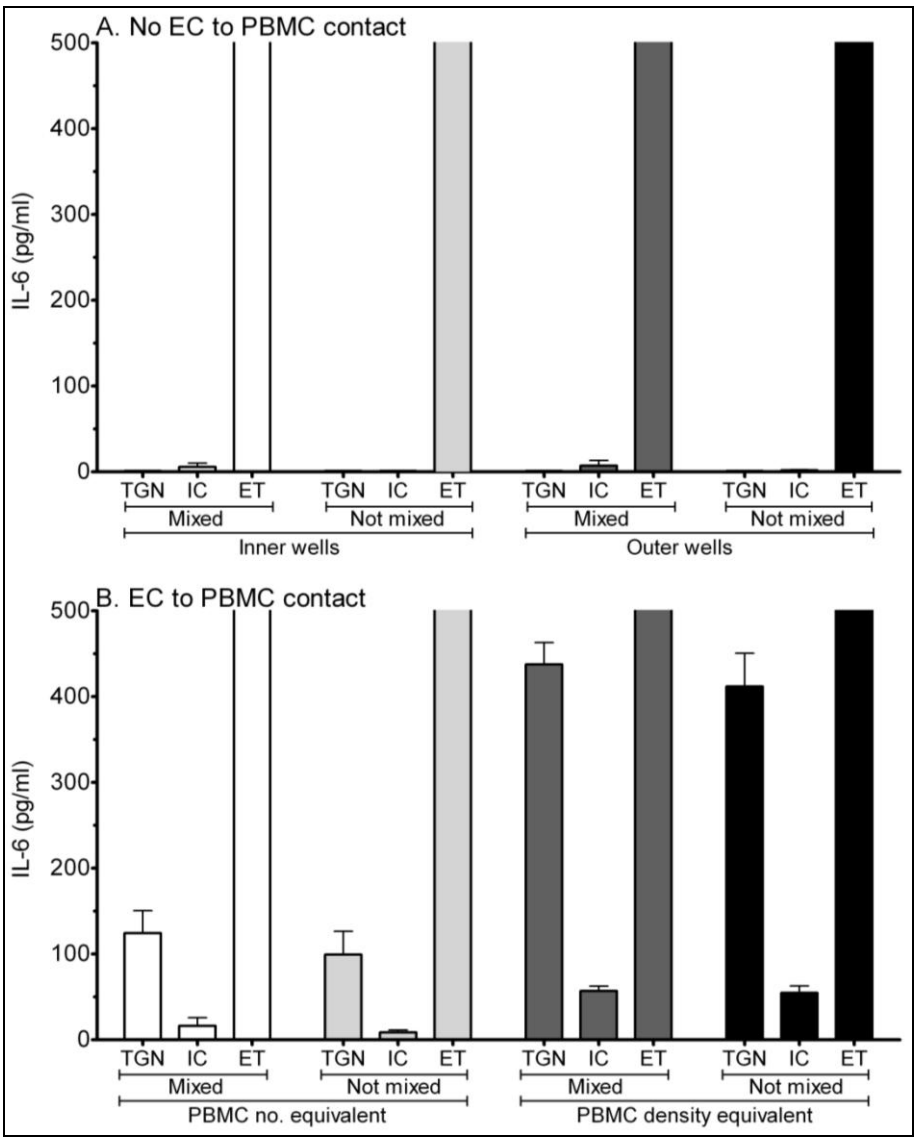


Figure 39. Effect of mixing (to achieve a homogenous environment within the well) on IL-6 responses to TGN1412 in 24 h co-culture assays in the absence and presence of endothelial cell (EC) to PBMC contact. HUVECjr2 (ECs) and PBMC were incubated in transwell plates in which the PBMC seeded in the inner wells above the HUVECjr2 which were in the outer wells were separated by a porous membrane to prevent EC to PBMC contact (see Figure 36). Alternatively, PBMC and HUVECjr2 were incubated together in just the outer wells to allow endothelial cell to PBMC contact. In both situations, cells were incubated with 3.3 µg/ml TGN1412, an isotype-matched negative control (IC) at 3.3 µg/ml or with 2.5 IU (250 pg)/ml endotoxin (ET) as a positive control. The contents of the inner and outer wells were either mixed or not mixed. Where the membrane was inserted and endothelial cell to PBMC contact was not permitted, IL-6 was measured in cell-conditioned medium sampled from both sides of the membrane (i.e. from the inner and outer wells). Where no membrane was used and EC to PBMC contact was permitted, PBMC were seeded either at an equivalent density (385,000 PBMC/cm^2 = 280,000 PBMC/well) or cell number (55,000 PBMC/well) to PBMC seeded over transwell membranes in inner wells when they were used (see above). HUVECjr2 were seeded at 45,000 cells/well in outer wells. Values are means and S.E.M of 4 donors of PBMC.

6.3.2 Effect of endothelial cell to PBMC contact on TNFα and IL-2 responses to TGN1412 in co-culture assays

It was not possible to measure TNFα and IL-2 responses to TGN1412 in cell-conditioned medium taken from above and below the membrane in transwell plates separately since only 70 µl of cell-conditioned medium could be removed from above the membrane (i.e. from the inner wells) and most of this (50 µl) was used to measure IL-6. Therefore, after establishing that soluble factors were able to pass through the membrane even with PBMC covering its surface, it was decided to measure TNFα and IL-2 concentrations produced in response to TGN1412 in cell-conditioned medium pooled from above and below the membrane (i.e. from inner and outer wells): these were the remaining aliquots of the solutions assayed to generate the data shown in Figure 37. Figure 40 shows that TGN1412 was not able to stimulate TNFα and IL-2 responses significantly above responses to the isotype-matched negative control when incubated for 24 h with HUVECjr2 which were below the membrane in outer wells and PBMC which were above the membrane in inner wells ($p>0.05$ for TNFα; however it was not possible to statistically analyse IL-2 responses since all were below the range of the IL-2 ELISA standard curve). In contrast, TNFα responses to the endotoxin positive control were marked. Under the same experimental conditions except for the absence of a porous membrane (thus permitting endothelial cell to PBMC contact), TGN1412 stimulated TNFα and IL-2 responses significantly above those to the isotype-matched negative control. This was the case when PBMC were seeded in the outer wells (with HUVECjr2) either at an equivalent density (385,000 PBMC/cm^2 = 280,000 PBMC/well) as PBMC seeded over transwell membranes in inner wells when these were used or at an equivalent cell number (55,000 PBMC/well); $p<0.05$ for TNFα. (It was not possible to statistically analyse IL-2 responses since for the isotype-matched control, all were below the range of the IL-2 ELISA standard curve.) Therefore, the lack of TNFα and IL-2 responses to TGN1412 when cells were separated by a membrane was not due to a small number of PBMC in the inner wells. Of note: the number of PBMC added above the membrane

was that which gave an equivalent cell density to that of conventional co-culture assays in NUNC plates as described in Chapter 5.

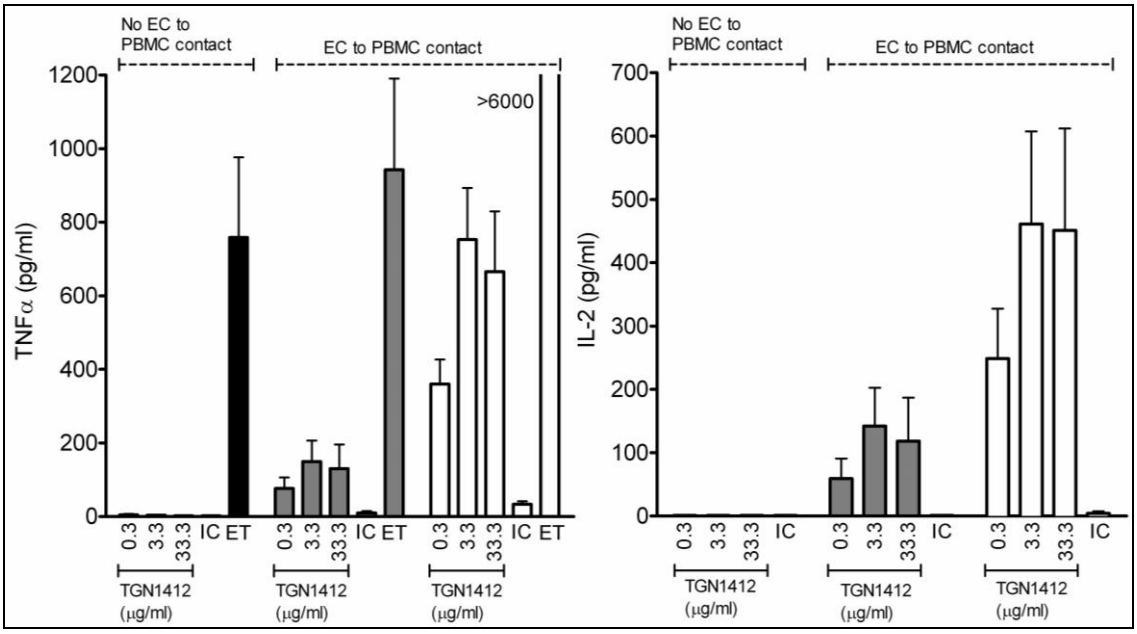


Figure 40. Effect of endothelial cell (EC) to PBMC contact on TNFα (left panel) and IL-2 (right panel) responses to TGN1412 in 24 h co-culture assays. HUVECjr2 (ECs) and PBMC were incubated either separately within transwell plates (i.e. PBMC were separated from HUVECjr2 by a porous membrane to prevent EC to PBMC contact) or together in just the outer wells of transwell plates (permitting EC to PBMC contact) with TGN1412 at the doses shown, an isotype-matched negative control (IC) at 3.3 μg/ml or with 2.5 IU (250 pg)/ml endotoxin (ET) as a positive control (TNFα only). In wells where a membrane was used, cell-conditioned medium from both sides of the membrane (from inner and outer wells) was pooled and mixed. In wells where no membrane was used and EC to PBMC contact was permitted, PBMC were seeded either at an equivalent density (385,000 PBMC/cm^2 = 280,000 PBMC/well, white columns) or cell number (55,000 PBMC/well, grey columns) as PBMC seeded over transwell membranes in inner wells when they were used. HUVECjr2 were seeded at 45,000 cells/well in outer wells. Values are means and S.E.M of 4-6 donors of PBMC in 2-3 independent experiments.

6.3.3 Comparison of cytokine and cell proliferative responses to superagonistic and agonistic anti-CD28 mAbs in co-cultures of PBMC and C-HUVEC

This experiment was carried out to investigate the possibility that allogeneic MHC class II molecules expressed by endothelial cells played a role in mediating responses to TGN1412 in co-culture assays. If this had been the case, it would have been expected that conventional agonistic anti-CD28 antibody substituted for TGN1412 would stimulate cytokine/proliferative

responses. Figure 41 shows that superagonistic TGN1412 stimulated TNFα, IL-6, IL-2 and cell proliferative responses of PBMC and C-HUVEC co-cultured for 48 h significantly above responses to an agonistic anti-CD28 mAb (not a superagonist) and to a negative control antibody, $p<0.001$. The responses stimulated by the agonistic anti-CD28 were not significantly above responses to the negative control antibody, $p>0.05$. (A one-way analysis of variance [ANOVA] with Bonferroni's multiple comparison test for TNFα, IL-6 and cell proliferation and Kruskal-Wallis test with Dunn's multiple comparisons test for IL-2, responses to 1 μg/well for all 3 antibodies were compared.) The results generated when HUVECjr2 were used as the monolayer instead of C-HUVEC were similar to those for C-HUVEC presented in Figure 41.

The small increase in TNFα and IL-6 responses to agonistic CD28 antibody at 10 μg/well may be attributable to a low level of endotoxin contamination of the antibody as the antibody used was not of clinical grade.

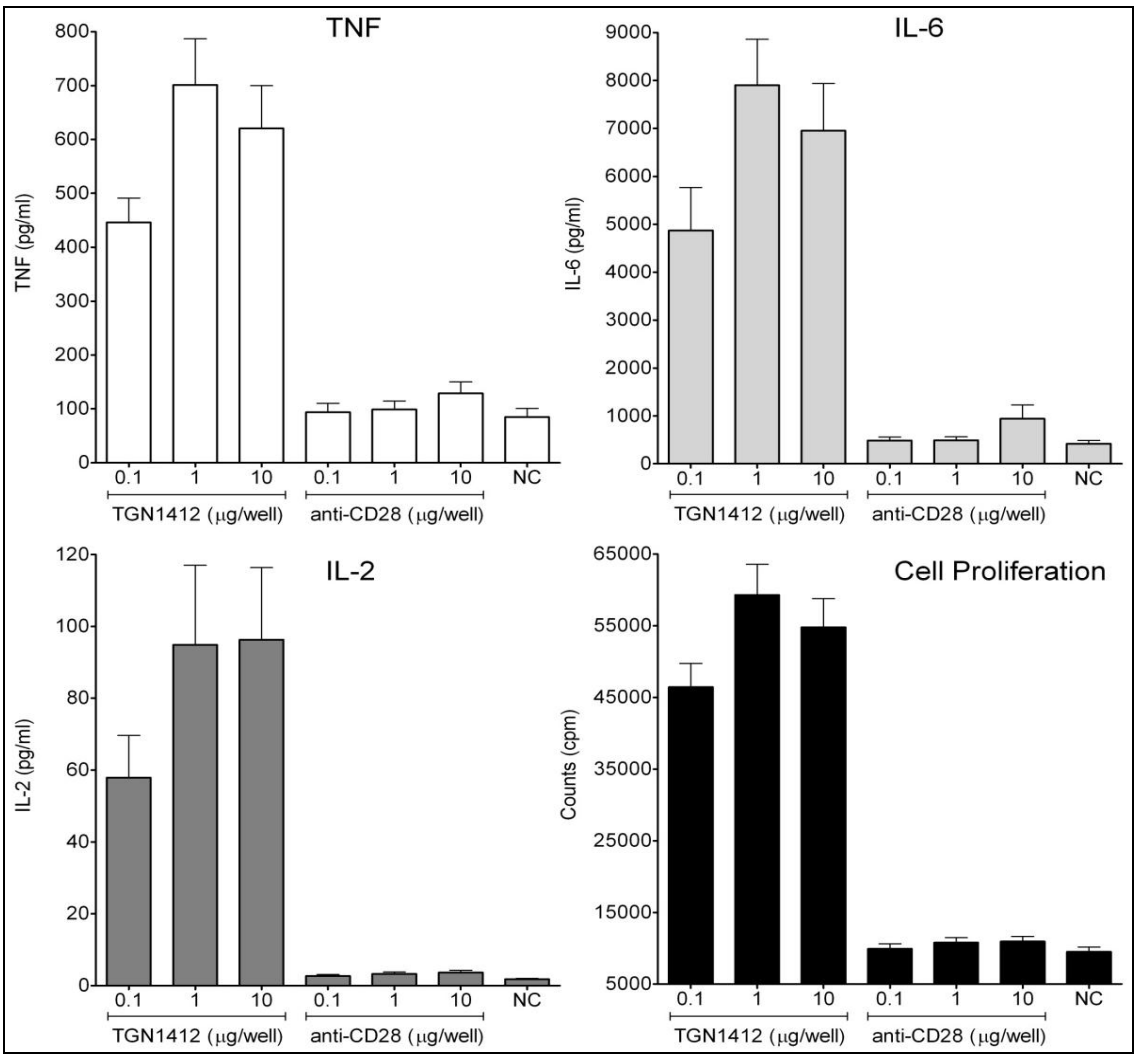


Figure 41. TNFα, IL-6, IL-2 and cell proliferative responses to superagonistic (TGN1412) and agonistic anti-CD28 mAbs and a negative control mAb (NC, 1 μg/well) in 48 h co-cultures of PBMC and C-HUVEC. Values for cytokine and cell proliferative responses are means ± S.E.M of 22-28 donations of PBMC (from 15 different donors) and 5 different umbilical cords from 4 independent experiments.

6.3.4 Cytokine and cell proliferative responses to TGN1412 pulsed with endothelial cells prior to the addition of PBMC in co-culture assays

This experiment was carried out to investigate the possibility that TGN1412 was immobilised by endothelial cells in co-culture assays. Figure 42 shows that when TGN1412 at 0.1, 1 and 10 μg/well was incubated with a monolayer of HUVECjr2 for approximately 5 h prior to washing the monolayer and adding PBMC (black bars), TNFα, IL-6, IFNγ, IL-2 and cell proliferative responses were very small and no different from responses to the isotype-matched negative control. In contrast, when TGN1412 was incubated with a co-culture of

HUVECjr2 and PBMC, after washing the monolayer, TNFα, IL-6, IFNγ, IL-2 and cell proliferative responses were notably larger than responses to the isotype-matched negative control, as can be seen from Figure 42.

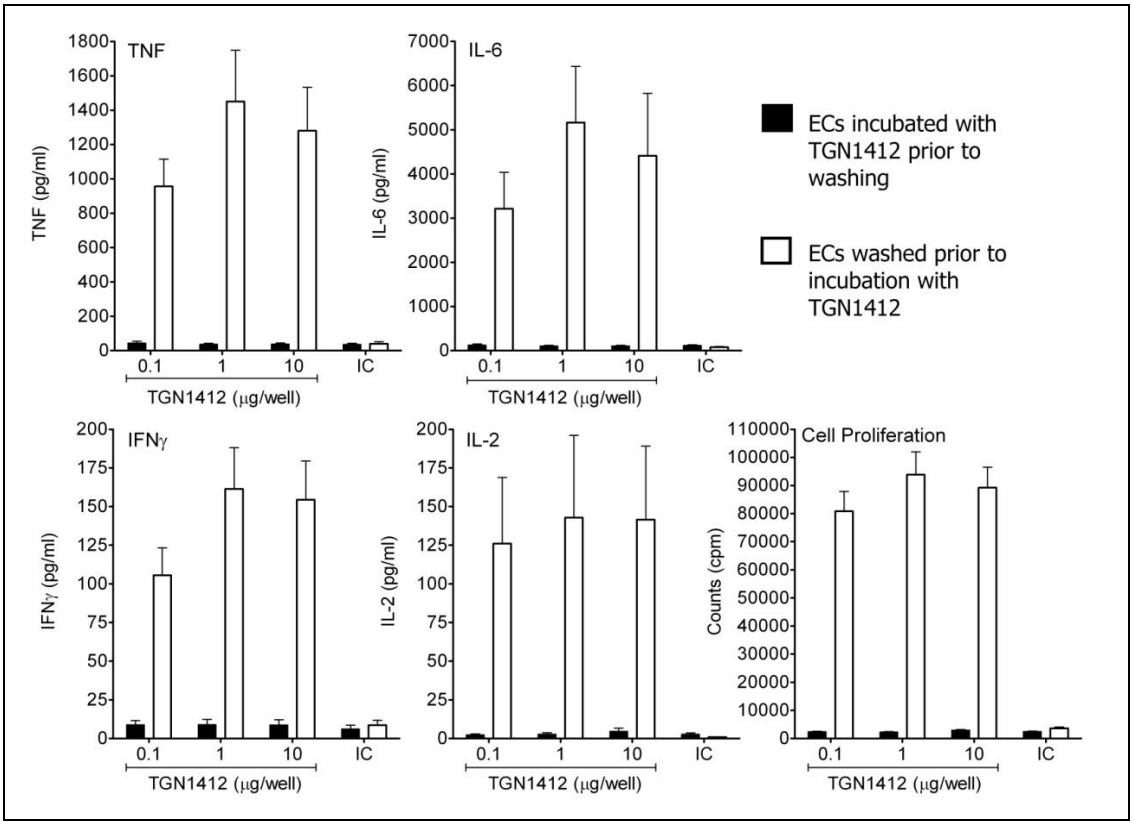


Figure 42. Effect of pulsing endothelial cells (ECs) with TGN1412 prior to the addition of PBMC in 48 h co-culture assays on TNFα, IL-6, IFNγ, IL-2 release and cell proliferative responses. TGN1412 or an isotype-matched negative control (IC, 1 μg/well) were either incubated with a monolayer of HUVECjr2 (ECs) for approximately 5 h prior to washing the monolayer and adding PBMC (black bars) or were incubated with HUVECjr2 and PBMC, i.e. after washing the monolayer (white bars). Values are means and S.E.M. of 8 donors of PBMC in 2 independent experiments.

6.3.5 Inhibitory affects of LFA-3 blocking antibody on responses to TGN1412

First, in order to determine if LFA-3 is expressed on PBMC and endothelial cells used in co-culture assays, flow cytometric analysis of the cells was carried out. This analysis showed that the fluorescence intensity emitted by the majority of HUVECjr2 in a population labelled with goat (IgG) anti-LFA-3 antibody and stained with FITC-conjugated anti-goat IgG antibody was greater than that of HUVECjr2 labelled with a control antibody (goat IgG with irrelevant

specificity) and stained in the same way. This was also true for PBMC from 2 donors confirming the expression of LFA-3 by HUVECjr2 and PBMC (see Figure 43).

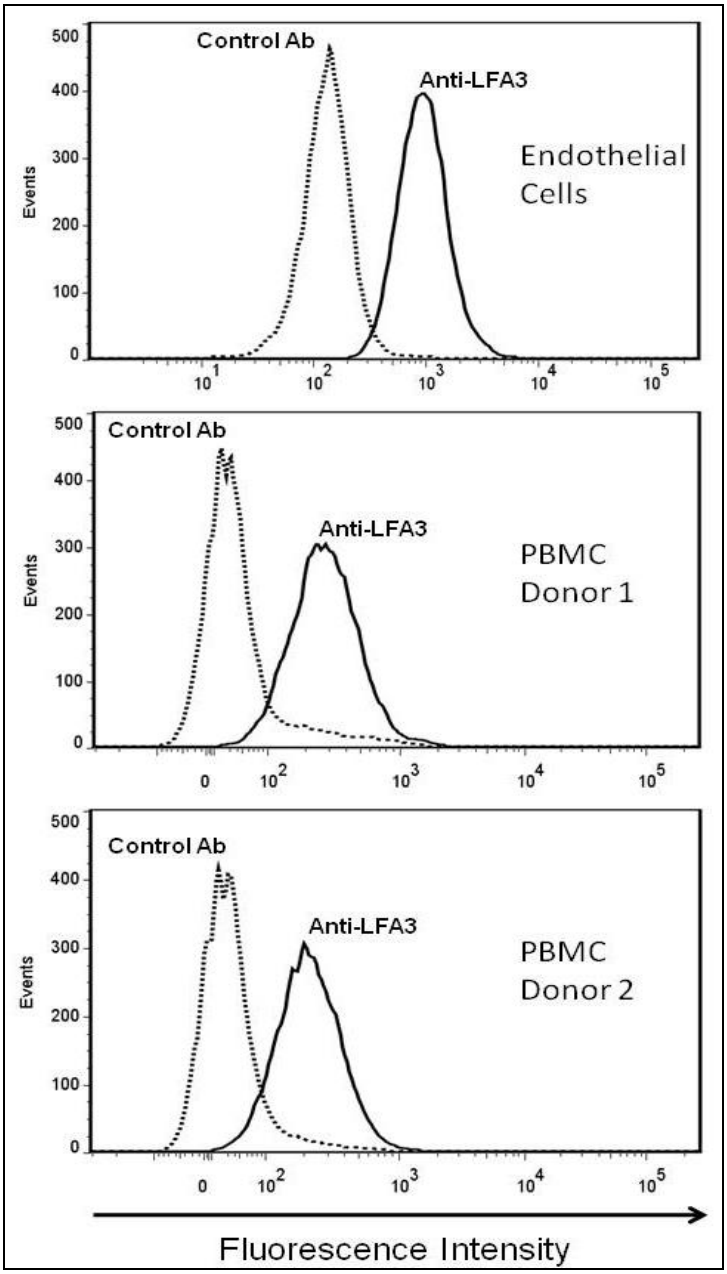


Figure 43. LFA-3 expression by endothelial cells (HUVECjr2) and PBMC measured by flow cytometry. Non-activated HUVECjr2 and PBMC from two different donors were labelled in suspension with goat (IgG) anti-human LFA-3 blocking antibody (Anti-LFA3) or goat IgG with an irrelevant specificity (Control Ab). Flow cytometric analysis of labelled cells after staining with a FITC-conjugated anti-goat IgG antibody was used to create histograms showing the number of cells in each population (events) emitting fluorescence at a range of intensities.

To determine whether or not LFA-3 played a role in responses stimulated by aqueous TGN1412 in co-culture assays, endothelial cells were incubated with a goat IgG LFA-3 blocking antibody specific for the extracellular region of human LFA-3 that prevented interaction of LFA-3 with its ligand CD2. Endothelial cells were also incubated with a control antibody (goat IgG with irrelevant specificity) or culture medium alone. Incubations were for 5 h, after which time PBMC and TGN1412 were added (LFA-3 blocking antibody and control antibody were not removed at this stage). TNFα, IL-6, IL-2 and cell proliferative responses stimulated by 1 μg/well TGN1412 in the absence of the LFA-3 blocking or control antibody were substantially larger than those to an isotype-matched (to TGN1412) negative control antibody. However, TNFα, IL-2 and, to a lesser extent, cell proliferative responses (but not IL-6 responses) to TGN1412 were inhibited by LFA-3 blocking antibody in a dose-dependent manner. In contrast, TGN1412-stimulated responses (TNFα, IL-6, IL-2 and cell proliferation) were not inhibited by a control antibody (goat IgG with irrelevant specificity). This suggests that inhibition was a result of the blocking antibody's specificity for LFA-3 and not merely because of the presence of goat IgG in the culture. The data is shown in Figure 44 where values are means of individual donor responses expressed as percentages of the response to TGN1412 alone. TNFα, IL-2 and cell proliferative responses stimulated by TGN1412 were significantly smaller when 0.1 μg/ml LFA-3 blocking antibody was present compared with those when 0.1 μg/ml control antibody was present ($p<0.01$, paired t test), but this was not the case for IL-6 responses ($p>0.05$, paired t test). All donors of PBMC tested gave a mean absolute response (of 4 replicates) to TGN1412 in the absence of blocking antibody that was at least double the mean response to the IgG4 control. There was negligible difference between TNFα, IL-6, IL-2 and cell proliferative responses of cells with LFA-3 blocking antibody and control antibody in the absence of TGN1412, compared with responses in the absence of either antibody, i.e. in culture medium alone. This shows that any (direct) stimulatory affect of these antibodies did not confound their effects on inhibition of TGN1412-stimulated responses. These data therefore suggest that ligation of LFA-3 with its ligand CD2 plays a role in responses stimulated by aqueous TGN1412 incubated in co-culture assays.

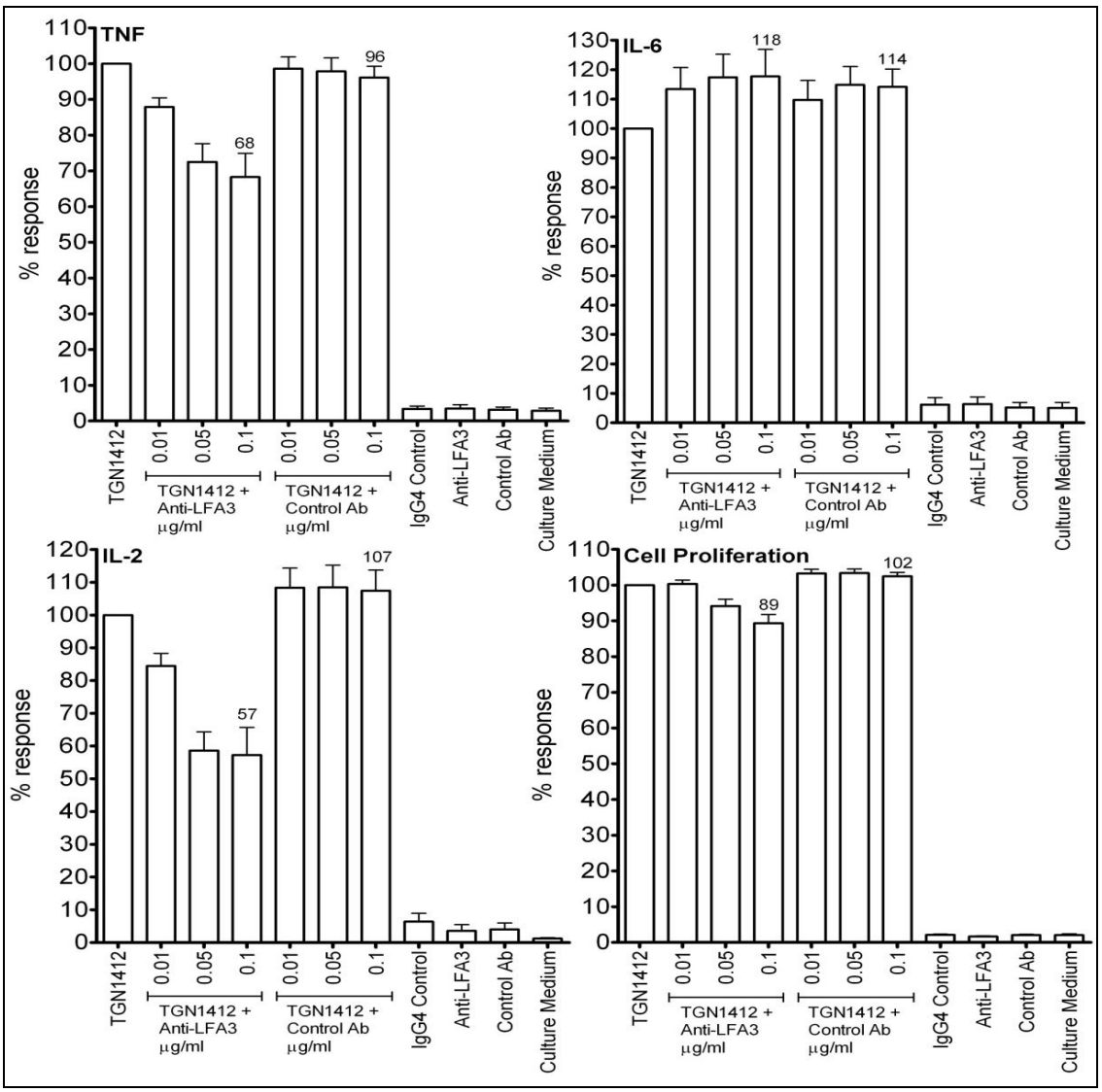


Figure 44. Inhibition of TGN1412-stimulated responses by LFA-3 blocking antibody in co-culture assays. TGN1412 (1 µg/well) was incubated in the absence and presence of a polyclonal goat (IgG) anti-human LFA-3 blocking antibody (Anti-LFA3) or goat IgG with an irrelevant specificity (Control Ab), at doses of 0.01 – 0.1 µg/ml, for 48 h with PBMC cultured over a monolayer of endothelial cells (HUVECjr2). Other controls included an isotype-matched (to TGN1412) negative control (IgG4 Control) at 1 µg/well, LFA-3 blocking antibody alone (Anti-LFA3) at 0.1 µg/ml and the control antibody alone (Control Ab) at 0.1 µg/ml. TNFα, IL-2 and cell proliferative responses are means and S.E.M of 15-16 individual PBMC donor responses expressed as percentages of the response to TGN1412 alone, from 4 independent experiments.

However, TNFα, IL-2 and cell proliferative responses to TGN1412 could not be abolished even when larger concentrations of LFA-3 antibody were used, as shown in Figure 45. In the presence of the anti-LFA-3 antibody, TNFα responses were reduced by a maximum of 51%, IL-2 responses by a maximum of 68% and proliferative responses by a maximum of

22%. Increasing the concentration of anti-LFA-3 antibody from 1 to 10 µg/ml did not further inhibit TNFα and cell proliferative responses suggesting that responses would not be further inhibited by even larger concentrations of LFA-3 blocking antibody.

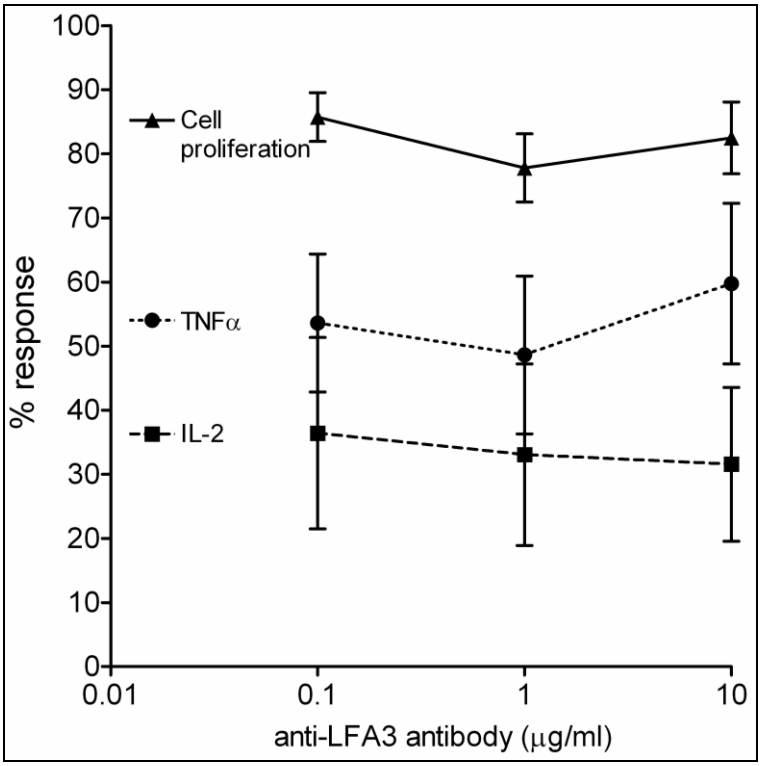


Figure 45. Effect of LFA-3 blocking antibody over a 3-log dose range on responses to TGN1412 in co-culture assays. TGN1412 was incubated at 1 µg/well in the absence and presence of LFA-3 blocking antibody at doses 0.1 – 10 µg/ml for 48 h with PBMC cultured over a monolayer of endothelial cells (HUVECjr2). TNFα, IL-2 and cell proliferative responses are means and S.E.M of 4 individual PBMC donor responses expressed as percentages of the response to TGN1412 alone, from 1 experiment.

There was no difference between optical density values given by cytokine-specific ELISAs for a range of TNFα, IL-6 and IL-2 standard concentrations prepared with either LFA-3 blocking antibody or control antibody at the maximum concentration used to block LFA-3 (10 µg/ml) or when prepared alone (Figure 46). This suggests that the effects of LFA-3 blocking antibody and control antibody on responses to TGN1412 in co-culture assays were not because they interfered with the detection of cytokines in cytokine-specific ELISAs.

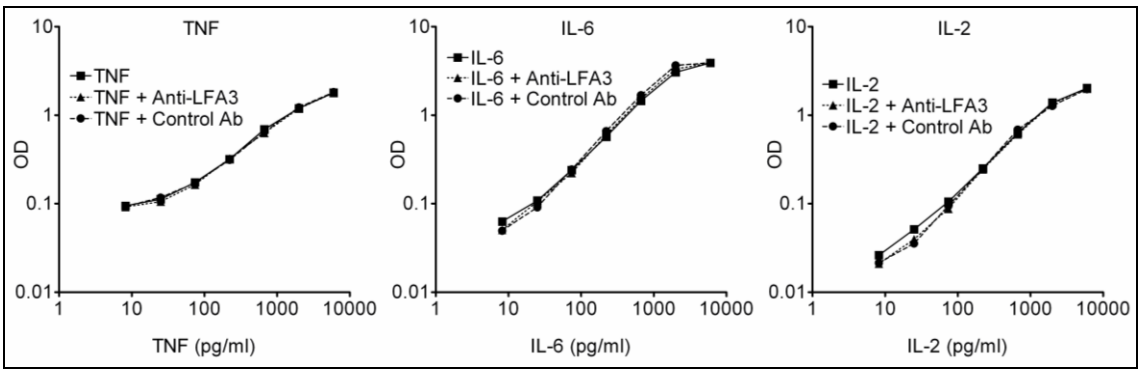


Figure 46. Effect of LFA-3 blocking antibody and control antibody on the detection of cytokines by cytokine-specific ELISAs. Dilution series of TNFα, IL-6 or IL-2 recombinant cytokine standards were prepared in the presence and absence of 10 µg/ml LFA-3 blocking antibody or control antibody. Each of the dilution series prepared was assayed by ELISA. Values are means of duplicates for each concentration of cytokine tested in one assay.

To determine whether or not ligation of LFA-3 to its receptor played a role in PBMC responses stimulated by immobilised TGN1412 (i.e. in the absence of endothelial cells), 24 h assays were carried out in which PBMC were incubated with immobilised TGN1412 and with either a goat IgG LFA-3 blocking antibody, a control antibody (goat IgG with irrelevant specificity) or culture medium alone. TNFα, IL-2 and cell proliferative responses stimulated by 10 µg/well TGN1412 in the absence of the LFA-3 blocking or control antibodies were substantially larger than those to an isotype-matched (to TGN1412) negative control antibody. These responses stimulated by TGN1412 were not inhibited by the addition of LFA-3 blocking antibody to an extent significantly greater than the extent of inhibition caused by control antibody, at any of the concentrations tested (0.001 – 1 µg/ml, $p>0.05$, one-way ANOVA with Bonferroni's Multiple Comparisons Test). This is shown in Figure 47 where values are means of 4 individual PBMC donor responses expressed as percentages of the response to immobilised TGN1412 alone. The four donors of PBMC selected for the experiment to determine the effect of LFA-3 blocking antibody on the effects of immobilised TGN1412 (Figure 47) were chosen because they had all been shown to yield PBMC that were sensitive to blockade by the LFA-3 blocking antibody of TGN1412-evoked responses in co-culture assays (Figure 44). These data therefore suggest that LFA-3 does not play a role in PBMC responses stimulated by immobilised TGN1412 in the absence of endothelial cells. Furthermore, these data rule out the

possibility that the inhibitory effects of LFA-3 blocking antibody on TGN1412-mediated responses in co-culture assays was a result of cytotoxicity caused by the blocking antibody since immobilised TGN1412 stimulated marked cell proliferation in the presence of LFA-3 blocking antibody.

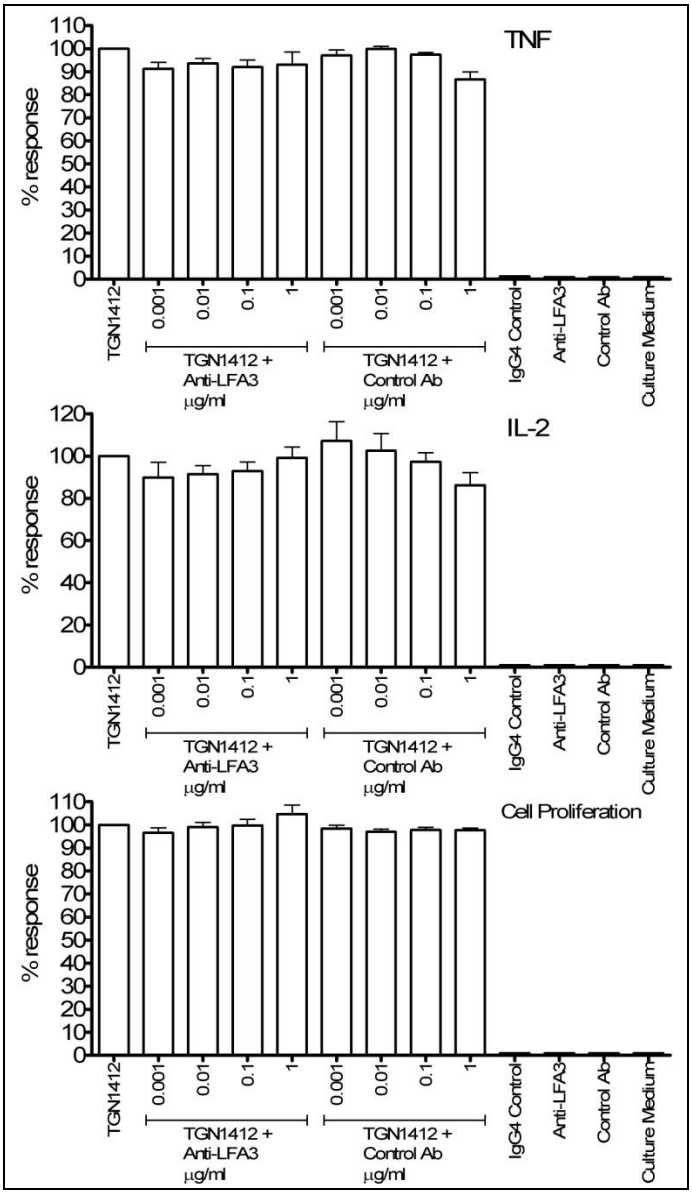


Figure 47. Effects of LFA-3 blocking antibody on PBMC responses to immobilised TGN1412. TGN1412 (10 µg/well) was immobilised by air-drying and incubated with PBMC in the absence and presence of a polyclonal goat (IgG) anti-human LFA-3 blocking antibody (Anti-LFA3) or goat IgG with an irrelevant specificity (Control Ab), at doses of 0.001 – 1 µg/ml for 24 h. Other controls included an immobilised isotype-matched (to TGN1412) negative control (IgG4 Control) at 10 µg/well, LFA-3 blocking antibody alone (Anti-LFA3) at 1 µg/ml and the control antibody alone (Control Ab) at 1 µg/ml. TNFα, IL-2 and cell proliferative responses are means and S.E.M of 4 individual donor responses expressed as percentages of the response to TGN1412 alone, from 1 experiment.

6.3.6 Inhibitory affects of ICAM-1 blocking antibody on cell proliferative responses to TGN1412.

To determine whether or not ligation of ICAM-1 to its receptor plays a role in responses stimulated either by aqueous TGN1412 in co-culture assays (with PBMC and endothelial cells) or by immobilised TGN1412 in assays with PBMC only, responses stimulated by TGN1412 in both types of assay were measured in the absence and presence of a mouse monoclonal (IgG1) anti-human ICAM-1 blocking antibody (Anti-ICAM-1). Also included in these experiments was a mouse monoclonal IgG1 antibody with an irrelevant specificity (Control Ab) at a range of concentrations. In co-culture assays, endothelial cells were incubated with ICAM-1 blocking antibody and control antibody or culture medium alone for 5 h, after which time PBMC and TGN1412 were added (i.e. in the presence of ICAM-1 blocking antibody and control antibody). Cell proliferative responses stimulated by 1 µg/well TGN1412 in the absence of the ICAM-1 blocking antibody or control antibody were substantially larger than those to an isotype-matched (to TGN1412) negative control antibody. However, cell proliferative responses to TGN1412 were inhibited by ICAM-1 blocking antibody in a dose-dependent manner and, to a much lesser extent, by control antibody. This is shown in Figure 48 where values are means of 4 individual PBMC donor responses expressed as percentages of the response to TGN1412 alone. Cell proliferative responses stimulated by TGN1412 were statistically significantly smaller when 3.3 µg/ml ICAM-1 blocking antibody was used than responses when 3.3 µg/ml control antibody was used ($p<0.0001$, paired t test). In contrast to the inhibitory affects of ICAM-1 blocking antibody in co-culture assays, this antibody and control antibody did not inhibit cell proliferative responses of PBMC to immobilised TGN1412. In addition, there was no significant difference statistically between TGN1412-stimulated cell proliferative responses using ICAM-1 blocking antibody and control antibody ($p>0.05$ at all concentrations tested, one-way ANOVA with Bonferroni's Multiple Comparisons test).

For both types of assay (co-culture and immobilised antibody), there was negligible difference between cell proliferative responses of cells to ICAM-1 blocking antibody and control antibody in the absence of TGN1412, compared with responses in the absence of any

antibody, i.e. in culture medium alone. Therefore, the extent of inhibition of TGN1412-stimulated responses caused by these antibodies was not confounded by any (direct) stimulatory affect of these antibodies. Therefore, these data suggest that ICAM-1 plays a role in cell proliferative responses stimulated by aqueous TGN1412 incubated in co-culture assays but not in responses stimulated by immobilised TGN1412 incubated with PBMC alone. Furthermore, the same 4 donors of PBMC were used for both types of assay, ruling out donor to donor variation as a reason for the lack of inhibition of immobilised TGN1412-stimulated responses of PBMC alone. These data also rule out the possibility that the inhibitory effect of ICAM-1 blocking antibody on TGN1412-mediated responses in co-culture assays was a result of cytotoxicity caused by the blocking antibody since immobilised TGN1412 stimulated profound T cell proliferation in the presence of ICAM-1 blocking antibody.

Unfortunately, it was not possible to determine the affects of ICAM-1 blocking antibody on TNFα and IL-6 responses to TGN1412 as the antibody used stimulated substantial IL-6 and TNFα responses when incubated in co-culture assays or with PBMC alone, in the absence of TGN1412. A test for the presence of bacterial endotoxins (Limulus Amebocyte Lysate test carried at NIBSC) revealed that 3.3 µg/ml ICAM-1 blocking antibody (i.e. the largest concentration tested in the present study) contained 0.2 IU (20 pg)/ml endotoxin, which, when considering the endotoxin dose response curve shown in Figure 11, is a sufficient amount of endotoxin to stimulate the release of substantial concentrations of pro-inflammatory cytokines such as TNFα and IL-6.

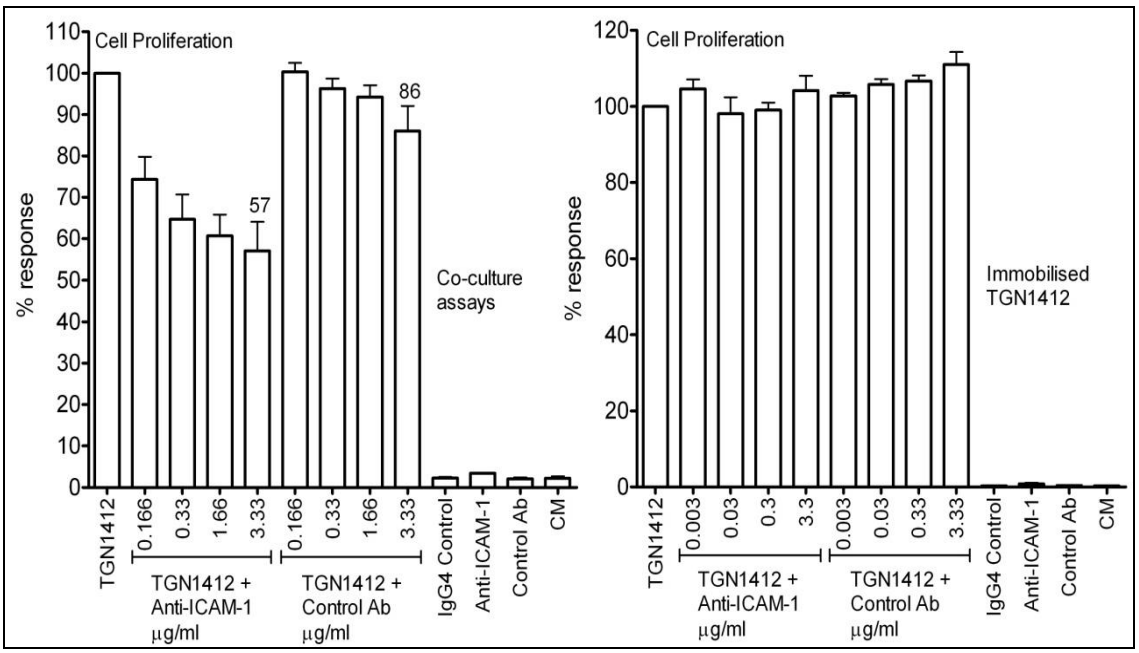


Figure 48. Inhibition of responses by ICAM-1 blocking antibody, to aqueous TGN1412 in co-culture assays and to immobilised TGN1412 incubated with PBMC alone. Co-culture assays: TGN1412 (1 μg/well) was incubated in the absence and presence of mouse monoclonal (IgG1) anti-human ICAM-1 blocking antibody (Anti-ICAM-1) or mouse monoclonal IgG1 antibody with an irrelevant specificity (Control Ab), at concentrations ranging between 0.166 – 3.33 μg/ml for 48 h with PBMC cultured over a monolayer of endothelial cells (HUVECjr2). Immobilised TGN1412: TGN1412 (10 μg/well) immobilised by air-drying onto polypropylene was incubated with PBMC in the absence and presence of ICAM-1 blocking antibody or control antibody at concentrations ranging between 0.003 – 3.3 μg/ml for 24 h. Other controls in both types of assay included an isotype-matched (to TGN1412) negative control (IgG4 Control) at 1 μg/well for co-culture and 10 μg/well when immobilised, ICAM-1 blocking antibody alone and control antibody alone, both at 3.3 μg/ml for both types of assay. Cell proliferative responses are means and S.E.M of 4 individual PBMC donor responses expressed as percentages of the response to TGN1412 alone, from 1 experiment for each type of assay. The same 4 donors of PBMC were used for both assays.

6.3.7 Inhibitory effects of α4 integrin blocking antibody (Tysabri) on responses to a TGN1412 analogue in co-culture assays

To determine whether or not integrins VLA-4 and α4:β7 play a role in mediating TGN1412-stimulated responses in co-culture assays following ligation to their receptors, VCAM-1 and MAdCAM-1 respectively, the therapeutic IgG4 mAb Tysabri was used as a blocking antibody owing to its specificity for the α4 integrin subunit of VLA-4 and α4:β7. Twenty-four hour co-culture assays (with HUVECjr2 as the monolayer) were carried out in

which a TGN1412 analogue (NIB1412-S228P) was used. This analogue was rendered incapable of forming and exchanging half molecules with other IgG4 molecules (unlike TGN1412 itself) as described in section 6.2.5. NIB1412-S228P was incubated in the absence and presence of Tysabri, or control antibody (IgG4 with irrelevant specificity) at concentrations of 0.1 – 10 μg/well. The purpose of using NIB1412-S228P rather than TGN1412 itself was to prevent the possible exchange of half molecules between TGN1412 and Tysabri when they were incubated together since this would have confounded the objective of the experiment by introducing a novel molecule. TNFα, IL-6, IL-2 and cell proliferative responses stimulated by 1 μg/well NIB1412-S228P were not inhibited by the addition of α4 integrin subunit blocking antibody (Tysabri) at any of the concentrations tested or by a control antibody. In most cases mean responses to NIB1412-S228P in the presence of α4 integrin subunit blocking antibody or control antibody were marginally larger than responses to NIB1412-S228P alone, as shown in Figure 49. Inhibition of NIB1412-S228P-stimulated TNFα and IL-2 responses caused by α4 integrin subunit blocking antibody and control antibody was not confounded by any (direct) stimulatory effect of these antibodies since responses to these antibodies in the absence of NIB1412-S228P were negligible. The small (direct) stimulatory effects of α4 integrin subunit blocking antibody and control antibody in terms of IL-6 release and cell proliferative responses would not have confounded inhibitory effects to any appreciable extent. These data suggest that interaction of α4 integrin subunit-containing integrins (VLA-4 and α4:β7) with their ligands does not play a role in responses stimulated by TGN1412 in co-culture assays.

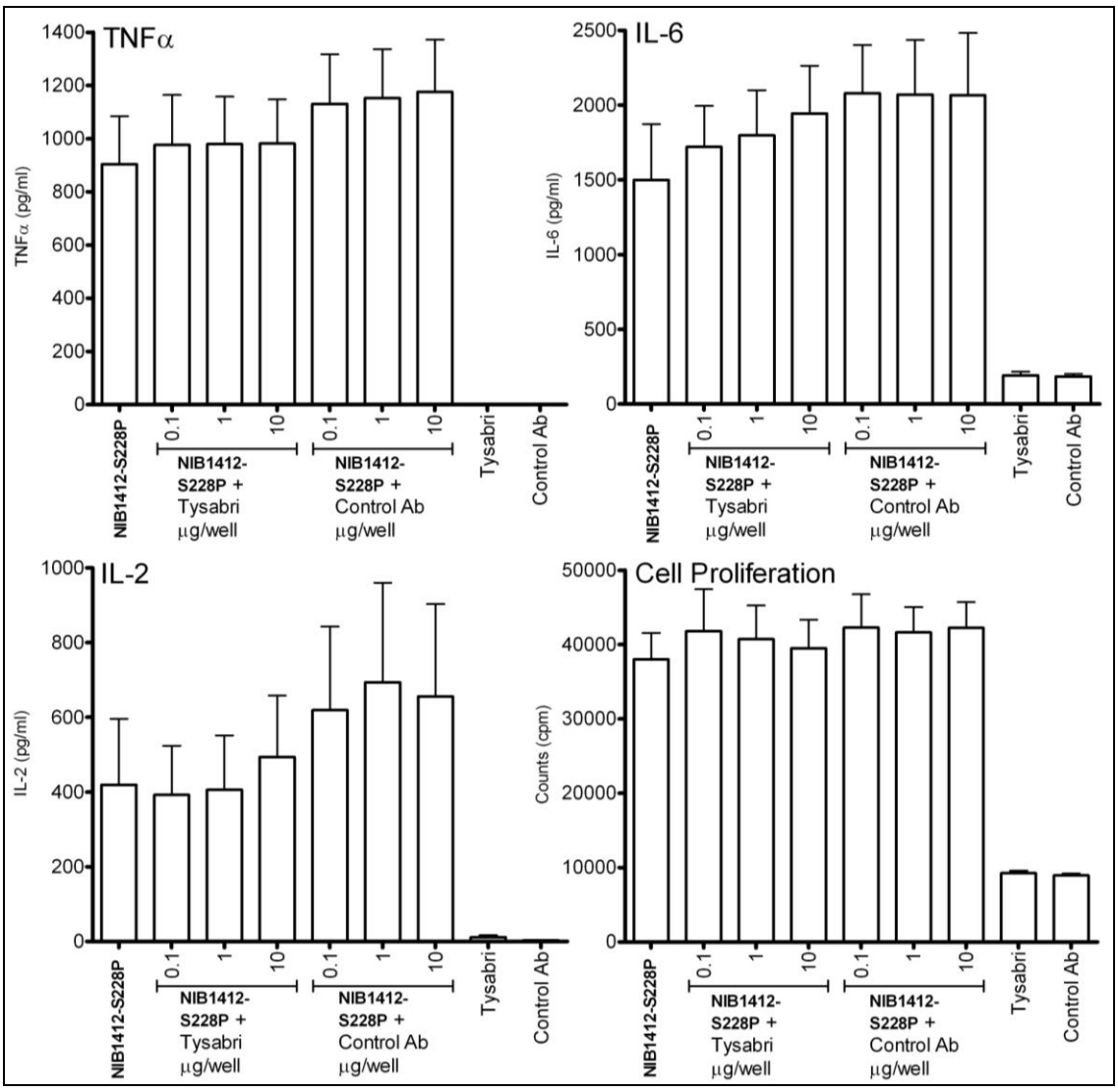


Figure 49. Effects of an α4 integrin subunit blocking antibody on responses to a TGN1412 analogue. A TGN1412 analogue (NIB1412-S228P) rendered incapable of forming and exchanging half molecules with other IgG4 molecules (unlike TGN1412 itself) was incubated at 1 µg/well in the absence and presence of Tysabri, an IgG4 α4 integrin blocking antibody, and a control antibody (IgG4 with an irrelevant specificity) at concentrations between 0.1 – 10 µg/well for 24 h with PBMC cultured over a monolayer of endothelial cells (HUVECjr2). Tysabri and control antibody were also incubated in the absence of the TGN1412 analogue under the same experimental conditions. TNFα, IL-6, IL-2 and cell proliferative responses are means and S.E.M of 4 donors of PBMC from 1 experiment.

6.3.8 Inhibitory effects of an anti-human IL-2 antibody on cell proliferative responses to TGN1412 in co-culture assays

To investigate the dependence of TGN1412-stimulated cell proliferative responses in co-culture assays on the presence of IL-2, TGN1412 was incubated in 48 h co-culture assays using

HUVECjr2 as the monolayer in the absence and presence of a mouse (IgG2a) anti-human IL-2 antibody (0.1 – 30 μg/ml) or an isotype-matched (IgG2a) negative control with an irrelevant specificity. Figure 50 shows that cell proliferative responses stimulated by TGN1412 (1 μg/well) were inhibited by the anti-human IL-2 antibody in a dose-dependent manner, the maximum reduction being approximately 40%. In contrast, cell proliferative responses were not inhibited by control antibody suggesting that inhibition was the result of the specificity of the anti-IL-2 antibody rather than by the mere presence of a mouse IgG2a antibody. These data suggest that TGN1412-stimulated cell proliferation is at least partially dependent on IL-2 production in co-culture assays.

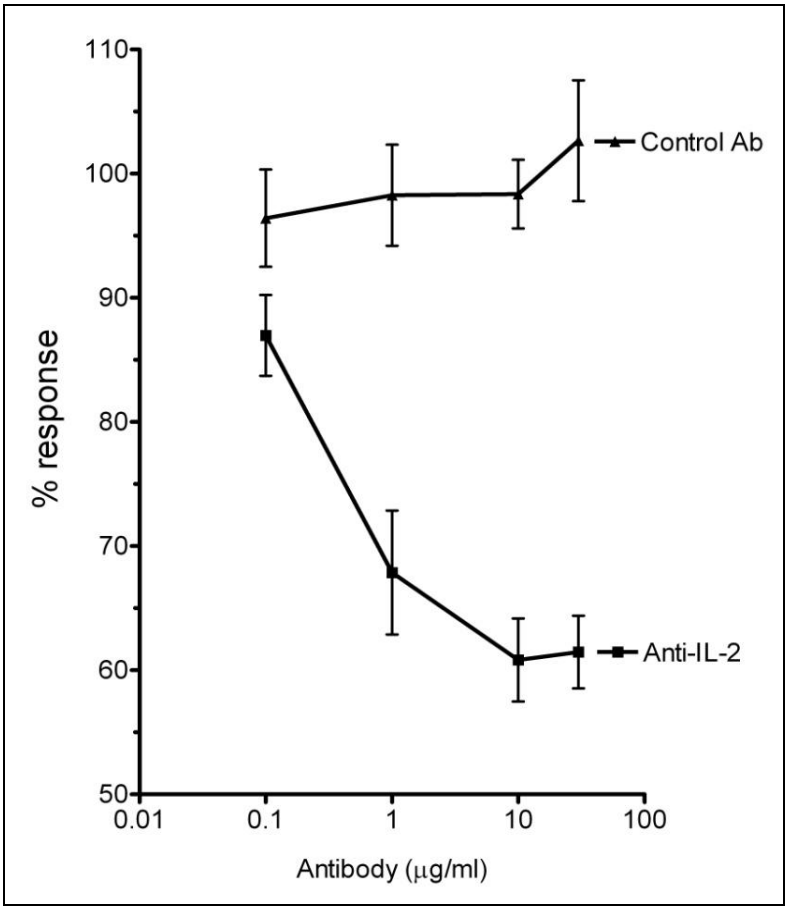


Figure 50. Effect of anti-IL-2 antibody on TGN1412-stimulated cell proliferative responses in co-culture assays. TGN1412 was incubated at 1 μg/well in the absence and presence of a mouse (IgG2a) anti-human IL-2 antibody and an isotype-matched (mouse IgG2a) negative control antibody with an irrelevant specificity, at concentrations between 0.1 – 30 μg/ml for 48 h with PBMC cultured over a monolayer endothelial cells (HUVECjr2). Cell proliferative responses are means and S.E.M of 4 individual PBMC donor responses expressed as percentages of the response to TGN1412 in the absence of anti-IL-2 or control antibody.

6.3.9 The effect of co-culturing PBMC over a monolayer of fibroblasts on TNFα, IL-6 and IL-8 responses to TGN1412

Figure 51 shows that TNFα, IL-6 and IL-8 responses to TGN1412 incubated in aqueous phase for 16 – 24 h with either HGF or HFF were very small and were not significantly different from responses to the isotype-matched negative control ($p>0.05$ when comparing responses to TGN1412 at 1 µg/well with responses to the isotype-matched negative control at 1 µg/well). In addition, cytokine responses to TGN1412 incubated for 16 – 24 h with PBMC over a monolayer of HGF, despite being larger than responses of HGF alone, were not significantly different from responses to the isotype-matched negative control ($p>0.05$ when comparing responses to TGN1412 at 1 µg/well with responses to the isotype-matched negative control at 1 µg/well). In contrast cytokine responses to the positive control IL-1α, incubated either with HGF alone or with PBMC co-cultured over a monolayer of HGF, were marked. For comparison, in co-culture assays of PBMC over a monolayer of HUVECjr2 rather than HGF (using 2% FCS and 2% hAB together in the culture medium rather than 2% hAB serum alone) TNFα, IL-6 and IL-8 responses to 1 µg/well TGN1412 were notably larger than those to 1 µg/well isotype-matched negative control. This suggests that the small TGN1412-specific responses in co-culture assays using HGF were unlikely to have been a result of using a mixture of human and bovine serum. The mixture was chosen because HGF were found to be unable to adhere to plates in serum containing hAB alone and because, as shown in Chapter 5, TGN1412 was unable to stimulate responses when FCS was used as the supplement.

TNFα, IL-6 and IL-8 responses to TGN1412 incubated for 24 h with PBMC co-cultured over a monolayer of HFFs, despite being larger than responses of HFFs alone, were not substantially different from responses to the isotype-matched negative control ($p>0.05$ when comparing responses to TGN1412 at 1 µg/well with responses to the isotype-matched negative control at 1 µg/well). In contrast, cytokine responses to the positive control, IL-17, incubated either with HFFs alone or with PBMC co-cultured over a monolayer of HFFs, were marked.

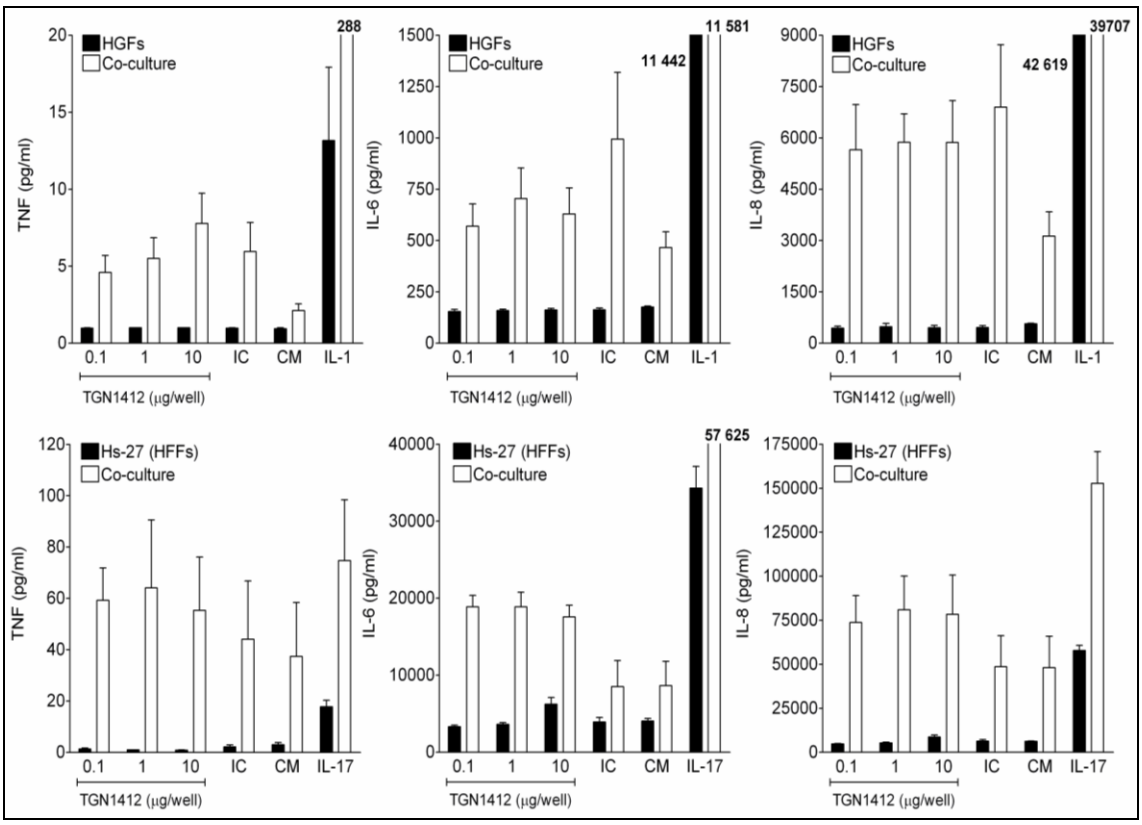


Figure 51. The effect of co-culturing PBMC over a monolayer of HGFs (top) or HFFs (bottom) on TNFα, IL-6 and IL-8 responses to TGN1412. TGN1412 at doses of 0.1, 1 and 10 µg/well, an isotype-matched negative control (IC) at 1 µg/well, culture medium alone (CM) and either IL-1α at 100 pg/ml or IL-17 at 200 pg/ml were incubated for 16 - 24 h in aqueous phase with either HGFs or HFFs alone (filled columns) or with PBMC co-cultured over a monolayer of HGFs or HFFs (open columns). For HGFs, values for the cytokine responses (pg/ml) are means ± S.E.M of 8 donors of PBMC and 1 donor of HGFs from 2 independent experiments. For HFFs, values are means ± S.E.M of 4 donors of PBMC and 1 donor of HFFs.

6.3.10 Stimulation by TNFα of IL-6 release from human umbilical vein endothelial cells.

Figure 52 shows that TNFα stimulates dose-dependent release of IL-6 when incubated for 48h with C-HUVEC or HUVECjr2 (30,000 cells/well), i.e. under the same culture conditions that were used for co-culture assays (of PBMC over C-HUVEC or HUVECjr2). IL-6 release from C-HUVEC and HUVECjr2 was very similar when they were stimulated with doses of TNFα up to 400 pg/ml; above this dose, IL-6 release was greater from C-HUVEC than from HUVECjr2. When stimulated with 25,600 pg/ml TNFα (i.e. the maximum dose tested), IL-6 release was approximately 970 pg/ml from C-HUVEC and 420 pg/ml from HUVECjr2. The

concentrations of TNFα released in response to TGN1412 in co-culture experiments (approximately 1100 pg/ml TNFα for HUVECjr2 and 600 pg/ml TNFα for C-HUVEC), stimulated only approximately 170 pg/ml IL-6 release from HUVECjr2 alone and 190 pg/ml IL-6 release from C-HUVEC alone, respectively. These concentrations of IL-6 released from HUVECjr2 alone and C-HUVEC alone fall a long way short of the concentrations of IL-6 released in co-culture experiments (approximately1750 pg/ml for HUVECjr2 and 7380 pg/ml for C-HUVEC). This suggests that IL-6 release from endothelial cells, stimulated by TNFα alone, is only partially responsible for the IL-6 concentrations measured in cell-conditioned medium in co-culture assays.

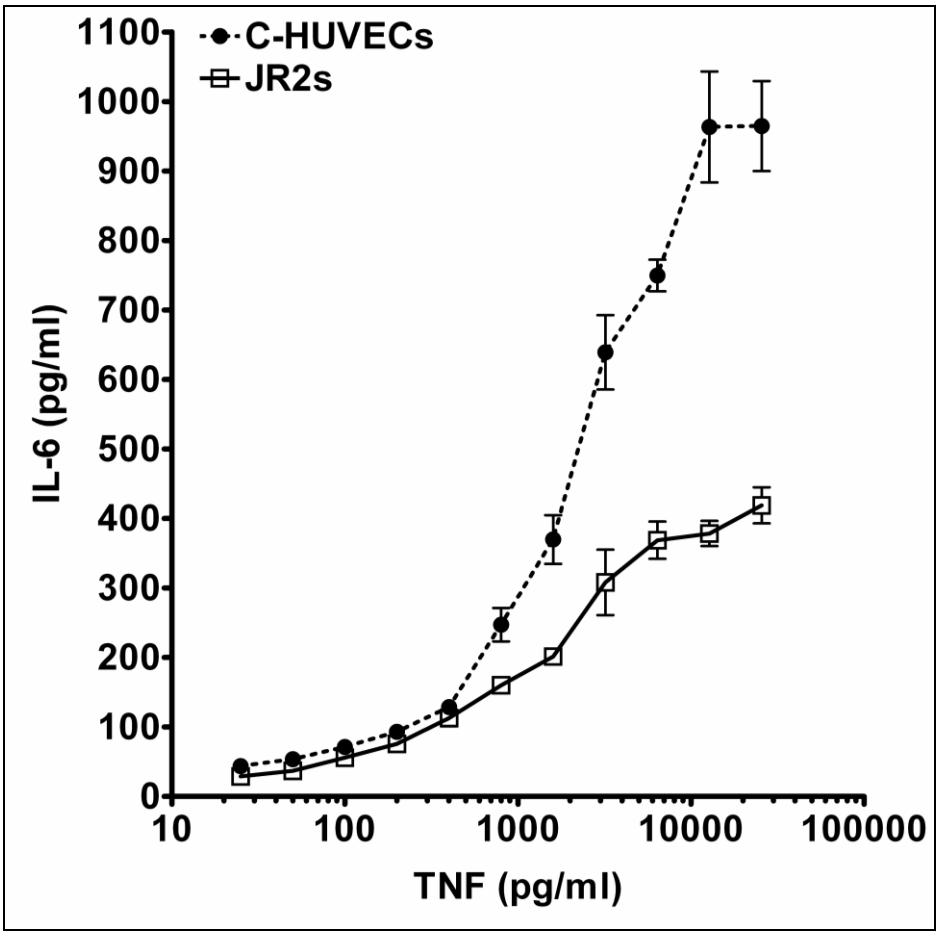


Figure 52. Stimulation of IL-6 release from C-HUVEC and HUVECjr2 by TNFα. IL-6 release stimulated by TNFα (2nd IS 88/786) at concentrations ranging 25 – 25,600 pg/ml, when incubated with 30,000 HUVECjr2 or C-HUVEC per well for 48 h under the same experimental conditions as for co-culture assays. Values are means and S.E.M of 4 replicates for each dose of TNFα (from 1 umbilical cord for C-HUVEC data).

6.4 Discussion

Following on from the previous chapter, the obvious question was: what do endothelial cells contribute to PBMC-based assays that allows TGN1412 to stimulate cytokine and cell proliferative responses without prior immobilisation of this antibody onto a plastic surface? To answer this, a number of possibilities of how endothelial cells mediate TGN1412-stimulated responses in co-culture assays were investigated. Since HUVECjr2 were readily available and had been shown (in Chapter 5) to be a satisfactory substitute for fresh endothelial cells (C-HUVEC), HUVECjr2 were the endothelial cells of choice for further experiments with confirmatory experiments conducted with C-HUVEC when these were available.

6.4.1 The role of PBMC to endothelial cell contact in mediating TGN1412-stimulated responses

In co-culture assays PBMC settle under the force of gravity over the monolayer of endothelial cells and so are in direct contact with the endothelial cells. To determine whether or not endothelial cell to PBMC contact was required for TGN1412 stimulation of PBMC, transwell plates were used in which HUVECjr2 cultured as monolayers in outer wells were separated from PBMC in inner wells by a porous membrane (see Figure 36). It was shown that TGN1412 stimulated IL-6, TNFα and IL-2 responses in co-culture assays only when endothelial cell to PBMC contact was permitted, i.e. when endothelial cells and PBMC were not separated by a porous membrane.

It is unlikely that the membrane itself in some way hampered the capability of cells to respond because when the membrane was inserted above a co-culture of endothelial cells and PBMC that were directly in contact, IL-6 responses to TGN1412 were not affected. Similarly, PBMC cultured alone over the porous membrane in the absence of endothelial cells produced substantial concentrations of IL-6 in response to endotoxin.

The large number of PBMC settling on a membrane within such a small area could have prevented the free-flow across the membrane of crucial soluble factors, produced by either

PBMC or endothelial cells or both. This may have been the reason for the absence of TGN1412-stimulated responses when endothelial cell to PBMC contact was prevented by the membrane. However, since IL-6 was measured at a range of concentrations in cell-conditioned medium from above and below the membrane when spiked either into cell-conditioned medium above or below the membrane, it is unlikely that PBMC were preventing the movement of soluble factors across the membrane. IL-6 was chosen as a representative soluble cytokine for measuring the capability of soluble factors to pass through the membrane. With a molecular weight of 26 KDa (Naka et al., 2002), it is one of the larger cytokines, thus if IL-6 was able to pass through, it was reasoned that others were likely to be able to pass as well. In addition, endotoxin spiked into cell-conditioned medium above the membrane stimulated IL-6 release that was measured in cell-conditioned medium above and below the membrane. The same was true when endotoxin was spiked into cell-conditioned medium below the membrane. Furthermore, mixing the cell-conditioned medium above and below the membrane in an attempt to achieve a more homogenous medium throughout the entire well did not restore TGN1412-stimulated IL-6 responses. These results therefore suggest that TGN1412-stimulated responses in co-culture assays were not initiated by soluble factors such as cytokines unless the soluble factors responsible were very short-lived and lost all biological activity during the time that it took for them to pass through the membrane.

Once it had been established that endothelial cell to PBMC contact was required in co-culture assays for TGN1412 to stimulate TNFα, IL-6 and IL-2 responses, a number of mechanisms that could potentially underlie TGN1412-stimulation of PBMC, involving endothelial cell to PBMC contact, were investigated.

6.4.2 The role of allogeneic MHC Class II antigens expressed by endothelial cells in mediating TGN1412-stimulated responses

One possible theory was that a primary signal for CD4+ T cell activation, the pre-dominant T cell subset reported to be stimulated by immobilised TGN1412 (Stebbings et al., 2007) and which is also stimulated by TGN1412 in co-culture assays (unpublished data,

NIBSC), was provided by stimulation of the TCR by allogeneic MHC class II expressed by endothelial cells and that TGN1412 provided a co-stimulatory signal. Most of the evidence suggests that this is an unlikely theory (reviewed in the Introduction to this chapter), however, to further investigate this notion, a conventional agonistic CD28 antibody was substituted for TGN1412 in co-culture assays. Responses to this agonist (rather than superagonist) antibody were negligible when either C-HUVEC or HUVECjr2 were used as the monolayer. Since this co-stimulatory antibody can only activate T cells in the presence of a primary signal, for example through antigen recognition by the TCR, it was unlikely that allogeneic MHC class II antigen stimulation of the TCR was occurring. Thus, it can be concluded that stimulation of cells in co-culture assays is specific to TGN1412 superagonists and not conventional agonistic CD28 antibodies.

In organ transplant recipients, the primary signal for T cell activation is provided by recognition of allogeneic MHC class II molecules on donor cells. An important co-stimulatory signal required for T cell activation by donor endothelial cells is provided by the interaction of LFA-3 (expressed by endothelial cells) with CD2 (expressed by T cells) since endothelial cells lack CD80 and CD86 expression, the co-stimulatory CD28 ligands expressed by specialised APCs (McDouall et al., 1996; Rose, 1998). Therefore, if allogeneic MHC class II antigens expressed by endothelial cells were stimulating T cells to any extent in co-culture assays, stimulation would have been expected in the absence of TGN1412, especially since LFA-3 had been shown to be expressed by HUVECjr2 in this study. The negligible IL-2 responses of cells in co-culture assays in the absence of TGN1412 clearly show this was not the case.

Taken together, these findings suggest that it was unlikely that endothelial cells mediated TGN1412-stimulated responses in co-culture assays through a mechanism involving allogeneic stimulation of the TCR and so this possibility was not pursued further.

6.4.3 The role of TGN1412 immobilisation by endothelial cells in mediating TGN1412-stimulated responses

The notion that endothelial cells in co-culture assays immobilise TGN1412, perhaps through Fc to Fc receptor interaction, to present the molecule to PBMC in a manner similar to that achieved by air-drying or wet-coating the antibody on plastic surfaces, was investigated. Had cytokine responses of cells been large in co-culture assays, where endothelial cells were pre-incubated for 5 h with TGN1412 prior to gentle washing (to remove unbound TGN1412) and the addition of PBMC, this would have suggested that TGN1412 was, in some manner, being immobilised by endothelial cells. However, responses were negligible for all 8 PBMC donors tested. These data suggest either that any remaining TGN1412, i.e. immobilised by endothelial cells, was not capable of stimulating cytokine responses or that antibody was not bound to endothelial cells (and hence not immobilised) with an affinity strong enough to withstand washing of the endothelial cells. Since IgG4 antibody has a low affinity for Fc receptors (Bugelski et al., 2009), it is possible that any Fc receptor-bound TGN1412 had been washed away. Similarly, in a different study at NIBSC, binding of TGN1412 to C-HUVEC or HUVECjr2 was not detected using immunocytochemistry or cell ELISA techniques (Findlay et al, 2011). Since most experiments to show that TGN1412 is immobilised by endothelial cells would involve washing these cells, this is a difficult concept to prove.

However, when 1 µg/well of a TGN1412 analogue (NIB1412-S228P) rendered incapable of forming and exchanging half molecules with other IgG4 molecules (used for the reasons described in section 0) was incubated in co-culture assays in the presence of Tysabri (IgG4) or an isotype-matched (to Tysabri) negative control at 0.1 – 10 µg/well, responses to NIB1412-S228P were not inhibited. Although the primary purpose of this experiment was to investigate the role of α4 integrin subunit in TGN1412-stimulated responses, as discussed later, this lack of inhibition suggests that immobilisation of NIB1412-S228P by endothelial cells was not crucial to responses stimulated by this TGN1412 analogue. If immobilisation had been crucial, the presence of excess amounts of the other IgG4κ molecules in the culture (Tysabri or its isotype control) would have likely inhibited responses to the TGN1412 analogue through competition

for occupancy of endothelial cell surface components responsible for immobilising the TGN1412 analogue (i.e. Fc receptors). Furthermore, Tysabri or its isotype-control were incubated with endothelial cells and PBMC for at least 1 h prior to the addition of the TGN1412 analogue giving Tysabri and the control an advantage over the TGN1412 analogue with regard to receptor occupancy. Moreover, human AB serum was added to co-cultures and since IgG1 is the predominant subclass in serum, with its higher affinity for Fc receptors than IgG4 (Bugelski et al., 2009), it was likely to have "blocked" any unbound Fc receptor. In one study, it was suggested that aggregated target-bound TGN1412, with its putative higher affinity for Fc receptors compared with unbound TGN1412, may displace any Fc receptor bound IgG in co-culture assays (Sandilands et al., 2010). However, more recently, unpublished data from our laboratory has revealed that another TGN1412 analogue produced by NIBSC with a mutated Fc portion incapable of binding to Fc receptors (including FcγRIa, FcγRIIa, FcγRIIb, FcγRIIIa, FcγRIIIb and FcRn) was still able to stimulate cytokine responses in co-culture assays (manuscript in preparation). Taken together, these data suggest that immobilisation of TGN1412 through Fc receptor binding is unlikely to be the mechanism by which TGN1412 stimulated responses in co-culture assays.

6.4.4 The role of adhesion molecules in mediating TGN1412-stimulated responses

Since endothelial cell to PBMC contact is required in order for TGN1412 to stimulate IL-6, TNFα and IL-2 responses of cells in co-culture assays, and since TGN1412-stimulated responses are unlikely to be allogeneic or dependent on TGN1412 immobilisation by endothelial cells, an alternative possible role for endothelial cells was investigated. Recruitment of specific immune cell subsets from the bloodstream into tissues usually occurs in venules. Recruitment is mediated by the interaction of specific combinations of adhesion molecules expressed by vascular endothelium (forming the venules) and the immune cells (Pittet and Mempel, 2008). Some of these receptor/ligand combinations are known to provide co-stimulatory signals in T cell activation pathways. It is (at least in part) for this reason that there has been much debate on the role of endothelial cells as non-specialised APCs in T cell

activation (Westphal et al., 1993a). Endothelial cells have been shown to promote TCR/CD3-induced T cell activation and proliferation though a cell to cell contact-dependent mechanism involving the interaction of LFA-3 expressed by endothelial cells and CD2 expressed by T cells (Hughes et al., 1990; Hughes and Pober, 1993; Westphal et al., 1993b). It was therefore conceivable that this receptor/ligand combination might have played a role in TGN1412-stimulated responses. HUVECjr2 and PBMC isolated from two donors were shown, by flow cytometric analysis, to constitutively express LFA-3. LFA-3 blocking antibody which prevents ligation of LFA-3 with its ligand CD2, partially inhibited TNFα, IL-2 and cell proliferative responses to TGN1412 in co-culture experiments. However, these responses could not be entirely inhibited when larger concentrations of blocking antibody were used and for cell proliferative and TNFα responses, 10 μg/ml did not inhibit responses to a greater extent than 1 μg/ml, suggesting that LFA-3 was saturated with blocking antibody at such concentrations. Under these (saturated) conditions, LFA-3 blocking antibody reduced TGN1412-stimulated cell proliferation (-22%), TNFα release (-51%) and IL-2 release (-68%) suggesting that LFA-3 augments responses to TGN1412 in co-culture assays but that LFA-3 alone is not responsible for the initiation of TGN1412-stimulated responses.

An assumption was made that if LFA-3 blocking antibody was pre-incubated with endothelial cells and removed by washing prior to addition of PBMC and TGN1412, LFA-3 molecules expressed post addition of PBMC/TGN1412 (i.e. upregulated as a result of cytokines produced by endothelial cells/PBMC) would not be blocked by LFA-3 antibody. Consequently, LFA-3 blocking antibody was left in co-cultures throughout the duration of the incubation period. However, flow cytometric analysis clearly showed that PBMC, in addition to endothelial cells, express LFA-3 and this is consistent with findings from other studies (Hviid et al., 1993). Therefore, the inhibitory effects of the LFA-3 blocking antibody in co-culture assays in theory could have been a result of blocking LFA-3 expressed by either endothelial cells or PBMC or both. However, the findings from the studies referenced above (Hughes et al., 1990; Hughes and Pober, 1993; Westphal et al., 1993b) suggest that LFA-3 expressed by endothelial cells may be more likely to have been the source of the stimulatory activity in co-cultures of endothelial cells and PBMC. The stimulatory activity of LFA-3 was specific to co-culture assays since, when

LFA-3 blocking antibody was incubated with PBMC alone, responses to immobilised TGN1412 were not inhibited. Perhaps responses of PBMC stimulated by immobilised TGN1412 were so large that they could not be further augmented by co-stimulatory molecules expressed by PBMC.

The mechanism by which LFA-3 augments responses to TGN1412 in co-culture assays is unclear. Of course, as a CD28 superagonist, TGN1412 is able to stimulate T cells without the requirement for a primary signal through ligation of the TCR. The signalling pathways leading to T cell activation by CD28 superagonists and how this differs from conventional CD28/TCR co-stimulation is still the subject of debate and is reviewed in the General Introduction. What is clear from the past ten years of research is the involvement of Src-family protein tyrosine kinases in pathways activated by both conventional and superagonistic (TGN1412) CD28 mAbs (Waibler et al., 2008; Schraven and Kalinke, 2008) as discussed in Chapter 1. It is generally accepted that Src-family protein tyrosine kinases can attach to lipid rafts after post-translational modification with palmitate, allowing them to be recruited close to cytoplasmic domains of membrane-bound signalling receptors (Tavano et al., 2004; Murphy et al., 2008). It has been reported that antibodies used to block CD2 expressed on the surface of T cells prevented endothelial cell-induced aggregation of lipid rafts and IL-2 release by T cells (Mestas and Hughes, 2001). It was suggested by Mestas and Hughes that lipid raft aggregation may lead to the rearrangement of signalling molecules involved in T cell activation upon stimulation via the TCR. In addition, engagement of CD28 expressed by T cells promoted lipid raft aggregation at the immunological synapse, resulting in a reorganisation of signal molecules involved in T cell activation (Tavano et al., 2006). Taken together, these findings support the notion that in co-culture assays, TGN1412-stimulated lipid raft aggregation in the T cell membrane is enhanced by ligation of LFA-3, expressed by endothelial cells, to CD2, expressed by T cells, leading to an increase in the number of Src-family kinases in the immunological synapse. This in turn could amplify phosphorylation events of relevant signalling molecules leading to larger T cell responses. Further evidence to support this theory was that ligation of an LFA-3/IgG fusion protein with CD2 augmented anti-CD3 induced tyrosine kinase activity and T cell proliferation (Kanner et al., 1992).

Furthermore, as discussed in the Introduction to this chapter, signalling events within T cells resulting from LFA-3/CD2 interactions in the absence of TCR stimulation have been reported to involve ITAM motifs in the ζ chain of the TCR, Lck, ZAP70, LAT, PLC-γ1 and SLP-76. These are the same signalling processes that follow TCR ligation (Kaizuka et al., 2009). It was also reported that the presence of the TCR, ZAP-70 and LAT are required for CD28 superagonistic activation, despite the fact that ligation of the TCR is not required. It was suggested that superagonistic CD28 activation was reliant on low level constitutive "background" signals emanating from unligated TCRs (Hunig and Dennehy, 2005) and that amplification of background TCR signalling by CD28 superagonists occurs at the level of SLP76 involvement (Dennehy et al., 2007). Therefore, in co-culture assays and thus in the putative presence of the TCR but in the absence of TCR ligation/stimulation, signals emanating from LFA-3/CD2 interaction could, in fact, provide (additional) signals for bivalently bound TGN1412 (as a CD28 superagonist) to "boost" but not for monovalently bound CD28 agonists (as illustrated in Figure 7). The word "additional" is used here in brackets because it is not known whether or not constitutive "background" signals are coming from the TCR in co-culture assays. Furthermore, signals emanating from LFA-3/CD2 interaction alone were not solely responsible for the initiation of TGN1412-stimulated responses because TGN1412-stimulated activity could not be entirely blocked with LFA-3 blocking antibody. (This is discussed in more detail below.) However, additional signals resulting from LFA-3/CD2 interaction may lower the T cell activation threshold.

In contrast to TNFα, IL-2 and cell proliferative responses, IL-6 responses to TGN1412 in co-culture assays were not inhibited by the LFA-3 blocking antibody. This supports the notion that receptor/ligand combinations other than LFA-3/CD2 are involved in mediating TGN1412-stimulated responses since transwell experiments showed that IL-6 responses stimulated by TGN1412 are dependent on endothelial cell to PBMC contact. It is unclear why reduced concentrations of TNFα as a result of inhibition caused by LFA-3 blocking antibody did not lead to reduced IL-6 release since TNFα stimulated IL-6 release by endothelial cells in a dose-dependent manner, and because TNFα has the same effect on monocytes (Murphy et al., 2008).

ICAM-1 expressed by endothelial cells plays a pivotal role in mediating leukocyte adhesion and migration across endothelial cells through ligation with LFA-1 expressed by leukocytes (Petruzzelli et al., 1998; Faveeuw et al., 2000; Muller, 2002; Pittet and Mempel, 2008). ICAM-1 is also expressed by specialised APCs and functions as a co-stimulatory molecule after ligation with LFA-1, promoting T cell activation (Wingren et al., 1995; Chirathaworn et al., 2002; Wang et al., 2009). In addition, the co-stimulatory effects of endothelial cells on T cell activation have been reported to be mediated through the interaction of ICAM-1 expressed by endothelial cells and LFA-1 expressed by T cells (Westphal et al., 1993a; Westphal et al., 1993b; Westphal et al., 1993c). It was therefore conceivable that in co-culture assays, ICAM-1 expressed by endothelial cells may have co-stimulated with TGN1412 to activate cells via interaction with LFA-1. It has been shown that both HUVECjr2 and C-HUVEC used in this study constitutively express ICAM-1 (Findlay et al., 2011b). It has also been reported elsewhere that endothelial cells constitutively express ICAM-1 (Rose, 1998). Preliminary experiments in the current study have shown that ICAM-1 blocking antibody, which prevents ligation of ICAM-1 with its ligand LFA-1, reduced TGN1412-stimulated cell-proliferative responses by 43%. This suggests that ligation of this receptor ligand pair is involved in TGN1412 activation of cells in co-culture assays. However, because ICAM-1 is expressed by cells within the PBMC fraction (Murphy et al., 2008) and because the ICAM-1 blocking antibody was left in the co-culture (as for LFA-3), the inhibition of TGN1412-stimulated responses could have resulted from blocking ICAM-1 expressed by endothelial cells or PBMC or both. The stimulatory activity of ICAM-1 was specific to co-culture assays as when ICAM-1 blocking antibody was incubated with PBMC alone, cell proliferative responses to immobilised TGN1412 were not inhibited. This is possibly for the same reason described for LFA-3 above.

Unfortunately, due to the presence of bacterial endotoxin (a potent stimulus for pro-inflammatory cytokine release) in the blocking antibody, it was not possible to use it in experiments in which pro-inflammatory TNFα and IL-6 responses were measured. An endotoxin-free ICAM-1 blocking antibody could not be sourced to permit the investigation of

the contribution, if any, of ICAM-1 to TGN1412-stimulated pro-inflammatory cytokine responses in co-culture assays.

Ligation of VCAM-1 expressed by specialised APCs to the integrin VLA-4 expressed by T cells plays a pivotal role in T cell adhesion and, not surprisingly, can provide a co-stimulatory signal for T cell activation (Pober and Cotran, 1991; van Seventer et al., 1991a; Nguyen et al., 2008). Since endothelial cells can express VCAM-1, it was conceivable that this receptor/ligand interaction played a role in TGN1412-stimulated responses in co-culture assays. Although VCAM-1 was not constitutively expressed by resting HUVECjr2 or C-HUVEC (Findlay et al., 2011b) eliminating the possibility that VCAM-1/VLA-4 interaction might have played a role in initiating responses to TGN1412, VCAM-1 expression was induced after these HUVEC were treated with TNFα (Findlay et al., 2011b). This finding was in agreement with other reports that its expression can be upregulated by IL-1 and TNFα treatment (Rose, 1998). Consequently, VCAM-1/VLA-4 interaction could conceivably have promoted responses to TGN1412 after their initiation. The therapeutic mAb Tysabri is a humanised anti-α4 integrin subunit IgG4 antibody which blocks the interaction of integrins containing the α4 subunit with their ligands. VLA-4 consists of subunits α4:β1 and so, in this study, Tysabri was used to block potential VLA-4/VCAM-1 interaction. However, this antibody was unable to inhibit responses to a TGN1412 analogue in co-culture assays. This suggests that VCAM-1/VLA-4 ligation was not involved in promoting responses to TGN1412 after their initiation since Tysabri, which was present in wells for the full co-culture incubation, should have prevented interaction between newly expressed VCAM-1 and VLA-4 molecules. This was consistent with the finding of another study which also showed that blocking the interaction of VCAM-1 expressed by endothelial cells with VLA-4 expressed by T cells did not inhibit T cell activation (Westphal et al., 1993a), albeit activation that was via stimulation of the TCR/CD3 complex rather than with a CD28 superagonist.

In addition to VLA-4, the integrin α4:β7 clearly has the α4 subunit, thus interaction with its ligand MAdCAM-1, also expressed by endothelial cells at least *in vivo*, should be blocked by Tysabri. Although α4:β7/MAdCAM-1 interaction has been reported to provide a co-stimulatory

pathway in T cell activation (Lehnert et al., 1998), the data indicates that it did not contribute to responses stimulated by a TGN1412 analogue in co-culture assays.

Of note, the finding that the TGN1412 analogue stimulated responses in co-culture assays suggests that half-molecule formation of the original TGN1412 and Fab arm exchange is not required for these effects to occur. In addition, the finding that the inclusion of Tysabri in co-culture experiments with TGN1412 had very little effect on responses to TGN1412 validates Tysabri as a suitable isotype-matched [to TGN1412] negative control.

Cell proliferative responses in co-culture assays were, at least, partially dependent on the production of IL-2 since anti-human IL-2 antibody inhibited TGN1412-stimulated cell proliferative responses in a dose-dependent manner through putative binding of IL-2, preventing engagement with IL-2 receptor expressed by cells in co-culture. However, cell-proliferative responses were not entirely blocked by anti-IL-2 mAb. It is possible that not all of the IL-2 in the cell-conditioned medium was bound by anti-IL-2 mAb but instead was perhaps bound to the IL-2 receptor resulting in some cell proliferation. The IL-2Rαβγ complex, following its putative expression by TGN1412-activated T cells, would have a competitive advantage over the anti-IL-2 mAb for binding to IL-2 due to its very high affinity for this cytokine. It is also possible that other cytokines may have stimulated cell proliferation. For example, IL-4, IL-15 and GMCSF are all capable of stimulating cell proliferation (Cheng et al., 2002) although it is generally accepted that most T cell proliferation is usually through signalling induced by IL-2 and its high affinity receptor.

Inhibition of TGN1412-stimulated cell proliferative responses by LFA-3 and ICAM-1 blocking antibodies was likely to have been the result of inhibition of IL-2 responses. However, although IL-2 responses could be reduced by a maximum of 68% when LFA-3 was blocked, cell proliferative responses could only be reduced by a maximum of 22%. It is likely that the IL-2 released, albeit in smaller amounts when LFA-3 was blocked, was still sufficient to stimulate cell proliferation. In other studies, it was reported that endothelial cells augmented IL-2 production, albeit from mitogen-stimulated T cells. This augmentation was reported to be dependent on CD2/LFA-3 interaction (Hughes et al., 1990; Savage et al., 1991) which is consistent with the findings in the present study. Although the concentrations of IL-2 released in

co-culture assays were small in comparison with concentrations measured in the blood of the trial volunteers, it appears that the IL-2 which was produced *in vitro* was having a biological affect.

As discussed in the introduction to this chapter, there is evidence to suggest that the area of interface between endothelial cells and T cells is highly organised and resembles the immunological synapse which forms during APC-activation of T cells (Choi et al., 2004). The function of an immunological synapse is to promote the organisation of molecules required for T cell activation (Davis and Dustin, 2004). A fundamental purpose of co-stimulatory activation pathways in T cell activation is to promote transportation of cell surface molecules distributed around the T cell into the immunological synapse. This is achieved by promoting the aggregation of lipid rafts which bring associated proteins with them (as described for LFA-3 above), and through cytoskeletal re-arrangements (Huppa and Davis, 2003). Co-stimulatory combinations of LFA-1:ICAM-1 and CD2:LFA-3 have been reported to form part of the immunological synapse in a number of studies (Huppa and Davis, 2003; Davis and Dustin, 2004; Dustin, 2007; Dustin, 2009; Kaizuka et al., 2009). Kaizuka et al. (2009) reported that signalling events activated upon LFA-3/CD2 ligation were accompanied by the reorganisation and clustering of CD2 and associated signalling molecules into microdomains and that this was dependent on the actin network. This formation of microdomains was thought to be a requirement for the activation of Lck. Other groups have suggested that the organisation of signalling complexes in the synapse promoted by co-stimulatory pathways is based, to a certain extent, on size, which favours the aggregation of various tyrosine kinases involved in signalling but leads to exclusion of CD45, a phosphatase which would act as an antagonist of tyrosine kinases (Dustin and Shaw, 1999). In summary, it would appear that the spatial organisation of signalling molecules within the immunological synapse for T cell activation is very important. It is conceivable that in co-culture assays, adhesion/co-stimulatory molecules expressed by endothelial cells promote a favourable organisation of relevant signalling molecules/molecular targets within the immunological synapse for T cell activation. This optimised spatial organisation of signalling molecules may negate the requirement for TGN1412 to be presented

in the manner achieved by immobilisation in order for it to activate T cells. It is likely that a threshold level of cross-linking of CD28 by TGN1412 is required and that this threshold can be achieved by concentrating TGN1412 on a surface, i.e. by air-drying or wet-coating on plastic as discussed in Chapter 4. LFA-3, ICAM-1 and other co-stimulatory/adhesion molecules expressed by endothelial cells (discussed below) may act in concert to induce the formation of an immunological synapse in which the spatial organisation of signalling molecules reduces the threshold of CD28 cross-linking required for T cell activation. This reduced threshold may be small enough such that aqueous TGN1412 can provide sufficient cross-linking. Indeed, elsewhere it has been suggested that ICAM-1/LFA-1 interaction lowers the threshold amount of foreign antigen required to activate T cells (Bachmann et al., 1997), albeit for activation via TCR recognition of antigen and not by a CD28 superagonist.

In the present study LFA-3 alone was not entirely responsible for the initiation of TGN1412-stimulated responses since such responses could not be completely blocked by LFA-3 blocking antibody. Therefore, LFA-3 (and possibly ICAM-1) may simply augment responses to aqueous TGN1412 while an alternative pathway involving endothelial cell/PBMC interaction is responsible for the initiation of TGN1412-stimulated responses. However, on the contrary, it is also possible that if one adhesion/costimulatory molecule is entirely blocked by antibody, cells are still able to make use of other adhesion/costimulatory molecules (although the threshold of cross-linking may increase). If this was the case with TGN1412, no single co-stimulatory adhesion molecule would be exclusively responsible for allowing TGN1412 to stimulate responses but together they could make this possible. In order to entirely block responses to TGN1412 in co-culture assays, it may be necessary to use antibodies specific for all of the co-stimulatory/adhesion molecules involved. In a published study, endothelial cell-mediated augmentation of IL-2 responses to PHA-stimulated T cells could not be entirely blocked by anti-LFA-3 antibody. However, when endothelial cells were substituted with purified LFA-3, augmentation could be completely blocked with anti-LFA-3. So, it was concluded that adhesion molecules other than LFA-3 were involved in the co-stimulatory activity of endothelial cells (Savage et al., 1991). In another study, mixtures of antibodies specific for a number of endothelial cell co-stimulatory/adhesion molecules (LFA-3, ICAM-1

and VCAM-1) resulted in greater inhibition of T cell responses than the individual antibodies suggesting that these adhesion molecules acted in concert. However, in that study, T cell activation was through antibody stimulation of the TCR/CD3 complex and not through superagonistic anti-CD28 activation (Westphal et al., 1993a).

Many of the adhesion molecules/co-stimulatory molecules expressed by endothelial cells are also expressed by PBMC. Therefore, if this notion regarding the formation of a "favourably organised synapse" promoted by co-stimulatory/adhesion molecules were true, one could ask why PBMC alone are unable to respond to aqueous TGN1412. Furthermore, why didn't LFA-3 and ICAM-1 blocking antibodies inhibit responses of PBMC alone to immobilised TGN1412? The answer to these questions could be explained by differences in the spatial arrangement of PBMC and endothelial cells in the well and by differences in the density of relevant molecules expressed on the surface of endothelial cells and PBMC within the well.

Adhesion molecules reported to have co-stimulatory effects on T cell activation may not be expressed at high levels by non-activated endothelial cells *in vivo* and *in vitro*. Although one of their biological functions is to mediate the adhesion and migration of immune effector cells into tissue, their expression is usually upregulated in response to cytokines during the inflammatory response. LFA-3 and ICAM-1 are both constitutively expressed by non-activated endothelial cells but ICAM-1 in particular is expressed only at low levels (Smith and Thomas, 1990; Rose, 1998; Findlay et al., 2011b). Nevertheless, LFA-3 and ICAM-1 expression is upregulated by pro-inflammatory cytokines, including TNFα and IFNγ (Karmann et al., 1995; Omari and Dorovini-Zis, 1999; Murphy et al., 2008). A low level expression of ICAM-1 and LFA-3 may be sufficient for TGN1412-stimulated TNFα release by T cells to occur, resulting in upregulated expression of co-stimulatory molecules by endothelial cells, further promoting T cell activation. LFA-1 exists in a low affinity form on non-activated T cells but conformational changes leading to a high affinity state can be stimulated by several mechanisms including cross-linking of CD2 and TCR/CD3 (Petruzzelli et al., 1998). Therefore, in co-culture assays, LFA-3/CD2 interaction may have activated conformational changes in LFA-1 molecules. The outcome of this change is expression of high affinity state LFA-1 molecules which would enhance ICAM-1 ligation promoting TGN1412-stimulated T cell activation. However, it has

been suggested that although naive CD4+ T cells express large numbers of inactive LFA-1 molecules, CD4+ memory T cells express large numbers of active LFA-1 molecules (Parra et al., 1993).

Although the co-stimulatory properties of LFA-3/CD2, ICAM-1/LFA-1 and VCAM-1/VLA-4 interactions are the most studied, there are other cell surface proteins expressed by T cells that also have been reported to provide co-stimulatory pathways, such as CD5, CD44, CD9, CD27, CD30 (Mestas and Hughes, 2001; Mestas et al., 2005). Since some of the ligands for these receptors may be expressed by endothelial cells, T cell activation in co-culture assays might be mediated by cell surface proteins of endothelial origin not investigated in the present study. For example, OX40 expressed by T cells can be co-stimulatory and endothelial cells express OX40L, the ligand for OX40. It has been reported that ligation of OX40 with OX40L expressed by endothelial cells augmented T cell responses to stimulation of the TCR/CD3 complex (Kunitomi et al., 2000; Mestas et al., 2005). In addition, ICOS expressed by T cells can be co-stimulatory in T cell activation following ligation with its ligand LICOS (Simpson et al., 2010). Indeed LICOS expression by endothelial cells has been confirmed. Furthermore, co-culture of endothelial cells with CD4+ memory T cells in the presence of a superantigen led to the production of Th1 and Th2 cytokines which was inhibited by a mAb specific for LICOS (Khayyamian et al., 2002).

TGN1412 was unable to stimulate cytokine responses in co-culture assays significantly above those to an isotype control when monolayers of HGF or HFF were used. This suggests that not any cellular substrate can be used to elicit TGN1412-stimulated responses in co-culture assays and that, most likely, the endothelial phenotype is required. This has been confirmed in a a more comprehensive study at NIBSC where the capability of cell monolayers to elicit TGN1412-stimulated responses in co-culture assays (using the method developed in this thesis) correlated with endothelial phenotype, as defined by the expression of constitutive and induced endothelial markers (Findlay et al., 2011). In addition, it has been reported elsewhere that fibroblasts were unable to co-simulate T cells activated through the TCR/CD3 complex but that, under the same experimental conditions, endothelial cells were able to do so (Page et al.,

1994b). It has been suggested that fibroblasts do not express the relevant co-stimulatory molecules, at least at the densities required (Rose, 1998).

The capability of endothelial cells to act as APCs has been debated for many years. Allogeneic endothelial cells expressing MHC class II molecules play a crucial role in transplant rejection but in co-culture assays it seems that TGN1412-stimulated responses were not due to allogeneic stimulation by endothelial cells, and obviously this could not have been the case in the clinical trial volunteers. However, expression of co-stimulatory molecules by endothelial cells is taken by some scientists as evidence to suggest that they can act as non-specialised APCs *in vivo*, activating the memory T cell subset resulting in a rapid localised recall response (as discussed in the introduction to this chapter). This APC function may have provided these cells with the necessary "machinery" required to elicit TGN1412-stimulated responses in co-culture assays and *in vivo*. Of course, the requirement for the monolayer in co-culture assays to have an endothelial phenotype further validates the co-culture assay as "physiological" method for detecting TGN1412-like activity. However, it does not rule out the possibility that other cell-types were involved *in vivo*.

6.4.5 TGN1412-stimulated IL-6 release in co-culture assays is, at least in part, from HUVEC

Although it is most likely that TGN1412 initiated responses in co-culture assays through a contact-dependent mechanism between endothelial cells and PBMC, it is expected that a number of soluble factors released after the initial stimulation intensified/amplified responses. IL-6 responses to TGN1412 in co-culture experiments (with HUVECjr2 or C-HUVEC) were much greater than responses to air-dried TGN1412 by PBMC alone, even though the same PBMC cell density was used in both methods. It was therefore conceivable that IL-6 was released from endothelial cells as well as from PBMC in co-culture experiments. In the present study, C-HUVEC or HUVECjr2 cultured alone under the same conditions as in co-culture assays released IL-6 in response to added TNFα in a dose-dependent manner. It is likely therefore that TNFα, produced by PBMC in response to TGN1412, stimulated endothelial cells

to release IL-6. (Of note, the fact that C-HUVEC released more IL-6 in response to TNFα than HUVECjr2 may, in part, explain why IL-6 responses to TGN1412 in co-culture assays were larger when C-HUVEC formed the monolayer.) However, IL-6 responses of HUVECjr2 and C-HUVEC alone, stimulated by added TNFα at a dose equivalent to the amount released in response to TGN1412 in co-culture experiments, were much smaller than TGN1412-evoked IL-6 responses in co-culture assays with PBMC. It is possible that endothelial cells produced IL-6 in response to soluble factors other than TNFα and it is to be expected that cells within the PBMC population produced IL-6 in co-culture assays, especially as IL-6 (albeit at smaller concentrations) was produced in assays using PBMC alone with immobilised TGN1412. Elsewhere it has been reported that IL-6 production by endothelial cells can be stimulated by IL-1 (Sironi et al., 1989). In addition, endothelial cells and monocytes cultured together produced synergistic IL-6 responses when endothelial cells were pre-treated with platelet-activating factor (PAF), a potent inflammatory mediator also produced by endothelial cells (Lacasse et al., 1996) and which could well have been produced in response to TGN1412 *in vivo* and *in vitro*. Taken together, these data strongly suggests that endothelial cells contributed to IL-6 production in responses to TGN1412 *in vivo*.

The inclusion of human endothelial cells in PBMC based assays has improved the capability of the *in vitro* test to predict the clinical effects caused by TGN1412 by giving a test system in which TGN1412 in aqueous phase (i.e. not dried onto plastic) stimulates cytokine release. In this chapter, the mechanisms that may underlie TGN1412-stimulated responses in co-culture assays, and thus potentially *in vivo*, have been investigated. It would, of course, be of great benefit if this co-culture assay could be used as part of a testing programme to accurately predict pro-inflammatory clinical infusion reactions caused by therapeutic mAbs other than TGN1412. The utility for this purpose of this co-culture assay in comparison with other *in vitro* tests for predicting clinical responses to therapeutic mAbs is evaluated in the next chapter.

CHAPTER 7: Comparison of novel methods for predicting the risk of pro-inflammatory clinical infusion reactions during monoclonal antibody therapy

CHAPTER 7: Comparison of novel methods for predicting the risk of pro-inflammatory clinical infusion reactions during mAb therapy

7.1 Introduction

So far, the present study has focused on TGN1412 but clinical infusion reactions during mAb therapy are far from exclusive to TGN1412. Depending on the antibody, a significant number of clinical infusion reactions may occur, usually on the first or second infusion, and these may be severe enough to constitute what has been termed 'cytokine release syndrome' (Breslin, 2007; Chung, 2008; Wing, 2008; Hansel et al., 2010). The response to TGN1412 was, by far, the most extreme case of 'cytokine release syndrome', causing it to be termed a 'cytokine storm' (Suntharalingam et al., 2006). The TGN1412 incident prompted a re-think of the conduct of first-in-human clinical trials (Expert Group on Phase One Clinical Trials, 2006; European Medicines Agency, 2007; Stebbings et al., 2009) and the role of cytokine release assays in the pre-clinical safety testing that precedes such trials (Wing, 2008). Subsequently, in November 2009, a European Medicines Agency-sponsored workshop was held to consider the merits of *in vitro* cytokine release assays for predicting cytokine release syndrome (Vidal et al., 2010). The overall conclusion from the workshop was that cytokine-release assays have a place in predicting the risk of pro-inflammatory clinical infusion reactions during mAb therapy and that efforts should be made to further develop and refine the assays. During the present study, the co-culture method resulted in much smaller cytokine responses to aqueous TGN1412 than those of PBMC alone to TGN1412 air-dried onto polypropylene (the antibody immobilisation technique for which the largest cytokine responses to TGN1412 were observed). The latter technique better mimicked the magnitude of *in vivo* responses in humans to TGN1412 (Suntharalingam et al., 2006). However, air-drying an antibody onto plastic to stimulate cells does not have an obvious *in vivo* analogue. Consequently, the experiments described in this chapter were carried out to evaluate the suitability of the aqueous mAb/PBMC/endothelial co-culture assay for predicting the risk of clinical infusion reactions to therapeutic mAbs other than TGN1412 and to compare the mAb/PBMC/endothelial co-culture assay with the air-drying (of

the mAb) method. The panel of mAbs included in this evaluation comprised, in addition to TGN1412 (essentially a positive control), mAbs associated with a significant incidence of pro-inflammatory clinical infusion reactions, i.e. Herceptin and Campath-1H, and mAbs not associated with a significant incidence of pro-inflammatory clinical infusion reactions, i.e. Tysabri and Avastin (Dillman, 1999; Chung, 2008; Hellwig et al., 2008; Hansel et al., 2010). The chosen readouts for the two methods were TNFα, IL-6, IL-8, IL-2 and cell proliferation, readouts associated with pro-inflammatory clinical infusion reactions (Wing, 2008; Bugelski et al., 2009) and with TGN1412 activity.

7.2 Methods

7.2.1 Monoclonal antibodies

The clinical grade mAbs used in this study (Tysabri, Avastin, Herceptin, Campath-1H, TGN1412 and an IgG4κ negative control) are described in 2.6.

7.2.2 General procedures

Aseptic technique was used for antibody manipulations and for the cell culture procedures. Antibodies and reagents were stored according to the manufacturers' instructions and antibodies, reagents and lab ware for cell culture procedures were obtained as free from detectable pyrogen/endotoxin. Culture medium for all experiments was RPMI 1640 (Sigma R0883) supplemented with 2mM L-glutamine (Sigma G7513), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma P0781) and non-essential amino acids (Gibco 11140). Values (i.e. each of the 4 replicates) in pg/ml or counts per min (cpm) for each donor's response to mAbs/controls were baseline corrected by deducting background values (i.e. the mean of 4 replicates for responses of cells from the same donor but where no mAb was added to wells) and a mean of the 4 baseline corrected replicates calculated. Statistical analyses were conducted using the Kruskal-Wallis Test with the Dunn's Multiple Comparisons procedure.

7.2.3 Comparison of cytokine responses of PBMC incubated with mAbs immobilised by air drying (Method 1)

Monoclonal antibodies Tysabri, Avastin, Herceptin, Campath-1H, TGN1412 and a control mAb were immobilised by coating onto the surfaces of wells of untreated 96-well, U-bottomed polypropylene microtitre plates (Corning #3790, now replaced with #3879). To coat, 50 µl of the antibody solution, diluted in PBS B, was added to each well. Plates were left overnight in a class II laminar flow cabinet with the lids removed to allow the solutions to evaporate. Coated plates were washed twice with 200 µl PBS B using a multi-channel pipette to remove salt crystals and unbound antibody. Antibodies were air-dried onto polypropylene plates as the largest absolute cytokine responses to mAbs were observed using this technique in Chapter 4. PBMCs were isolated from fresh human blood as described in section 2.2. These cells were incubated (125,000 cells/well) for 24 h at 37 °C, 5% CO_2 in the antibody-coated wells of plates containing 250 µl culture medium and 2% donor's own plasma or in wells containing 250 µl aqueous antibody (diluted with culture medium) or lectin PHA (positive control) and 2% donor's own plasma. After incubation, cell-conditioned medium was removed from the PBMCs and assayed in cytokine-specific ELISAs for TNFα, IL-6, IL-8 and IL-2 as described in section 2.4. Standard curves for each cytokine were prepared in culture medium containing 2% (final) pooled donor plasma (i.e. plasma from the four donors of PBMC, used for any one assay). Cell proliferative responses of the remaining cells were quantified by measuring 3H-thymidine incorporation as described in section 2.5. 3H-thymidine was diluted in culture medium containing 2% donor's own plasma prior to addition to the cells.

7.2.4 Comparison of cytokine responses of human cells to mAbs incubated in aqueous phase with PBMCs cultured over a monolayer of human umbilical vein endothelium derived cells (Method 2)

HUVECjr2 were the endothelial cells used in the experiments described here and were maintained as described in section 5.2.2. HUVECjr2 were from a single working cell bank

expanded from the original source and were used between passage 2 and passage 13. HUVECjr2 were seeded in 96-well flat bottomed tissue culture treated microtitre plates (Nunc 167008) at 30,000 cells/well in 100 µl of culture medium containing 10% hi-hAB serum. After an overnight incubation at 37 °C in 5% CO_2, 100 µl culture medium was removed. PBMCs were isolated from fresh human blood as described in section 2.2. Monoclonal antibodies Tysabri, Avastin, Herceptin, Campath-1H, TGN1412, control mAb and PHA (positive control) were incubated in aqueous phase with the attached endothelial cells and 125,000 PBMCs/well in 300 µl culture medium containing 2% hi-hAB serum for 46-48 h at 37 °C in 5% CO_2. Under the same experimental conditions, the mAbs and controls were also incubated with PBMCs alone. Supernatants were harvested and assayed using cytokine-specific ELISAs for TNFα, IL-6, IL-8 and IL-2 as described in section 2.4. Standard curves for each cytokine were prepared in culture medium containing 2% hi-hAB serum. Cell proliferative responses of the remaining cells were quantified by measuring 3H-thymidine incorporation as described in section 2.5. 3H-thymidine was diluted in culture medium containing 2% hi-hAB serum prior to addition to the cells.

7.3 Results

Tables 9 and 10 and figures 53 and 54 show TNFα, IL-6, IL-8, IL-2 and cell proliferative responses of human cells cultured with the mAbs Tysabri, Avastin, Herceptin, Campath-1H, TGN1412 and control mAb using two different methods. Method 1 was to incubate PBMCs with mAbs immobilised by air-drying them onto the surfaces of wells of 96-well polypropylene plates. Method 2 was to incubate mAbs added in aqueous phase to PBMCs cultured over a monolayer of human umbilical vein endothelium-derived cells (co-culture). In Methods 1 and 2 antibodies were also incubated in aqueous phase with PBMCs alone for comparison. In Method 2, antibodies were added to the monolayer of human umbilical vein endothelium-derived cells alone and none of the mAbs stimulated measurable cytokine release from these cells, i.e. in the absence of PBMCs. For the methods where the mAbs were incubated in aqueous phase (i.e. in co-culture experiments and when mAbs were incubated with PBMCs alone) the mAbs would have been transferred in the cell-conditioned medium to the cytokine ELISA plates possibly

causing interference in the ELISAs. However, there was no detectable difference between the values for absorbencies for TNFα, IL-6, IL-8 and IL-2 cytokine standard curves prepared in the absence and presence of either the control mAb, Tysabri, Avastin, Herceptin, Campath-1H or TGN1412 (400 μg/ml). This shows that none of the mAbs in aqueous phase inhibited the detection of cytokines by ELISA (see section 2.6 and Appendix II).

The mAbs Tysabri and Avastin are two mAbs that are rarely associated with clinical infusion reactions (Chung, 2008; Hellwig et al., 2008) and one of the two, Avastin, which stimulated larger responses than Tysabri, was chosen as the comparator for the mAbs that are more frequently associated with pro-inflammatory clinical infusion reactions. For all of the mAbs tested, cytokine and cell proliferative responses were larger when the antibody was immobilised or incubated in co-culture than when incubated in aqueous phase with PBMCs alone. Values in the tables and figures are means and S.E.M of the largest responses (calculated from the largest responses for each donor) obtained from responses to 0.1–100 μg/well mAbs in antibody immobilisation experiments and to 0.01–10 μg/well mAbs in co-culture experiments.

7.3.1 TNFα, IL-6 and IL-8 responses for Method 1: antibody immobilisation by air-drying

TNFα, IL-6 and IL-8 responses of PBMCs incubated for 24 h with mAbs, either in aqueous phase or immobilised by air-drying, and PHA (positive control) are given in Table 9 and the responses are summarised in Figure 53. TNFα responses to immobilised TGN1412 were 6x larger than responses to Avastin ($p<0.01$); in contrast, responses to immobilised Tysabri, Herceptin and Campath-1H were less than twice the responses to Avastin and these small differences were not statistically significant ($p>0.05$). Small TNFα responses to mAbs Herceptin and Campath-1H added in aqueous phase were not statistically significantly different from TNFα responses to any of the other mAbs tested in aqueous phase ($p>0.05$).

IL-6 responses to immobilised TGN1412 were 6x larger than responses to Avastin ($p<0.05$). IL-6 responses to immobilised Campath-1H were 5x larger than responses to Avastin.

However, this difference was not statistically significant ($p>0.05$), possibly due to the large donor to donor variation of responses to Campath-1H. IL-6 responses to immobilised mAbs Tysabri and Herceptin were less than twice the responses to Avastin and these small differences were not statistically significant ($p>0.05$). Small IL-6 responses to mAbs Campath-1H and TGN1412 tested in aqueous phase were not significantly different from IL-6 responses to Avastin tested in aqueous phase ($p>0.05$).

IL-8 responses to immobilised TGN1412 and Campath-1H were 2.5x larger than responses to Avastin ($p<0.05$). Responses to immobilised mAbs Tysabri, Herceptin and the control mAb were less than twice the responses to Avastin and these small differences were not statistically significant ($p>0.05$). Small responses stimulated by Campath-1H incubated in aqueous phase were 6x larger than responses to Avastin in aqueous phase ($p<0.05$).

Despite the non-statistically significant ($p>0.05$) cytokine responses of PBMCs (alone) to mAbs incubated in aqueous phase (except for the IL-8 response to Campath-1H), TNFα, IL-6 and IL-8 responses to PHA (positive control) were marked.

7.3.2 TNFα, IL-6 and IL-8 responses for Method 2: co-culture of PBMCs over a monolayer of human umbilical vein endothelium-derived cells

TNFα, IL-6 and IL-8 responses to mAbs and PHA (positive control) incubated for 48 h in aqueous phase with PBMCs cultured over a monolayer of human umbilical vein endothelium-derived cells or with PBMCs alone are given in Table 10; these responses are also summarised in Figure 53. TNFα responses to TGN1412 in co-culture experiments were 69x larger than responses to Avastin ($p<0.01$). TNFα responses to Campath-1H in co-culture experiments, were 8x larger than responses to Avastin ($p>0.05$). TNFα responses of PBMCs alone stimulated by Campath-1H, were 10x larger than responses of PBMC alone stimulated with Avastin ($p<0.05$).

TGN1412 and Campath-1H were the only mAbs capable of stimulating marked IL-6 responses in co-culture experiments and these responses were both 112x larger than responses to Avastin, however this difference was statistically significant only for TGN1412 ($p<0.05$)

possibly due to the large donor to donor variation in responses to Campath-1H. Small IL-6 responses of PBMCs alone to Campath-1H were not significantly different from responses of PBMC to Avastin ($p>0.05$).

TGN1412 and Campath-1H stimulated IL-8 responses in co-culture experiments that were at least 32x larger than responses to Avastin in co-culture experiments ($p<0.05$). Very small responses to the mAbs Tysabri and Herceptin in co-culture experiments were not significantly larger than responses to Avastin ($p>0.05$). Only Campath-1H was able to stimulate IL-8 responses above responses to Avastin when PBMCs were cultured alone: the responses stimulated by Campath-1H were 22x larger than responses to Avastin ($p<0.05$).

TNFα, IL-6 and IL-8 responses to PHA (positive control) incubated with PBMCs cultured with endothelium derived cells or PBMCs alone were all marked.

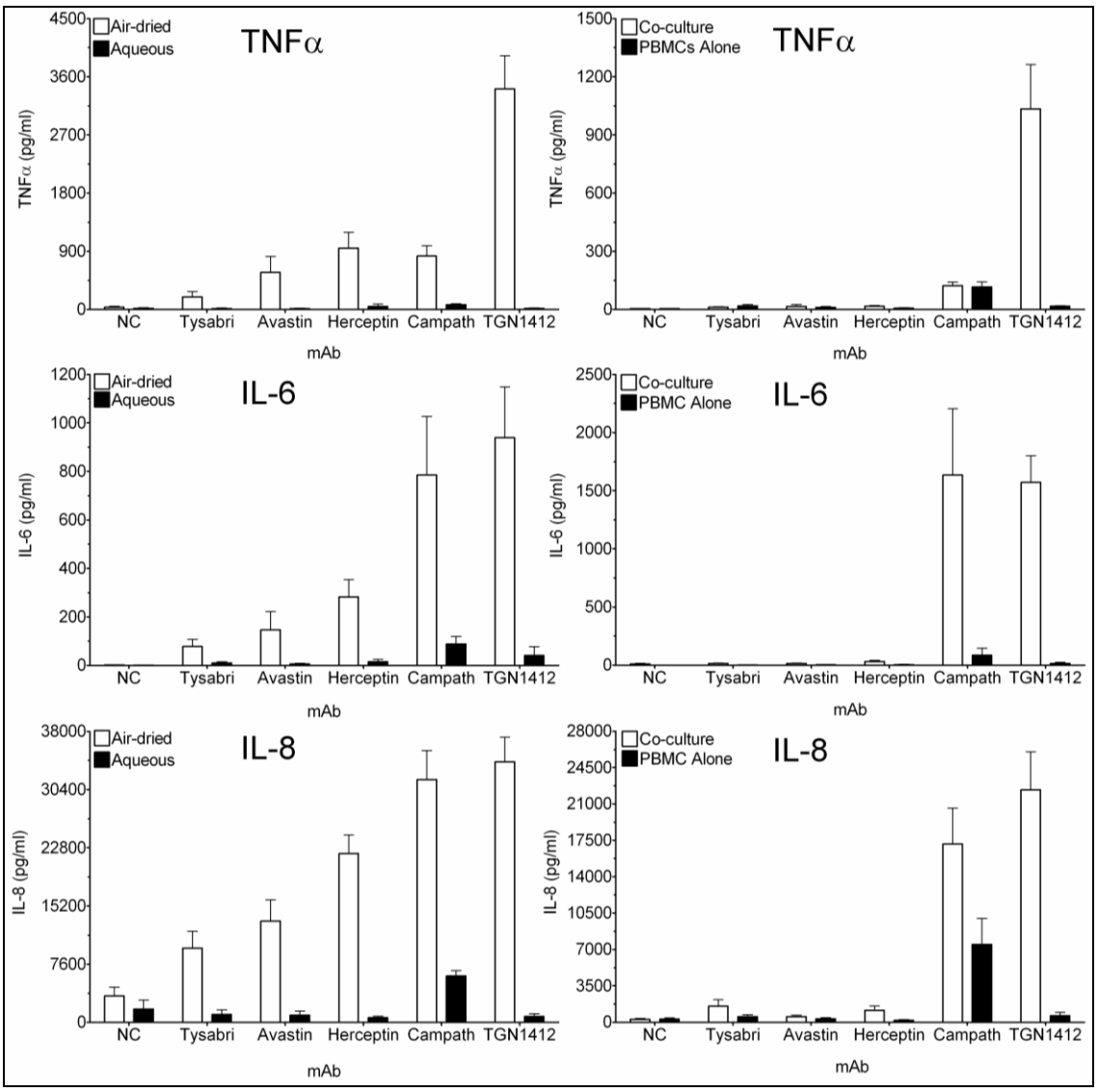


Figure 53. TNFα, IL-6 and IL-8 responses of human cells to mAbs Tysabri, Avastin, Herceptin, Campath-1H, TGN1412 and the control mAb (NC, Negative Control) using two different methods. Left-hand panels: PBMCs were cultured for 24 h with mAbs that had either been immobilised by air-drying onto wells of a 96-well polypropylene microtitre plate (white bars) or that had been added as an aqueous solution (black bars). Right-hand panels: PBMCs were cultured for 48 h with mAbs, in aqueous phase, either over a monolayer of human umbilical vein endothelium derived cells (white bars) or alone (black bars). The cytokine values (pg/ml) are means ± S.E.M of 4-18 donors of PBMC.

Table 9. Cytokine/cell proliferative responses of PBMCs to mAbs and PHA (10 μg/ml) incubated in aqueous phase and mAbs immobilised by air-drying.

	Control		**Tysabri**		**Avastin**		**Herceptin**		**Campath-1H**		**TGN1412**		**PHA**
	Aqu.	*Air-dried*	*Aqu.*	*Air-dried*	*Aqu.*	*Air-dried*	*Aqu.*	*Air-dried*	*Aqu.*	*Air-dried*	*Aqu.*	*Air-dried*	*Aqu.*
TNFα	18 ±8	36 ±15	14 ±9	196 ±79	14 ±8	572 ±248	47 ±37	948 ±247	73 ±17	826 ±160	18 ±5	3414 ±511	4524 ±1018
IL-6	<1	2 ±1.0	10 ±5	79 ±28	6 ±3	147 ±6	15 ±10	283 ±15	88 ±31	785 ±241	41 ±36	939 ±209	>4000
IL-8	1726 ±1185	3458 ±1123	1016 ±613	9700 ±2195	944 ±545	13235 ±2769	619 ±391	22064 ±2399	6050 ±710	31689 ±3812	781 ±350	34020 ±3233	39590 ±4453
IL-2	2 ±1	4 ±2	4 ±1	5 ±2	11 ±10	13 ±7	14 ±7	22 ±15	14 ±8	14 ±7	6 ±2	1350 ±226	238 ±55
Cell Prolif.	32 ±12	12 ±10	35 ±12	36 ±19	15 ±7	245 ±167	23 ±9	38 ±27	23 ±11	72 ±37	293 ±127	34148 ±4516	64807 ±6175

Values for cytokines (pg/ml) are means ± S.E.M of 8-12 donors of PBMC. Values for cell proliferative responses (counts per min) are means ± S.E.M of 4-8 donors of PBMC.

Table 10. Cytokine/cell proliferative responses of PBMCs alone or PBMCs and human umbilical vein endothelium derived cells in co-culture to mAbs and PHA incubated in aqueous phase.

	Control		**Tysabri**		**Avastin**		**Herceptin**		**Campath-1H**		**TGN1412**		**PHA**	
	PBMC	*Co-culture*	*PBMC*	*Co-culture*	*PBMC*	*Co-culture*	*PBMC*	*Co-culture*	*PBMC*	*Co-culture*	*PBMC*	*Co-culture*	*PBMC*	*Co-culture*
TNFα	5 ±	7 ±1	18 ±8	12 ±3	11 ±4	15 ±11	7 ±2	16 ±4	117 ±25	123 ±18	16 ±4	1055 ±288	>2000	>2000
IL-6	<1	11 ±6	<1	15 ±5	4 ±3	14 ±4	5 ±4	32 ±10	86 ±60	1633 ±571	14 ±11	1572 ±230	3766 ±1146	2341 ±719
IL-8	306 ±111	282 ±95	537 ±197	1540 ±628	339 ±107	531 ±138	191 ±75	1152 ±407	7478 ±2517	17154 ±3462	638 ±232	22359 ±3675	>30000	>30000
IL-2	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	2 ±1	191 ±47	515 ±141	278 ±116
Cell Prolif.	15 ±7	165 ±59	82 ±25	204 ±46	72 ±30	265 ±86	60 ±33	308 ±128	257 ±134	1759 ±531	374 ±177	57669 ±6823	117034 ±15953	93039 ±15106

Values for cytokines (pg/ml) and for cell proliferative responses (counts per min) are means ± S.E.M of 4-18 donors of PBMC.

7.3.3 IL-2 and cell proliferative responses to mAbs for Methods 1 (antibody immobilisation) and 2 (co-culture)

IL-2 and cell proliferative responses for methods 1 and 2 are given in tables 9 and 10 and are summarised in Figure 54. None of the mAbs incubated in aqueous phase with PBMCs alone in methods 1 and 2 stimulated notable IL-2 or cell proliferative responses. For methods 1 and 2, notable IL-2 and cell proliferative responses were stimulated only by TGN1412 and these responses to TGN1412 were at least 100x larger than responses to Avastin ($p<0.01$ for IL-2 responses in both methods, $p<0.05$ for cell proliferative responses in Method 2, $p>0.05$ for cell proliferative responses in method 1). IL-2 and cell proliferative responses to PHA (positive control) incubated with PBMCs cultured with endothelium derived cells or PBMCs alone were marked.

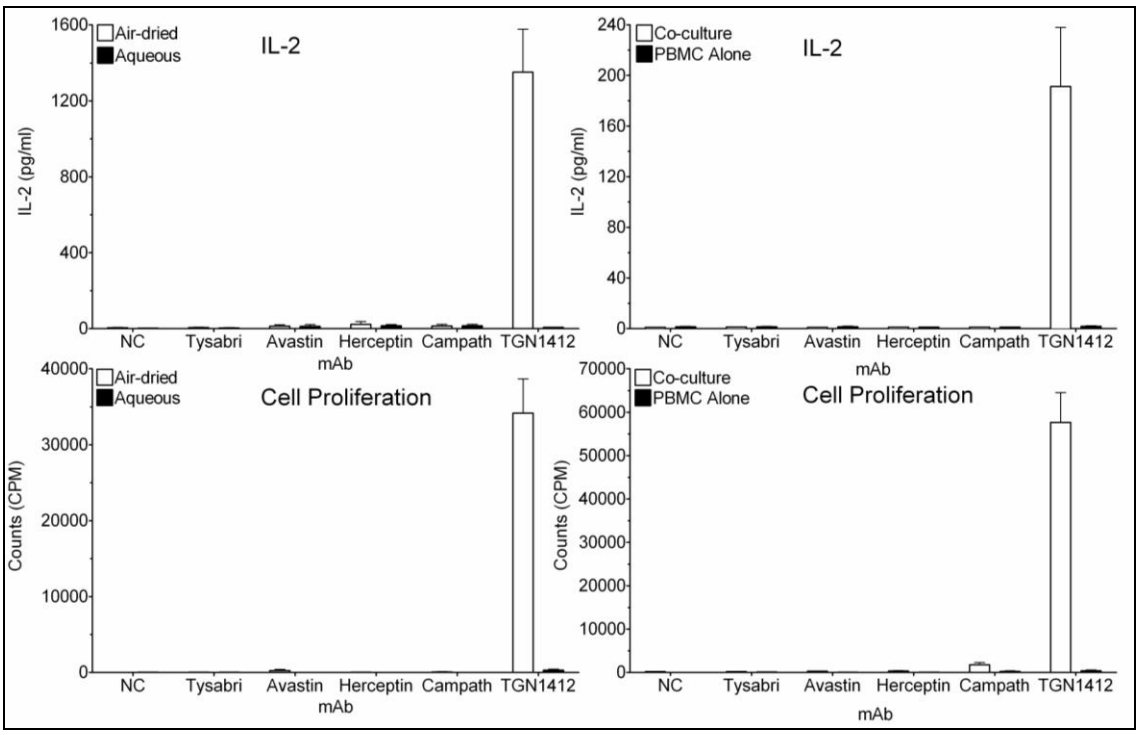


Figure 54. IL-2 and cell proliferative responses of human cells to mAbs Tysabri, Avastin, Herceptin, Campath-1H, TGN1412 and the control mAb (NC, Negative Control) using two different methods. Left-hand panels: PBMCs were cultured for 24 h with mAbs that had either been immobilised by air-drying onto wells of a 96-well polypropylene microtitre plate (white bars) or that had been added as an aqueous solution (black bars). Right-hand panels: PBMCs were cultured for 48 h with mAbs, in aqueous phase, either over a monolayer of human umbilical vein endothelium derived cells (white bars) or alone (black bars). The values (pg/ml or counts per min) are means ± S.E.M of 4-18 donors of PBMC.

7.4 Discussion

Cytokine release assays represent an important element of the pre-clinical safety testing of therapeutic mAbs and other medicines and vaccines in development (Vidal et al., 2010). The two methods developed in previous chapters for predicting the clinical effects of TGN1412 were compared with regard to their capability to predict the clinical effects of therapeutic mAbs other than TGN1412. Method 1 involved air-drying mAbs onto plastic (polypropylene) prior to incubation with PBMC, and Method 2 involved incubating mAbs in aqueous phase with a co-culture of PBMC and human umbilical vein endothelium-derived cells. The panel of mAbs included in this evaluation comprised mAbs associated with a significant incidence of pro-inflammatory clinical infusion reactions, i.e. Herceptin and Campath-1H, and mAbs not associated with a significant incidence of pro-inflammatory clinical infusion reactions, i.e.

Tysabri and Avastin (Dillman, 1999; Chung, 2008; Hellwig et al., 2008; Hansel et al., 2010). TGN1412 was chosen as the positive control for the two methods under evaluation since it had been shown previously to stimulate the release of TNFα, IL-6 and IL-8 in both methods.

The data obtained for TGN1412 in the present study can be summarised as follows: TGN1412 was, overall, the most potent stimulator of cytokine release of the therapeutic mAbs tested above and stimulated the release of TNFα, IL-6, IL-8, IL-2 and cell proliferation in both methods. These findings are broadly consistent with those in Chapter 4 and of previous studies (Stebbings et al., 2007; Findlay et al., 2010; Eastwood et al., 2010). With regard to the other antibodies tested, Campath-1H, when immobilised by air-drying, was the most potent stimulator of IL-6 and IL-8 release (after TGN1412) but was not a potent stimulus for TNFα release relative to TGN1412. This is contrary to previous studies where immobilised Campath-1H was a potent stimulus for TNFα release (Eastwood et al., 2010); however, different experimental conditions were used in the study of Eastwood et al. (2010), the main difference being the use of 15% FCS rather than 2% donors' own plasma. Similarly, when tested in the co-culture of PBMC over endothelial cells, Campath-1H stimulated the release of much less TNFα than TGN1412 but was its equal at stimulating the release of IL-6 and IL-8.

Unlike TGN1412 or, indeed, any of the other antibodies tested, when incubated in aqueous phase with PBMCs alone, Campath-1H stimulated both IL-8 release and TNFα release, which was consistent with the findings of Wing et al. (1995) who investigated ex-vivo whole blood cultures. This is consistent with the finding that first dose cytokine release syndrome stimulated by Campath-1H (IgG1) is a consequence of Fc ligation of target-bound antibody (CD52) to FcγIII receptors (CD16) on natural killer cells, a function weakened in Campath-1H's IgG4 counterpart (Wing et al., 1995; Wing et al., 1996). The destruction of targeted tumour cells by Campath-1H is through antibody-dependent cell-mediated cytotoxicity (Waldmann and Hale, 2005; Hansel et al., 2010). In contrast, TGN1412, as an anti-CD28 IgG4 mAb, does not stimulate a "cytokine storm" by the same mechanism that Campath-1H stimulates cytokine release syndrome (Eastwood et al., 2010).

The lack of cytokine responses to the IgG1 molecules Avastin and Herceptin in aqueous phase with PBMCs alone, mediated by IgG1 Fc to Fc receptor ligation, is unclear especially for Herceptin since there is evidence to suggest that Herceptin induces ADCC through FcγIII receptor (CD16) ligation on natural killer cells (Arnould et al., 2006), as with Campath-1H. Low target antigen density is a possible explanation since it has been shown that the expression of lymphocyte surface antigens correlated approximately with the capability of human IgG1 antibodies against the surface antigens to stimulate cytokine release (Wing et al., 1995). Another important factor in Fc receptor mediated cytokine release is the ease of access of the antibody Fc region, once bound to its antigen, to the Fc receptor (Wing et al., 1996).

The release of IL-6 *in vivo* is delayed after infusion of Campath-1H, compared with the release of TNFα and IFNγ (Moreau et al., 1996) suggesting that IL-6 may be released in response to TNFα and IFNγ from cells other than T cells such as monocytes or endothelial cells (Wing et al., 1996). In support of this notion, IL-6 release stimulated by aqueous phase Campath-1H in the present study was much larger when PBMCs were cultured over a monolayer of endothelial cells than when cultured with PBMC alone, whereas TNFα release remained much the same in the absence or presence of endothelial cells. An as yet unexplained result, however, is the large IL-6 response of PBMCs stimulated by air-dried Campath-1H. Large donor to donor variation in IL-6 responses to Campath-1H, for both methods, may be a result of FcγIII receptor polymorphisms that modulate antibody dependent cell-mediated cytotoxicity as is the case for therapeutic mAb Rituximab (Treon et al., 2005). A small (albeit statistically significant) TNFα response to Campath-1H compared with responses to TGN1412 may have been the result of natural killer cells constituting only a small proportion of PBMCs.

Another difference between TGN1412 and Campath-1H was the finding (from both methods described in this study), that TGN1412 alone of the antibodies tested stimulated the release of IL-2 and caused cell proliferation. Indeed, the capability of TGN1412 to stimulate the release of IL-2 and to cause cell proliferation (and also the release of IFNγ) have previously been described as the cardinal features of TGN1412 stimulation, with the major cell type stimulated being $CD4^+$ effector memory cells (Eastwood et al., 2010) in contrast to the $CD8^+$ and natural killer cells stimulated by Campath-1H (Wing et al., 1996).

Although the two methods evaluated were capable of distinguishing responses to TGN1412 and Campath-1H from each other and from the responses to the other antibodies tested, neither method adequately distinguished Herceptin, an antibody reported to cause a significant incidence of pro-inflammatory clinical infusion reactions, from Avastin and Tysabri, antibodies not associated with a significant incidence of pro-inflammatory clinical infusion reactions, though Herceptin did stimulate more TNFα, IL-6 and IL-8 release than Avastin and Tysabri when immobilised by air-drying. Indeed, both the IL-6 and the IL-8 responses to the immobilised antibodies tested showed a graded increase in the release of IL-6 and IL-8 with the frequency/severity of clinical infusion reactions/cytokine release syndrome reported in the literature (see above), as can be seen from Figure 53.

Perhaps not surprisingly, neither of the two methods evaluated offers perfect diagnostic capability for cytokine release syndrome across the range of mAbs tested. What the two methods do offer, however, particularly when used in conjunction and when IL-2 release and cell proliferation are included as readouts, is a relatively straightforward means to discriminate antibodies likely to precipitate a cytokine storm (like TGN1412) from antibodies that are likely to stimulate cytokine release syndrome (like Campath-1H) without the need for expensive, state of the art flow cytometry equipment. Furthermore, these methods were able to predict cytokine release to antibodies with two distinct underlying mechanisms for causing cytokine release, i.e. through activation of the target receptor and through Fc mediated effector functions. Further work to investigate additional antibodies with a diverse range of mechanisms and PBMC/endothelial cell interactions would further refine the methods, however, the methods as described above are here shown to have value in predicting the potential for a therapeutic antibody to trigger cytokine release syndrome in humans and its likely clinical consequences. Data from such methods should therefore be integrated into the risk–benefit analysis for candidate medicines in line with the principles described in the Committee for Medicinal Products for Human Use Risk Mitigation Guideline (European Medicines Agency, 2007).

Chapter 8:
General discussion & conclusions

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8.1 Failure of pre-clinical tests to predict the clinical effects of TGN1412

In March 2006, the therapeutic mAb TGN1412 was administered to human volunteers during its "first time in man" phase I clinical trial. Infusion of this mAb stimulated a "cytokine storm" resulting in life-threatening clinical effects (Suntharalingam et al., 2006). In the pre-clinical testing phase, TGN1412 itself and TGN1412 orthologues were investigated using animal models (rodents and macaques) and in *in vitro* experiments using human PBMC, purified T cells and T cell subsets in which the CD28 superagonists were incubated with aqueous TGN1412. None of the results from the pre-clinical testing had been interpreted as suggesting that TGN1412 would cause severe adverse events in humans, despite the testing revealing TGN1412's capability to induce T cell proliferation and the production of pro- and anti-inflammatory cytokines. The failure of pre-clinical testing to predict the toxic effects of TGN1412 provided the impetus for this thesis, the hypothesis of which was that c*ytokine-driven adverse effects of therapeutic monoclonal antibodies and the mechanisms involved can be better predicted with novel in vitro procedures using human cells*.

Following the failed clinical trial, it was established at NIBSC that the syringe material administered to the trial volunteers was indeed of clinical grade, that it complied with its specification and that no mistakes had been made in quality control tests to establish this status (Expert Group on Phase One Clinical Trials, 2006). Although the tests conducted after the trial included a rabbit pyrogen test and a bacterial endotoxins test, it was conceivable that TGN1412 contained a non-endotoxin pyrogenic contaminant to which rabbits were insensitive but to which humans were sensitive. In the present study, when the cell-based assay optimised for the detection of (all) pyrogenic contaminants was carried out as described in Chapter 3 (using PBMC and whole blood), the results indicated that TGN1412 was free from (endotoxin and 'non-endotoxin') pyrogenic contaminants and that the pro-inflammatory activity of TGN1412 was a result of the intrinsic nature of the molecule itself. Moreover, the negligible cytokine responses stimulated by TGN1412 in these experiments suggested that the cytokine release

assay optimised for detecting pro-inflammatory contaminants,was not appropriate for detecting the intrinsic pro-inflammatory activity of TGN1412 itself, in common with the animal models used. Consequently, there was an urgent need for new procedures better able to detect unwanted (cytokine-releasing activities) intrinsic activities of mAbs (and other biological medicines).

It is only recently that scientists have been able to explain the failure of animal models to predict the clinical effects of TGN1412 activity. In rodents, the removal of Tregs prior to treatment with a CD28 superagonist results in an increase in systemic cytokines. For this reason it has been suggested that IL-2 produced by T cells stimulates proliferation of Tregs which "quench" the production of cytokines. In humans, the prevalence of the main cytokine-producing cells stimulated by TGN1412 may be different than in rodents. For example, CD4+ memory cells are likely to be more abundant in humans than in laboratory rodents because humans are exposed to infection more frequently than laboratory animals. As a consequence, in humans, Tregs are unable to quench the overproduction of cytokines (Muller et al., 2008; Gogishvili et al., 2008; Pallardy and Hunig, 2010; Romer et al., 2011). As previously mentioned, the failure of macaques to predict the cytokine storm stimulated by TGN1412 is due to a lack of CD28 expression by CD4+ effector memory cells (Eastwood et al., 2010).

8.2 Monoclonal antibody immobilisation

Stebbings et al. (2007) showed that, *in vitro*, TGN1412 was able to stimulate cytokine responses if it was immobilised prior to incubation with human PBMC. The objective of the study described in Chapter 4 was to further develop the *in vitro* antibody immobilisation test. Six different methods of applying TGN1412 to 96-well microtitre plates were investigated to determine the capability of these *in vitro* methods to predict the observed *in vivo* cytokine-releasing activity of TGN1412. The methods included air-drying the mAb onto polystyrene or polypropylene plates, wet-coating the mAb onto polystyrene or polypropylene plates and incubating the mAb in aqueous phase in polystyrene or polypropylene plates. Immobilised mAb was subsequently incubated with PBMC. TGN1412 stimulated significant dose-dependent cytokine responses when immobilised onto both polystyrene and polypropylene plates but not

when incubated in aqueous phase in polystyrene or polypropylene plates. Overall, TGN1412 air-dried onto polypropylene plates gave the largest absolute cytokine responses. Wet-coating onto polystyrene plates resulted in the smallest cytokine responses.

Substituting PBMC with whole blood resulted in smaller cytokine responses to immobilised TGN1412 (air-dried). This was unexpected since whole blood assays more closely mimic the *in vivo* situation and a number cell types are removed when isolating PBMC, for example the granulocytes. It is possible that the isolation procedure somehow primed the PBMC or removed a negative influence or influences from the whole blood, or both. Erythrocytes undergo lysis when in culture, resulting in the release of haemoglobin which is toxic to other cells (Sheerin et al., 1999). This toxicity may have inhibited cytokine release in whole blood assays. Alternatively, it may simply be that because in the absence of blood flow, the immobilised TGN1412 is rapidly covered with the large excess of red blood cells in whole blood rather than with the lymphocytes that express the CD28 receptor. From a practical perspective, of course, whole blood (as opposed to PBMC) would be the preferred method as it is less time consuming. Others have found that whole blood assays give larger cytokine responses than PBMC. Recently it was reported that endotoxin stimulates larger TNFα responses in whole blood compared with PBMC (Chen et al., 2010). In addition, antigen-induced cytokine release by T cells was reported to be smaller for PBMC than whole blood (Li et al., 2010). It is likely that the most appropriate cell source (PBMC or whole blood) will depend upon the nature of the product being tested and should be assessed on a case by case basis. As with PBMC, TGN1412 air-dried onto polypropylene stimulated larger cytokine responses using whole blood than TGN1412 air-dried onto polystyrene plates.

Overall the differences in TGN1412-stimulated cytokine responses between the immobilisation methods could not be explained by different methods affecting either the quality or quantity, or both, of TGN1412 immobilised. (In this context, quality means orientated with the CD28-binding domain more readily available to bind CD28 expressed on T cells.) However, one exception to this result was the finding that less TGN1412 remained bound to polystyrene plates when immobilised by wet-coating, compared with the other methods. In addition, wet-coating to polystyrene favoured TGN1412 being immobilised with an orientation such that the

CD28-specific variable region was less available to bind to the CD28. It should be noted that a lack of binding of TGN1412 to polystyrene plates used in this study could be a result of the tissue culture treatment that they had been subjected to and was not due to the fact or solely to the fact that they were made of polystyrene.

To minimise clinical infusion reactions to other mAbs, it was important to determine whether or not any of the methods used to immobilise TGN1412 were better at predicting unwanted pro-inflammatory activities of mAbs other than TGN1412. It was only when the antibodies were air-dried onto polypropylene or polystyrene or wet-coated onto polypropylene (but not when wet-coated onto polystyrene), that the antibodies associated with pro-inflammatory clinical infusion reactions stimulated cytokine responses. It is likely that, as with TGN1412, the other mAbs tested were less able to bind to polystyrene plates when wet-coated. It can be concluded that if antibody immobilisation procedures are used in pre-clinical tests to predict the clinical effects of mAbs, caution must be exercised as not all plate types are equally suitable and some may give false negative or false positive results.

It is not fully understood why, *in vitro*, immobilisation of TGN1412 is required to stimulate cytokine responses. Immobilisation of TGN1412 may have presented this mAb to T cells such that it was able to bind to CD28 receptors within a concentrated (i.e. polarised) area of the cell surface and thus achieving a level of CD28 cross-linking above the threshold level required for T cell activation. The manner in which TGN1412 is presented to its target receptor *in vitro* is clearly crucial to its biological effect. Given that TGN1412 was administered as a solution, it is not known how this polarised binding could have been achieved *in vivo* or, indeed, if it occured at all. Perhaps, *in vivo*, other influences reduce the threshold of CD28 cross-linking required for T cell activation, negating the requirement for the polarised binding of TGN1412 to T cells.

It was also shown in Chapter 4 that immobilised TGN1412 was able to upregulate TNFα gene expression in PBMC after a 30 minute incubation and that TNFα protein was detected in cell-conditioned medium after a 60 min incubation of TGN1412 with PBMC. This finding suggests that the kinetics of TNFα release stimulated by TGN1412 air-dried onto polypropylene (chosen because this method stimulated the largest cytokine responses) mimicked the kinetics of

TNFα release *in vivo* in man (Suntharalingam et al., 2006), further validating this mAb immobilisation method for the prediction of TGN1412-like activity. Also in support of this *in vitro* method, elevated IL-6 and IL-8 responses of PBMC to immobilised TGN1412 could not be measured in terms of gene expression (RT-PCR) or protein production (ELISA) even after a 90 minute incubation. This was consistent with the kinetics of IL-6 and IL-8 responses *in vivo* which followed TNFα responses (Suntharalingam et al., 2006). It is possible that cells bearing CD28 receptor are directly stimulated by TGN1412 to release TNFα which subsequently stimulates other cells bearing TNFα receptor (CD120) to release IL-6 and IL-8. Such 'other cells' may include monocytes and endothelial cells. Consistent with the downstream role of other cell types is the finding (Chapter 6) that TNFα was capable of stimulating endothelial cells to release IL-6. It should also be noted that the kinetics of TNFα responses to immobilised TGN1412 measured by ELISA broadly mirrored the kinetics of TNFα gene expression. It would seem therefore that the TNFα ELISA used in this study, which is simpler and takes less time to carry out than RT-PCR, is sufficient for evaluating the kinetics of cytokine responses over short periods of stimulation. That said, cytokine levels after a 1 hr incubation with immobilised TGN1412 measured by ELISA were much smaller than those measured in the blood of the trial volunteers 1 h post infusion. The use of RT-PCR, however, was valuable as it was able to show that, *in vitro*, TGN1412 stimulated *de novo* TNFα synthesis within 30 min of its incubation with PBMC. Therefore, TNFα released by cells, at least in part, was not pre-formed (Suntharalingam et al., 2006).

Antibody immobilisation was the first method to be developed following the TGN1412 clinical trial that proved to be capable of predicting the cytokine responses measured in the blood of the trial volunteers. Had this test been carried out as part of the TGN1412 pre-clinical testing phase, it is likely that the trial would not have proceeded. Nevertheless, this mAb immobilisation method can be criticised for several reasons: immobilisation of a mAb onto a plastic surface has little if any physiological relevance and for some mAbs may give "false positives" if used as a pre-clinical safety test. For example, Avastin is a therapeutic mAb that is not associated with causing cytokine release syndrome (Chung, 2008) but when immobilised by air-drying in this study it was capable of stimulating some cytokine release. Therefore, it should

not be assumed that an antibody immobilised by air-drying that stimulates pro-inflammatory cytokine responses of PBMC would automatically do so in man. In contrast, an antibody that fails to stimulate pro-inflammatory cytokine responses of PBMC – even when it is immobilised by air-drying – would appear to be unlikely to do so *in vivo* in man.

The use of a mAb immobilisation method to predict a "safe starting dose" for future mAbs progressing to a phase I "first time in man" clinical trial would be questionable since it cannot be certain how much of the mAb is attached to the well following the washing steps. In addition, the amount that is attached may vary from one type of plate to another or even from one batch of plates to another. Indeed, a ten-fold shift in the maximum immunostimulatory dose (based on the amount added to wells) from 10 to 100 µg/well TGN1412 was observed in this study following the change from one batch of plates to another from the same manufacturer. Consequently, the mAb immobilisation method could be described as qualitative rather than quantitative. To make dose predictions, one could perhaps establish the percentage of added TGN1412 which remains bound to a specific batch of plates intended for use.

Following the TGN1412 clinical trial, the Secretary of State for Health set up an Expert Scientific Group which was chaired by Sir Gordon Duff. The Duff Report was published after ten Expert Scientific Group meetings and included considerations from 77 submitted viewpoints from experts. It recognised that "The need for better and safer medicines is clear" and that "pre-clinical development studies that were performed with TGN1412 did not predict a safe dose for use in humans" (Expert Group on Phase One Clinical Trials, 2006). A method that is able to predict the toxic effects of mAbs would clearly be even more valuable if it could be used to make accurate predictions of a safe starting dose. A method whereby TGN1412 could be incubated in aqueous phase was here considered a step in the right direction.

8.3 The co-culture method

In an attempt to develop a method which could predict the toxic effects of TGN1412 in aqueous phase, the co-culture assay was developed as described above: this assay incorporated a monolayer of human umbilical vein endothelial cells over which human PBMC and aqueous

TGN1412 were incubated. Since endothelial cells form the lining of blood vessels it was likely that infused TGN1412 stimulated T cells in the presence of endothelial cells. Furthermore, endothelial cells are involved in mediating inflammatory responses *in vivo* (Muller, 2002). Indeed, this method was able to predict clinical responses stimulated by TGN1412 in aqueous phase, with TGN1412 stimulating dose-dependent cytokine responses. The dose of aqueous TGN1412 which stimulated the largest cytokine and cell proliferative responses was 3.3 µg/ml (1 µg/well) which, in contrast to immobilised TGN1412, was a consistent maximally effective dose in all experiments.

Unfortunately for the TGN1412 trial volunteers, this maximum immunostimulatory dose *in vitro* (3.3 µg/ml) was close to the *in vitro* equivalent dose (2 µg/ml) of the dose given to the trial volunteers (0.1 mg/kg) as described in Chapter 3. This fact shows that the co-culture method is predictive of the nature of *in vivo* responses. The NOEL approach (no observed adverse effect level) was used to calculate a safe starting dose for TGN1412 at its clinical trial. Since no effects of TGN1412 were observed in Cynomolgus macaques following infusion of 50 mg/kg, this dose was used to calculate an equivalent safe starting dose for humans. What was not known at the time was that macaques are an inappropriate animal model for predicting TGN1412 activity since they do not respond as humans to TGN1412.

In light of the above findings, the MABEL approach (minimum anticipated biological effect level) is now considered to be more appropriate than NOEL (Expert Group on Phase One Clinical Trials, 2006) as the chosen starting dose is required to have some biological effect. The MABEL approach has now been incorporated into the European Medicines Agency/Committee for Medicinal Products for Human guidelines (European Medicines Agency, 2007). If data from the co-culture assay is used to calculate a safe starting dose using the MABEL approach, 0.04 µg/well/300 µl = 0.132 µg/ml could be considered the MABEL (see Figure 27) where cytokine release/cell proliferation are the measured biological effects. (This was the smallest dose of TGN1412 tested in this study and it should be noted that smaller doses may have given a biological effect).

Using the method to equate *in vitro* with *in vivo* doses described in section 3.2.1, 0.132 µg/ml would approximately equate to 396 µg/3 L blood plasma in a 70 kg adult, which gives a

starting dose of 0.0057 mg/kg. This is approximately 17 times smaller than the dose (0.1 mg/kg) actually given to the trial volunteers, even without the addition of a safety margin which is also usually applied (Stebbings et al., 2009). It should be noted however that this safe dose prediction does not account for all differences between *in vivo* and *in vitro* systems. TGN1412 would be distributed and compartmentalised in the body and so it is likely that less TGN1412 would be available to activate cells *in vivo* than cells cultured in a well when equivalent doses are applied. However this is less of a problem than if the reverse situation was to be the case. The cell density, cell-types present (granulocytes are excluded from the PBMC fraction) and cell flow-rate (nil *in vitro*) are examples of other conditions which differ between the *in vivo* and *in vitro* situations. Further work to determine the effects of these differences on the MABEL of TGN1412 would be of value. That said, if data from a co-culture assay had been used to calculate a starting dose prior to the clinical trial, the starting dose would have been at least 17x smaller than the dose given to the trial volunteers. It is likely that a smaller starting dose would have been safer than the dose given during the trial, that is, of course, if the trial had been allowed to go ahead in the face of significant TGN1412-stimulated cytokine release *in vitro*.

Despite efforts to optimize the experimental conditions for the co-culture assay, TGN1412-stimulated increases in TNFα, IL-2 and IFNγ concentrations in 24 and 48 h co-culture assays were considerably smaller than the concentrations measured in the blood of the trial volunteers. It is possible that endothelial cells derived from human umbilical vein do not have the same phenotype as endothelial cells involved in mediating TGN1412 responses *in vivo.* Indeed, the heterogeneity of endothelial cells is widely acknowledged (Garlanda and Dejana, 1997) and those involved *in vivo* obviously would not have been of umbilical vein origin. Unfortunately, when endothelial cells from a more physiologically relevant source, i.e. human saphenous vein, were used instead of HUVEC in co-culture assays, background cytokine were unacceptably large even in the absence of TGN1412 making it difficult to draw any conclusions from the experiments.

In vitro culture of HUVEC may alter the expression of various molecules key to eliciting TGN1412-stimulated responses. It was certainly found in the present study that different culture conditions (implemented in two different laboratories) used for HUVEC from the same source

(TCS Biologicals, batch ZHC-2101) greatly affected the capability of these cells to mediate TGN1412-stimulated responses in co-culture assays. This finding emphasises the importance of culture conditions in maintaining endothelial cell phenotype. Indeed, it was shown in the present study that an endothelial phenotype is important since fibroblasts from two different sources (human gingiva and human foreskin) were poor mediators of TGN1412-stimulated responses in co-culture assays. This is supported by an additional study conducted at NIBSC where the capability of cells to evoke TGN1412-responses in co-culture assays (using the method developed in this thesis) correlated with endothelial phenotype, defined by the expression of constitutive and induced endothelial markers (Findlay et al., 2011b).

The small cytokine responses to TGN1412 in co-culture assays may reflect the fact that only endothelial cells and PBMC were present. *In vivo*, endothelial cells may act in concert with other cell-types, such as fibroblasts, smooth muscle cells or specialised APCs such as dendritic cells, to mediate TGN1412-stimulated responses. Mature dendritic cells presenting antigen in secondary lymphoid organs such as the lymph nodes express a high level of co-stimulatory molecules required for T cell activation, in addition to the ligands for CD28. Such molecules include ICAM-1 and LFA-3 (Lyerly et al., 2000; Kim et al., 2006) which in the work described above were found to have a role in mediating TGN1412-stimulated cytokine responses in co-culture assays. However, dendritic cells only exist in an immature state in peripheral blood (and thus in PBMC) and these cells lack expression of co-stimulatory molecules (Lyerly et al., 2000). Therefore, mature dendritic cells may have contributed to the mechanism of action of TGN1412 *in vivo* but may not have done so in co-culture assays.

As discussed in section 5.4, it is also possible that the small TGN1412-stimulated responses in co-culture assays were the result of activation of different T cell subsets compared with those activated by immobilised TGN1412 and with those activated in the trial volunteers. It is likely that the mechanism by which TGN1412 stimulated responses in co-culture assays was only part of what happened in the trial volunteers. In light of this, caution must be excised when making ‘safe dose’ predictions for future mAbs with TGN1412-like activity entering into “first time in man” clinical trials.

The concentrations of IL-6 released in response to TGN1412 in co-culture assays were comparable to concentrations measured in the blood of the trial volunteers, unlike the concentrations of IL-6 produced in response to immobilised TGN1412. HUVEC stimulated with TNFα in this study released significant concentrations of IL-6 and so a likely mechanism is that TNFα released in co-culture assays in response to TGN1412 acted upon endothelial cells, stimulating the release of IL-6. It is likely that the smaller IL-6 responses of PBMC alone to immobilised TGN1412 were due to the absence of an IL-6 contribution from endothelial cells although clearly endothelial cells were not the sole source of IL-6 in co-culture assays as discussed in section 6.4.5. As with TGN1412, IL-6 responses to Campath-1H were much larger when the mAb was cultured with PBMC over a monolayer of endothelial cells. Therefore, it would appear that the inclusion of a monolayer of endothelial cells in a PBMC based assay improves the capability of the assay to predict IL-6 responses to medicines. In contrast to IL-6 responses, TNFα responses to TGN1412 in co-culture assays were smaller than TNFα responses to immobilised TGN1412. It has been found previously that IL-6 inhibited the release of TNFα (and IL-1) from PBMC (Schindler et al., 1990). Therefore, it is possible that smaller TNFα concentrations measured in co-culture assays compared with those measured in immobilisation assays was, at least in part, a result of more inhibition of TNFα release by the larger concentrations of IL-6 in the cell-conditioned media in co-culture assays.

Exactly why TGN1412 was able to stimulate responses in co-culture assays in aqueous phase but not when incubated with PBMC alone is unclear. A number of possibilities were investigated which led to a number of conclusions. Data from transwell experiments suggests that endothelial cell to PBMC contact is required to initiate responses to TGN1412 in co-culture and that the initial activation of T cells by TGN1412 is not through a soluble signal. The finding that a conventional agonistic CD28 antibody did not stimulate cytokine or cell proliferative responses in co-cultures assays suggests that a signal resulting from ligation of allogeneic MHC class II molecules expressed by endothelial cells with the TCR was unlikely. In which case, this interaction was unlikely to have played a role in mediating responses to TGN1412. It was also concluded that it is unlikely that endothelial cells immobilised TGN1412 through Fc to Fc receptor binding for reasons discussed in more detail in section 6.4.3.

What was found, however, was that the adhesion molecules LFA-3 and ICAM-1 promoted responses to TGN1412 in co-culture assays but not in assays in which immobilised TGN1412 was incubated with PBMC alone. The reason for the latter is not clear but perhaps responses of PBMC stimulated by immobilised TGN1412 were so large that they could not be further augmented by co-stimulatory molecules expressed by PBMC. TGN1412-stimulated responses in co-culture assays could not be completely inhibited by LFA-3 blockade suggesting that other molecules were involved in the initiation of TGN1412-stimulated responses. It is possible that T cells may make alternative use of adhesion/co-stimulatory molecules such that if interaction with one molecule is prevented others could still perhaps initiate responses to TGN1412. If this were to be the case, blocking antibodies specific for all adhesion/co-stimulatory molecules involved would be required to entirely block TGN1412-stimulated responses. Unfortunately the use of blocking antibodies is limited especially when pro-inflammatory cytokine responses are readouts since "pyrogen-free" and "sodium-azide free" (a preservative that is toxic to cells) antibodies are difficult to source. Of course when only pyrogen/endotoxin-contaminated blocking antibodies are available, the readouts are limited to those not affected by pyrogen/endotoxin such as IL-2 and cell-proliferation.

Findings from the co-culture experiments using blocking antibodies are consistent with the notion that the interaction of various molecules expressed on the surface of endothelial cells with their counterstructures expressed by T cells may promote the formation of an immunological synapse at the endothelial cell/T cell interface. Within this synapse, LFA-3/CD2 and ICAM-1/LFA-1 interactions (and likely other interactions) may organise molecular targets/signalling molecules in such a manner that negates the requirement for TGN1412 to be immobilised in order to stimulate responses. The mechanisms underlying the effects that LFA-3/CD2 interactions have on TGN1412-stimulated responses are not clear; this subject is discussed in more detail in Chapter 6. In summary, LFA-3/CD2 interactions may promote the aggregation of lipid rafts within the immunological synapse and thus signalling molecules such as tyrosine kinases linked to the lipid rafts (Mestas and Hughes, 2001). The aggregation of lipid rafts and associated signalling molecules within the immunological synapse may be mediated by microfilaments as a result of LFA-3/CD2 interaction (Kaizuka et al., 2009). LFA-3/CD2

interactions may also promote the aggregation of activators of the tyrosine kinase Lck in the immunological synapse but exclude inhibitors of Lck as suggested by Kaizuka et al. (2009) albeit for T cell activation by APCs and not TGN1412. Another possible mechanism is that TGN1412 may function by "boosting" signals resulting from LFA-3/CD2 interactions. This is supported by evidence to suggest that superagonistic CD28 activation is reliant on low level constitutive "background" signals emanating from unligated TCRs (Hunig and Dennehy, 2005) and by the finding that, in the absence of TCR ligation, LFA-3/CD2 signalling involves the same signalling molecules as TCR signalling (Kaizuka et al., 2009).

As discussed in Chapter 6, it has been suggested that, *in vivo*, microvessel endothelial cells, such as those constituting venules, present foreign antigen from re-infecting pathogens locally to memory T cells to stimulate a rapid recall response. The co-stimulatory molecules expressed by endothelial cells which allow them to act as APCs *in vivo* may have also allowed these cells to mediate T cell activation by TGN1412. Several studies have suggested that endothelial cells are only capable of stimulating CD4+ memory T cell subsets but not naive T cells (Pober and Cotran, 1991; Marelli-Berg et al., 1996; Pober et al., 1997; Ma and Pober, 1998; Murphy et al., 1999; Choi et al., 2004). It is thought that naive T cells have more stringent activation requirements than memory T cell subsets and require specialised APCs (Choi et al., 2004). One study suggested that microvascular endothelial cells are able to stimulate transendothelial migration of CD4+ effector memory T cells (Pober and Sessa, 2007). The finding that immobilised TGN1412 preferentially expands effector memory T cells (Eastwood et al., 2010) is consistent with the fact that rapid responses to TGN1412 occurred during the clinical trial (Suntharalingam et al., 2006). Effector memory T cells may therefore have been the first T cell subset to be activated by TGN1412 in the trial volunteers. Given that endothelial cells are believed to activate only memory T cell subsets during antigen presentation *in vivo*, perhaps in co-culture assays involving endothelial cells, it is memory T cells that are activated by TGN1412. Effector memory T cells exist in very low numbers in peripheral blood and this could account in part for small IFNγ responses to TGN1412 in co-culture assays. In the study of Eastwood et al. (2010), only after 48 – 72 h stimulations with immobilised TGN1412 were larger concentrations of IFNγ detected in cell-conditioned medium. This incubation period of 48

– 72 h was required to allow TGN1412-stimulated naive and central memory T cells (in PBMC) to mature into $CD4^+$ effector memory T cells. However, concentrations of IFNγ in co-culture assays were much smaller than those stimulated by immobilised TGN1412, even after 48 h stimulation with TGN1412. It is possible that while immobilised TGN1412 is able to stimulate both central memory and naive T cell maturation into effector memory T cells, in co-culture assays using endothelial cells, perhaps central memory T cell maturation into effector memory T cells but not naive T cell maturation is stimulated by TGN1412. *In vivo*, effector memory T cells reside in tissues and are particularly abundant in the lungs and gastrointestinal mucosa (Eastwood et al., 2010) but do not re-circulate through the lymph nodes, unlike central memory T cells (see section 1.5). What is not clear is the logistics of exactly how abundant effector memory T cells already in tissues could have interacted with endothelial cells forming venules supplying the tissues. Central memory T cells may have been activated by TGN1412 in microvessels during their circulation throughout the body, for example in the high endothelial venules draining into the lymph nodes. However, responses mediated by central memory T cells usually take longer (see section 1.5) and so these cells were unlikely to have been responsible for the initial responses to TGN1412 in the clinical trial.

8.4 Other studies involving TGN1412 following its clinical trial

The two methods developed in this study for predicting TGN1412 activity were the first to be published following the disastrous events of the TGN1412 clinical trial (Stebbings et al., 2007; Findlay et al., 2010; Eastwood et al., 2010; Findlay et al., 2011a; Findlay et al., 2011b). Studies have been carried out in other laboratories in an attempt to develop better methods for predicting the capability of therapeutic mAbs (including TGN1412) to stimulate cytokine release and in an attempt to better understand the mechanisms underlying TGN1412 toxicity. Some of these methods were based upon findings from the studies described in the references quoted above. After the discovery that immobilised TGN1412 can stimulate cytokine responses from PBMC, one group evaluated the use of immobilising mAbs (including ANC28.1/5D10, a mouse anti-human CD28 superagonist) on beads coated with Protein A to predict the capability

of these mAbs to stimulate cytokine release (Walker et al., 2011). Protein A binds with high affinity to the Fc portion of some IgG antibodies and so can be used to immobilise mAbs without disrupting their binding to target antigen. The authors showed that this method was able to predict TGN1412-like activity since cytokine release by human whole blood stimulated with the CD28 superagonist for 48 h were significantly larger than those in control wells where beads were coated with autologous plasma. However, cytokine concentrations were considerably smaller than those stimulated by TGN1412 immobilised directly onto plates in the present study. This finding might be explained by the use of a different CD28 superagonist. Unfortunately only whole blood was incubated with mAbs immobilised by Protein A-coated beads and so a comparison between whole blood and PBMC could not be made.

The use of Protein A-coated beads is a more laborious and complex procedure than directly coating the mAb onto plates. Furthermore, the use of Protein A-coated beads does not provide a more physiologically relevant means for predicting TGN1412-like activity than the mAb immobilisation methods already published (Stebbings et al., 2007). In addition to a CD28 superagonist, the authors tested 8 other therapeutic mAbs and used hierarchical cluster analysis to show that the profile of cytokines stimulated by the CD28 superagonist was distinct from profiles stimulated by other therapeutic mAbs. Consistent with the findings from both TGN1412 immobilisation assays and co-culture assays in the present study and the findings of Eastwood et al. (2010), Walker et al (2011) reported that IL-2 stimulation was a cardinal feature of TGN1412-like activity. As described in Chapter 6, the IL-2 released in co-culture assays in response to TGN1412 clearly had a biological effect since when IL-2 was neutralised with an anti-IL-2 mAb, cell proliferative responses were inhibited. During the clinical trial, approximately 25 hours post-infusion of TGN1412, the trial volunteers were treated with an anti-IL-2 receptor mAb because of the expected effects of TGN1412 on T cells (Suntharalingam et al., 2006). This mAb binds to the α chain of the IL-2 receptor expressed by T cells preventing IL-2 from binding to the receptor and subsequent T cell proliferation (Yang et al., 2010).

In addition to IL-2, however, Walker et al. (2011) showed that the CD28 superagonist stimulated larger IL-4, IL-12 and IL-17 responses than were obtained with the other mAbs tested which they suggested was also a feature of TGN1412-like activity. Indeed concentrations

of IL-4 were elevated in the blood of the trial volunteers though concentrations of IL-12 and IL-17 were not reported (Suntharalingam et al., 2006).

The use of mAb immobilisation by Protein A-coated beads to compare cytokine responses to therapeutic mAbs can be questioned. First, Protein A differentially binds different IgG subclasses (Eliasson et al., 1989) and so differential cytokine release between mAbs could reflect their affinity for Protein A. Second, it has been reported that Protein A itself (as a bacterial antigen) can stimulate pro-inflammatory cytokine responses from monocytes (Tufano et al., 1991). Third, binding of Protein A to the mAb is likely to affect Fc to Fc receptor binding-mediated effector functions as it binds to a site close to the Fc receptor binding region of IgG Fc (Deisenhofer, 1981; Walker et al., 2011). Therefore, cytokine release stimulated by mAbs as a result of Fc-mediated mechanisms, may be affected or even not detected. That said, Campath-1H, which works predominantly through ADCC, did stimulate IL-6 and IL-8 responses when immobilised by Protein A-coated beads.

In the study of Sandilands et al., (2010), mouse an anti-human CD28 superagonistic mAb was immobilised by goat anti-mouse IgG coated wells of a microtitre plate. The same method was previously described in the study of Stebbings et al., (2007), in which TGN1412 immobilised in this way stimulated lymphocyte proliferation and IL-2 release. This method was not pursued in the present study because, in addition to being more time consuming and labour intensive than coating TGN1412 directly onto wells, it did not prove possible to source a 'clean' TGN1412 capture antibody which did not itself stimulate pro-inflammatory cytokine release from PBMC.

Sandilands et al. suggested that the rapid onset of clinical reactions to TGN1412 was likely mediated by "faster-acting" cells such as monocytes (rather than lymphocytes) especially since TNFα is commonly associated with activated monocytes (Sandilands et al., 2010). Using the anti-IgG capture method, Sandilands et al. produced evidence to suggest that monocytes were responsible for the initial rapid release of TNFα in the trial volunteers. Antibody-captured CD28 superagonistic mAb stimulated TNFα release when incubated with purified monocytes for 2 h. Furthermore, granulocytes were shown to express CD28, and CD28 specific mRNA was upregulated following CD28 superagonist stimulation. These cells also underwent

morphological changes resulting in an appearance similar to those reported in the trial volunteers (Suntharalingam et al., 2006). Initially after the trial it was assumed that the early effects of TGN1412 were the result of T lymphocyte activation (as opposed to monocyte activation) as it is these cells which predominantly express CD28. Indeed, several studies have shown that lymphocytes release cytokines following 60 minute stimulations with immobilised TGN1412 (Stebbings et al., 2007; Eastwood et al., 2010). It is possible, of course, that both lymphocytes and granulocytes (including monocytes) are activated by TGN1412. In addition, natural killer cells (another subset of lymphocytes) have been shown to express a variant of the CD28 receptor (Galea-Lauri et al., 1999) and it is possible that TGN1412 may bind to this receptor. Activated natural killer cells are producers of IFNγ and may have contributed to the large quantities of IFNγ released in response to TGN1412 in the trial volunteers (Sandilands et al., 2010).

Another model proposed by Sandilands et al. (2010) was that TGN1412 is immobilised by FcγR1 expressed by endothelial cells but only when first bound to cells expressing CD28. The authors speculated that target-bound TGN1412 would displace passively bound (to FcR) IgG present within the blood. The authors showed that superagonistic anti-CD28-opsonised PBMC stimulated TNFα release when incubated with HUVEC in a co-culture assay. However, no comparison was made between superagonistic anti-CD28 mAb coated onto PBMC and superagonistic anti-CD28 mAb incubated in aqueous phase in the co-cultures. It is therefore difficult to determine if the anti-CD28 mAb used stimulated a response only because it was bound to CD28-expressing cells prior to its addition to endothelial cells. Also, the CD28 superagonist used in the study of Sandilands et al., (2010) was a mouse IgG2a antibody which is likely to have a different affinity for human FcR than TGN1412, a humanised IgG4 mAb. The findings discussed in Chapter 6 of the present study suggest that it is unlikely that TGN1412 is immobilised by endothelial cells.

Recently, a method has been reported by Romer et al., (2011) in which aqueous TGN1412 was able to stimulate cytokine responses of PBMC alone. The authors' speculated that the failure of pre-clinical tests using PBMC to predict the cytokine storm stimulated by TGN1412 was due to the loss of T cell priming which usually occurs in tissues and is mediated

by cell to cell contact. In tissues, when T cells scan MHC molecules expressed by other cells (in search of cognitive antigen), weak background signalling events are thought to occur via the TCR. This leads to the sub-threshold activation of T cells and is thought to improve the responsiveness of T cells to cognitive antigen when it is encountered. It is thought that background signalling via the TCR is lost by T cells in peripheral blood where cell to cell contact is less likely. Since CD28 superagonist activation is dependent upon background TCR signalling, as discussed earlier, the authors' suggested that the lack of TGN1412-stimulated responses of PBMC could be explained by the absence of background TCR signalling. It was speculated that the "primed status" of T cells isolated from PBMC could be restored by the introduction of a two-day high cell density pre-culture of PBMC prior to stimulation with TGN1412. This pre-culture did indeed restore the capability of T cells to respond to aqueous TGN1412. Furthermore, the inclusion of mAbs specific for MHC class I and II molecules in the pre-culture prevented the restoration of responses to aqueous TGN1412 (Romer et al., 2011). This theory points towards the notion that TGN1412 did not activate cells within the peripheral blood of the trial volunteers, at least at first.

If the theory proposed by Romer et al., (2011) is true, it is not clear exactly how immobilised TGN1412 bypasses the requirement for "background" signals via the TCR achieved by a high cell density pre-culture of cells. Perhaps background signals are present but are even weaker in assays using PBMC (without a high cell density pre-culture) with immobilised TGN1412 compared with those achieved by a high cell density pre-culture. The stronger background signals achieved by a pre-culture at high cell density may lower the threshold of CD28 cross-linking required for T cell activation, allowing aqueous TGN1412 to activate cells. Without the pre-culture, the higher CD28-cross linking threshold may only be achieved by concentrating TGN1412 onto a surface. Endothelial cells in co-culture assays may lower the activation threshold themselves by stimulating/augmenting background signals which TGN1412 then boosts, resulting in activation. These signals may originate from MHC/TCR interactions although this is questionable since constitutive expression of MHC molecules by endothelial cells is unlikely as discussed in Chapter 6. A more plausible theory is that interactions between co-stimulatory adhesion pairs expressed by endothelial cells and PBMC

stimulate/augment the background signalling required by TGN1412 as discussed earlier in section 6.4.4.

8.5 Conclusions

To address the hypothesis of this study that *cytokine-driven adverse effects of therapeutic monoclonal antibodies and the mechanisms involved can be better predicted with novel in vitro procedures using human cells*, two methods have been developed, both of which involved the use of human cells. Certainly both methods were better able to predict cytokine release syndrome and cell proliferative responses stimulated by TGN1412 during its phase I clinical trial than the *in vivo* and *in vitro* pre-clinical tests that were conducted.

Post-TGN1412, it is clear that animal models are not appropriate for the safety (and efficacy) testing of all therapeutic mAbs. Therefore, more emphasis should be placed on the use of *in vitro* tests using human tissue, given that TGN1412's toxicity could have been predicted by both of the *in vitro* methods developed in this study. The benefits gained from any procedure developed for routine safety testing of biological medicines is related to the cost of the procedure and its complexity to carry out. Time-consuming procedures requiring the use of expensive, complex equipment and associated service contracts would be less favorable as would procedures requiring a particularly high level of skill since the risk of error when conducting the procedure is increased. The methods developed in this study offer a relatively straightforward means to discriminate antibodies likely to precipitate a cytokine storm (like TGN1412) from antibodies that are likely to stimulate cytokine release syndrome (like Campath-1H) without the need for expensive, state-of-the-art equipment. Moreover, these methods were able to predict cytokine release to antibodies with two distinct underlying mechanisms for causing cytokine release, i.e. via activation of the target receptor and Fc-mediated effector function. In addition, mAb-stimulated cytokine release in both methods correlated with the frequency/severity of clinical infusion reactions/cytokine release syndrome reported in the literature (see Chapter 7), with the exception of Herceptin in co-culture assays.

Of course, like most procedures, those developed in the present study are not without their limitations. Immobilisation of an antibody onto a plastic surface has little physiological relevance. Nevertheless, this artificial system better mimicked the magnitude of responses stimulated by TGN1412 in the trial volunteers than the co-culture assay. Although the co-culture procedure is more physiologically relevant, responding as it does to TGN1412 in aqueous phase, the smaller cytokine responses suggest that it represents only part of the underlying *in vivo* mechanism(s) of action of TGN1412. Another limitation of both procedures is the difficulty often encountered in obtaining the human tissue required: relying on blood donations from willing colleagues is not an option for all scientists. Although immortalized T cell lines are commercially available, these may not behave in the same manner as T cells from freshly isolated PBMC. In addition, sourcing fresh umbilical cords is not always easy due to ethical constraints and logistical issues. For this reason, one objective of the present study was to identify an alternative to fresh HUVEC from cords: an objective that was met when it was shown that HUVECjr2 cells (pooled from multiple donors) responded to TGN1412 in much the same way as fresh HUVEC from cords. The use of SCID (severe combined immunodeficiency) mice, which can be successfully transplanted with human tissue, could be a potential tool for the future testing of therapeutic biological medicines (Ito et al., 2002).

Despite their limitations, the methods using human cells developed in this study have proved to be not only better than the usual pre-clinical tests at predicting cytokine release caused by TGN1412 and other therapeutic mAbs but they have also proved to have value in investigating the mechanism of action of TGN1412. The new methods are already being used in the pharmaceutical industry to identify unwanted cytokine-releasing activities of candidate new therapeutic mAbs and for identifying mitigation strategies. For example, the incubation of a steroidal anti-inflammatory drug together with the mAb being tested would allow the evaluation of the effectiveness of such drugs in diminishing cytokine release.

In addition, the new methods may be used to assess the effects of new mAbs given concomitantly with other therapies. TGN1412 was developed as a candidate for the treatment of arthritis. Pre-clinical studies had suggested that TGN1412 could be used safely in combination with methotrexate, a treatment for arthritis that induces apoptosis of activated peripheral T cells

(Genestier et al., 1998; Paillot et al., 1998), because of the absence of any apparent interaction (TeGenero AG, 2005a). However this suggestion was revealed to be flawed in light of the TGN1412 clinical trial when it became evident that pre-clinical tests had not predicted the clinical effects of TGN1412 alone.

The methods developed in this study may also be used to rank candidate therapeutic mAbs with the same target according to their (unwanted) cytokine releasing activity in order to identify the safest candidate. There is also potential for data from PBMC/HUVEC co-culture assays to be used (in conjunction with other data) to make dose predictions for first time clinical use since the mAb is incubated in aqueous phase (rather than being dried onto plastic which precludes dose calculations). However, caution must be excised when extrapolating data from an *in vitro* assay to the clinic.

The methods developed here may not be appropriate for predicting the toxic effects of all mAbs as exemplified by Herceptin. When choosing an appropriate method, consideration should be given to target location and its level of expression and the mechanism of action of the mAb. Such factors could influence the human cell type(s) used, cytokines measured and whether or not mAb immobilisation is necessary. Indeed it was a consensus decision made during a European Medicines Agency sponsored workshop by 90 experts from the pharmaceutical industry and regulatory agencies that the format of cytokine release assays for predicting the toxic effects of mAbs should be "tailor-made" for the mAb tested (Vidal et al., 2010). That said, the hypothesis that *cytokine-driven adverse effects of therapeutic monoclonal antibodies and the mechanisms involved can be better predicted with novel in vitro procedures using human cells* is strongly supported by the data described in this thesis, with the methods facilitating the passage of safe therapeutic mAbs into phase I clinical trials.

8.6 Summary of key findings from the present study

8.6.1 Antibody immobilisation experiments

- Procedures involving the incubation of TGN1412 in aqueous phase with human PBMC/whole blood were not predictive of its adverse effects when given in man.
- When immobilised onto plastic, TGN1412 was capable of stimulating responses from human PBMC which mimicked the *in vivo* responses.
- The technique used for mAb immobilisation (i.e. wet-coating or air-drying) and the type of microplate onto which the mAb was immobilised influenced the size of the responses. Wet-coating onto the polystyrene microplates described in section 4.2.1 resulted in a lower amount of immobilised TGN1412 orientated such that it was capable of binding to CD28, compared with other techniques.
- TGN1412-stimulated cytokine responses of whole blood were much smaller than those of PBMC.
- Aqueous TGN1412 inhibited TNFα responses of PBMC to immobilised TGN1412. This is consistent with the notion that polarised binding of TGN1412 with CD28 is required for this mAb to stimulate responses.
- The rapid release of TNFα within 30 minutes of adding PBMC to immobilised TGN1412 was, at least in part, a result of *de novo* synthesis of TNFα.
- Therapeutic mAbs in addition to TGN1412, when immobilised, stimulated a graded increase in IL-6 and IL-8 release with the frequency/severity of pro-inflammatory clinical infusion reactions reported in the literature.

8.6.2 Co-culture experiments

- The inclusion of a monolayer of human endothelial cells in PBMC-based assays allowed TGN1412 added in aqueous phase to stimulate cytokine and cell proliferative responses. The inclusion of a monolayer of fibroblasts did not have the same effect.

- HUVECjr2 can be used as a substitute for fresh HUVEC.
- Endothelial cell to PBMC contact was required in order for TGN1412 to stimulate TNFα, IL-6 and IL-2 responses.
- Cytokine and cell proliferative responses in co-culture assays were not stimulated by a conventional CD28 agonistic mAb suggesting that responses to TGN1412 were not a result of co-culturing allogeneic cells.
- Immobilisation of TGN1412 by endothelial cells was unlikely to be the mechanism through which TGN1412 was able to stimulate responses in co-culture assays.
- LFA-3 promoted TNFα, IL-2 and cell proliferative responses to TGN1412 in the co-culture method but not IL-6 responses. ICAM-1 promoted cell proliferative responses in the co-culture method. Neither LFA-3 nor ICAM-1 promoted responses stimulated by immobilised TGN1412.
- TGN1412-stimulated cell-proliferative responses were, at least in part, a result of IL-2 activity.
- Endothelial cells stimulated with TNFα released IL-6 into the culture medium and so were likely to have been, at least in part, responsible for the larger TGN1412-stimulated IL-6 responses in co-culture assays compared with those from PBMC alone stimulated by immobilised TGN1412.
- TNFα, IL-2 and IFNγ concentrations produced in response to TGN1412 in co-culture assays were small in comparison with those measured in the serum of the trial volunteers suggesting that this method is only partially representative of the *in vivo* mechanisms of cytokine release.
- As with the mAb immobilisation method, the co-culture assay was capable of predicting the cytokine release frequently associated with Campath-1H infusions and could discriminate these responses from the cardinal features of TGN1412-like activity.

8.7 Future studies

The findings from this study have opened a number of avenues for further work, of course, time permitting. The use of microscopy to visualise patterns of CD28 clustering (and that of associated signalling molecules) following ligation with immobilised and aqueous TGN1412 may help to elucidate the mechanisms involved in the activation of cells.

Further work to identify which endothelial cell-expressed, membrane-bound, co-stimulatory molecules (in addition to the ones identified here) activate signalling pathways in TGN1412-stimulated T-cells in co-culture assays would perhaps allow further conclusions to be drawn. This could be achieved with the use of blocking antibodies or with the use of inhibitory RNA to block the expression of specific co-stimulatory molecules. It may be necessary to incubate different "cocktails" of blocking antibodies with different specificities in co-culture assays to fully block TGN1412-stimulated responses. The importance of any one co-stimulatory molecule to the initiation of responses stimulated by TGN1412 in co-culture assays could be investigated by incubating TGN1412 with PBMC/T cells and with immobilised recombinant/purified preparations of the co-stimulatory molecules.

The role of the co-stimulatory molecules identified from the above experiments in promoting the formation of a "favourably organised" immunological synapse allowing aqueous TGN1412 to stimulate responses could subsequently be evaluated. Lipid raft aggregation and the movement of microfilaments are associated with the formation of the immunological synapse. The effect of, for example, LFA-3 blocking antibodies on lipid raft aggregation could be visualised at the T cell:endothelial cell interface using fluorescently labelled cholera toxin B which binds gangliosides associated with lipid rafts. The role of lipid raft aggregation in mediating TGN1412-stimulated responses could be confirmed using MβCD (methyl-beta-cyclodextrin) which disrupts lipid raft aggregation by removing cholesterol from the cell membrane (Mestas and Hughes, 2001). In addition, latrunculin B de-polymerises actin filaments (Kaizuka et al., 2009) and so could be used to correlate the inhibitory effects of, for example, LFA-3 blocking antibody on TGN1412-stimulated responses in co-culture assays, with microfilament activity.

Further experiments using flow cytometric techniques could be employed to investigate which cell populations/sub-populations are activated by TGN1412, at what stage they are activated and the cytokines that the different cell subsets produce. This would give further insight as to physiological relevance of co-culture assays and to the mechanisms involved in the superagonistic activity of anti-CD28 mAbs allowing their future potential as research tools or even as therapeutic mAbs to be assessed. It would also be useful to expand the range of therapeutic mAbs tested and cytokines measured (perhaps with the use of a multiplexing system) in the assays developed in the present study. This would further validate these methods for predicting the toxic effects of mAbs.

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Appendix I

The buffers listed in this appendix were usually prepared by NIBSC Scientific Support Services.

Table 11. Phosphate Buffered Saline (PBS) A (sterile and non-sterile)

Material	Amount	Supplier
Sodium chloride	10 g	Fisher Scientific S/3160/53
Potassium chloride	0.25 g	Merck 10198 4L
Disodium hydrogen orthophosphate	1.44 g	Merck 10249 4C
Potassium dihydrogen orthophosphate	0.25 g	Merck 10203
Ultra Pure Water	1000 ml	Barnstead

To sterilise, bottles were autoclaved for 15 min at 122°C, 1.05 bar.

Table 12. PBS B (sterile)

Material	Amount	Supplier
Sterile water	900 ml	Baxter UKF7114
Sterile 10 x PBS	100 ml	Gibco 70013016

Prepared in a class II laminar flow cabinet.

Table 13. Coating Buffer A

Material	Amount	Supplier
Sodium dihydrogen orthophosphate dihydrate	5.0 g	Fisher Scientific S/3760/53
Disodium hydrogen orthophosphate	2.9 g	VWR 102494C
Ultra Pure Water	400 ml	Barnstead

After mixing to dissolve the solids, the pH was adjusted to 7.5 using 1 M sodium hydroxide prior to making the volume up to 500 ml with Ultra Pure Water. Stored between 2 - 8°C.

Table 14. Coating Buffer B

Material	Amount	Supplier
Sodium chloride	8.18 g	Fisher Scientific S/3160/53
Potassium chloride	0.2 g	Fisher Scientific P/4280/53
Potassium dihydrogen orthophosphate	0.2 g	Fisher Scientific P/4760/53
Disodium hydrogen orthophosphate	1.15 g	VWR 102494C
Ultra Pure Water	1000 ml	Barnstead

Stored between 2 - 8°C.

Table 15. Blocking Buffer A

Material	Amount	Supplier
Tris (hydroxymethyl) aminomethane	12.1 g	VWR 103156x
Ultra Pure Water	400 ml	Barnstead
Albumin from bovine serum	5 g	Millipore 82-100-1

After mixing to dissolve the solids, the pH was adjusted to 7.5 using 4 M hydrochloric acid prior to making the volume up to 500 ml with Ultra Pure Water. Stored between 2 – 8°C.

Table 16. Detecting Antibody Dilution Buffer

Material	Amount	Supplier
Tris (hydroxymethyl) aminomethane	2.1 g	VWR 103156x
Ultra Pure Water	400 ml	Barnstead
Phenol	0.5 g	Sigma P5566
Heat-inactivated (30 minutes at +56°C) FCS	25 ml	Sera Laboratories Int. Ltd EU-000FI

After mixing to dissolve the solids, the pH was adjusted to 7.5 using 4 M hydrochloric acid prior to making the volume up to 500 ml with Ultra Pure Water. Stored between 2 - 8°C.

Table 17. Substrate Buffer A

Material	Amount	Supplier
Citric acid	6.3 g	Sigma C-2404
Ultra Pure Water	800 ml	Barnstead

After mixing to dissolve the solid, the pH was adjusted to 4.1 using 4 M potassium hydroxide prior to making the volume up to 1000 ml with Ultra Pure Water. Stored between 2 - 8°C.

Table 18. TMB Substrate Solution

Material	Amount	Supplier
3,3',5,5'Tetramethylbenzidine (TMB)	240 mg	Sigma T2885
Acetone	5 ml	VWR 20065.327
Ethanol	45 ml	Hayman Speciality Products F200238
Hydrogen peroxide solution	0.3 ml	Sigma H-1009

The TMB was dissolved in acetone prior to addition of the remaining materials. Stored between 15 – 25 °C protected from light.

Table 19. Substrate Buffer B

Material	Amount	Supplier
Citric acid	7.3 g	Sigma C-2404
Disodium hydrogen orthophosphate	9.47 g	VWR 102494C
Ultra Pure Water	800 ml	Barnstead

After mixing to dissolve the solids, the pH was adjusted to 5.0 with 1 M sodium hydroxide prior to making the volume up to 1000 ml with Ultra Pure Water. Stored between 2 - 8°C.

Table 20. Stop Solution

Material	Amount	Supplier
Ultra Pure Water	500 ml	Barnstead
Sulphuric acid	27.2 ml	VWR 20690.330

Stored between 15 – 25 °C.

Table 21. Wash Dilution Buffer

Material	Amount	Supplier
Sodium chloride	29.22 g	Fisher Scientific S/3160/53
Sodium dihydrogen orthophosphate dihydrate	0.39 g	Fisher Scientific S/3760/53
Disodium hydrogen orthophosphate	1.07 g	VWR 102494C
Ultra Pure Water	500 ml	Barnstead
Tween 20	1 ml	Sigma P5927

The solids were dissolved in Ultra Pure Water prior to the addition of Tween 20 (polyoxyethylene-sorbitan monolaurate) to avoid frothing whilst mixing. The pH was then adjusted to 7.2 using 2 M sodium hydroxide prior to the volume being made up to 1000 ml with Ultra Pure Water. Stored between 2 - 8°C.

Table 22. Tris Buffered Saline (TBS) 10x stock

Material	Amount	Supplier
Tris (hydroxymethyl) aminomethane	6 g	VWR 103156X
Sodium chloride	80 g	Fisher Scientific S/3160/53
Ultra Pure Water	800 ml	Barnstead

After mixing to dissolve the solids, the pH was adjusted to 7.2 with hydrochloric acid prior to making the volume up to 1000 ml with Ultra Pure Water. Stored between 2 - 8°C. The 10x stock was diluted 1 in 10 with Ultra Pure Water prior to use.

Table 23. FACS Fix Solution

Material	Amount	Supplier
PBS A	150 ml	See above
Formaldehyde	25 ml	Fisher Scientific F/14514/PB17
Ultra Pure Water	325 ml	Barnstead

Stored between 15 – 25 °C.

Appendix II

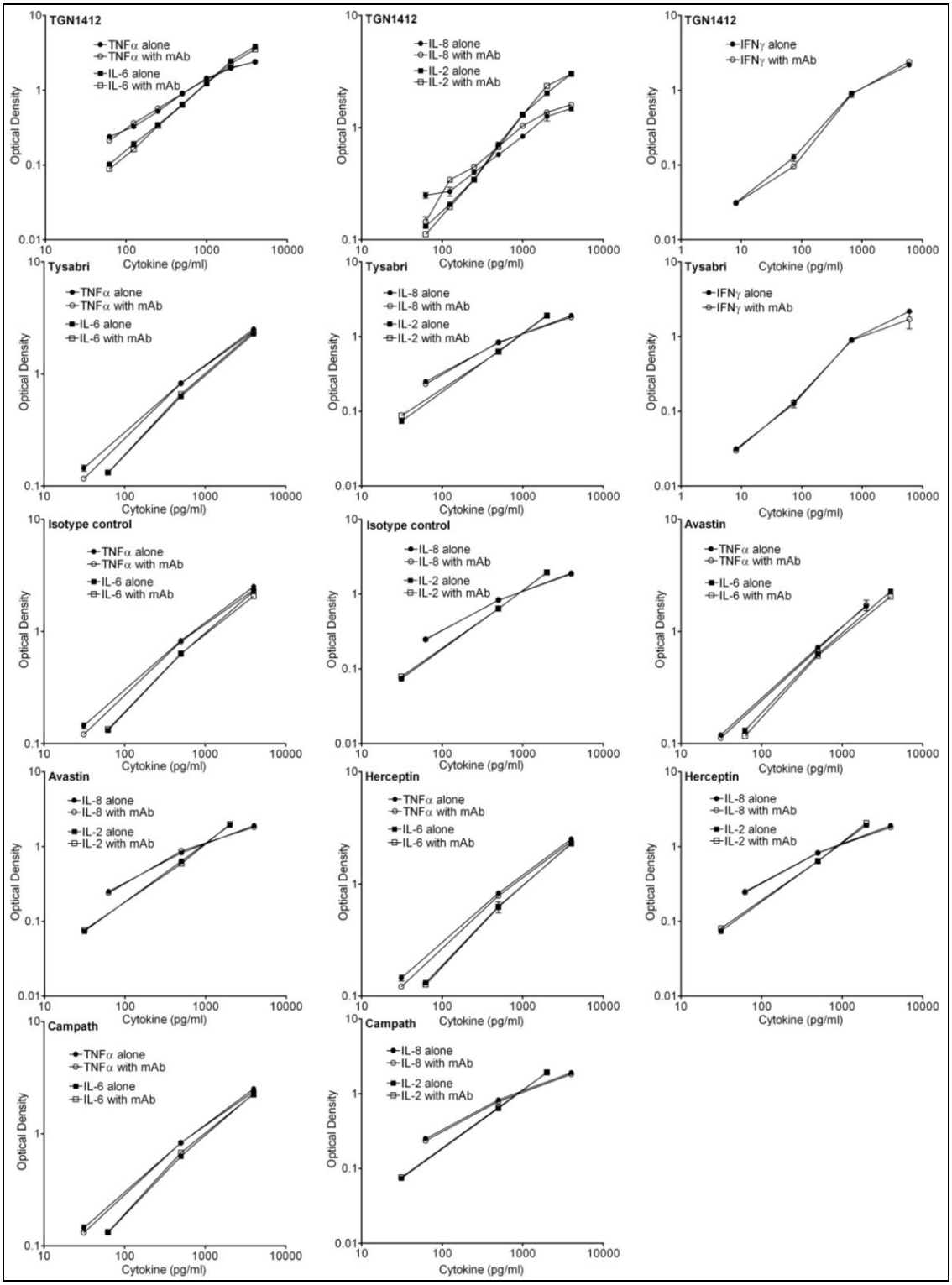

Figure 55. Effect of the presence of mAbs TGN1412, Tysabri, Isotype Control, Avastin, Herceptin and Campath-1H on the detection of cytokines TNFα, IL-6, IL-8, IL-2 and IFNγ by ELISA. A minimum of 3 concentrations of cytokine standard ranging concentrations typically used in experiments in this study to generate standard curves in ELISAs were prepared in the absence and presence of each mAb used in this study. The concentration of each mAb used reflected the highest dose incubated with cells in the methods investigated in this thesis, i.e. the maximum dose that was transferred to ELISA plates (see section 2.6). Values shown are means ± S.E.M of duplicates.