Analysis of the frequencies of cytokine producing lymphocytes at different developmental stages

Thesis submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine by

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February 2001
Abstract

The study of the frequencies of cytokine producing T cells at different developmental stages under different stimulation was performed at the single cell level. It was demonstrated that the frequency of production of cytokines by naïve (CD45RA⁺) T cells depends on the stimulation provided. Thus, there were high frequencies of IL-2, IFNγ and IL-4 producing CD45RA⁺ T cells derived from cord blood (CB) with T cell co-receptor independent signalling (using phorbol esters and calcium ionophore stimulation). MCSF, present in CB was found to be partially responsible for a reduced proliferative response to alloantigens. When co-receptor independent stimulation by PMA and ionomycin was used in established T cell lines and clones, there was unrestricted production of IFNγ and IL-4, resulting in high frequencies of all type 1 (defined by their exclusive production of IFNγ), type 2 (defined by their exclusive production of IL-4) and type 0 (defined by their simultaneous production of IFNγ and IL-4) T cells. Thus, stimulation with phorbol ester and calcium ionophore bypasses gene regulation and induces the expression of IFNγ and IL-4, even in differentiated T cells.

The frequency of cytokine producing alloantigen specific T cells was then determined using phorbol ester and CD3 stimulation. Using this stimulus, all samples had similar frequencies of type 1 cells regardless of the alloantigen (mismatch) used. Conversely, the enzyme linked immunospot assay (ELISPOT) was able to determine frequencies of different IFNγ producing T cells with single mismatches.

The expression of the Th2 marker CCR3 was considered to define T helper subsets. However it was found expressed in Th1 lines and discarded as a Th2 marker. These results suggest that:

A) CD45RA⁺ T cells from CB are not defective in their production of IL-2 or IFNγ,
B) Soluble factors may contribute to the low response to alloantigens in CB cells,
B) The use of non-T cell restricted mitogens to determine frequencies of cytokine producing cells may produce artificial profiles of cytokine producing cells and
C) ELISPOT maybe able to distinguish between different mismatches, which asses the frequencies of IFNγ producing cells, suggesting that T cell restricted stimulation should be used to study frequencies of cytokine producing alloreactive T cells.
D) CCR3 is not a Th2 cell marker.
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This thesis is dedicated to my mother, Gabriel, Alejandro, Claudia and Lalo.
Acknowledgements

All the work described in this thesis was performed by Isabel Perez-Cruz except the purification of CD45RA+ T cells from cord blood (Chapter 3 Section, 5.2.5 done by P. Fallen, ANRI), the activation of T cells for IL-2 production (Chapter 5 Section, 5.2.5 done by K. Bogunia-Kubik, ANRI), the detection of cytokines in serum (Chapter 5 Section 5.2. done by M. Wadhwa, NIBSC) and the HLA typing (Chapter 7 Section 7.2.2 done by M. Perez-Rodriguez, ANRI and the routine laboratories at the Anthony Nolan Round Table Laboratories).

I want to acknowledge the help of my supervisors, friend and colleges that made this work possible and enjoyable. First I want to thank to all the Anthony Nolan and the Royal Free Hospital staff, for the excellent work they perform. I want to thank Dr Alejandro Madrigal, director or the Anthony Nolan Research Institute, for his supervision and encouragement to learn and understand science. To Dr. Ann Margaret Little, Dr. Stephen Marsh, Dr. Anthony Dody and Dr Paul Travers, for their always useful comments and scientific discussions. Specially, I want to thank Dr. Linda Barber for her support and critical corrections of the content of this thesis. I am very grateful to Dr, Juan Carcamo (MSKCC) who’s aid was crucial at the final stages of the writing of this thesis. Specially, I want to thank Mauricio Plaza (NYU) for the technical assistance provided. Finally, I am very grateful to my direct supervisor, Dr. Shara Cohen, who always supported and taught me science in a very nice and patient way.

This work was possible thanks to the support of the Universidad Nacional Autonoma de Mexico (UNAM) and the Instituto the Investigaciones Biomedicas. I want to thank UNAM and my country, Mexico, for the opportunity I received to consolidate my studies.

I want to acknowledge the help and commitment of my mother, my brothers, my sister and Lalo, without whom life would have a tasteless meaning.
Publications

Peer Reviewed

1. I. Perez-Cruz, P. Fallen, A. Madrigal, S.B.A. Cohen (2000) Naïve T cells from cord blood have the capacity to make type 1 and type 2 cytokines. *Immunology letters* 75 (1) 85-88.


7. I. Perez-Cruz, E. Dominguez de Ortega, J.A. Madrigal, S.B.A. Cohen (1998). Foetal lymphocytes are more sensitive to freezing than adult lymphocytes within a mononuclear cell environment. *Bone Marrow Transplant* 22, S1, 41-43


9. I. Perez-Cruz, A. Madrigal, S.B.A. Cohen. The eotaxin receptor CCR3 is expressed in type 1 T cells (Submitted)

Book Chapters


Abstracts

1. Perez-Cruz, P. Fallen, J. A. Madrigal, S.B.A. Cohen (1999) CD3⁺CD45RA⁺ naïve lymphocytes can achieve IFNγ and IL-4 production to similar levels as CD3⁺CD45R0⁻ memory cells. *Blood* 94 (10) S1 part 2 pag 57b.


4. Perez-Cruz, P.R. Fallen, J. Madrigal, S.B.A. Cohen (1999). CD45RA+CD3+ naïve lymphocytes from cord blood can achieve high IL-2 and IL-4 production compared to their adult counterparts *European Journal of Immunogenetics.* 26, (1) p53 A4.3


**Reviews**

List of abbreviations:

A
Acute GvHD, aGvHD.
Antibody, Ab.
Ab dependent cell cytotoxicity, ADCC.
Alkaline phosphatase buffer, APB.
Altered peptide ligand, APL.
Antigen, Ag
Antigen presenting cell, APC.
B
Bone marrow transplant, BMT.
Bovine serum albumin, BSA.
C
CD28 responder element, CD28RE.
Cluster of differentiation, CD.
Complementary determining regions, CDR.
Concanavalin A, ConA.
Cord blood, CB.
Cord blood transplant, CBT.
Chronic GvHD, cGvHD.
Cyclosporin A, CsA.
Cytomegalovirus, CMV.
Cytotoxic T cell, Tc.
Cytotoxic T lymphocyte, CTL.
D
D-3 phosphatidylinositol, D-3-P.
Delay type hypersensitivity, DTH.
Diacylglycerol, DAG.
Double strand, ds.
Double positive lymphocyte, DP.
E
Enzyme linked immunoadsorbant assay, ELISA.
Enzyme linked immunospot, ELISPOT.
Endoplasmic reticulum, ER.
Ezrin, radixin and moesin, ERM.
Extracellularly regulated kinase, Erk
F
Foetal calf's serum, FCS.
G
GTP, guanosine triphosphatase protein.
Graft versus host disease, GvHD.
Granulocyte monocyte-colony stimulatory factor, GM-CSF.
H
Helper T cell, Th.
Hen egg lysozyme, HEL.
Human leukocyte antigen, HLA.
I
Intercellular adhesion molecule, ICAM.
Immunoglobulin, Ig.
Immunoreceptor tyrosine-based activation motifs, ITAM.
Inositol triphosphate, IP3.
Intracellular calcium, Ca^{2+}
Interleukin, IL.
IL-2 receptor, IL-2R
IL-4 receptor, IL-4R.
IL-12 receptor, IL-12R.
Interferon, IFN.
IFN regulatory transcription factors, IRF.
IFNγ receptor, IFNγ R.
Ionomycin, I.
J
Janus kinase, JAK.
K
Keyhole limpet hemocyanin, KLH.
L
Lymphocyte activation gene, LAG.
Lymphocyte-associated antigen, LFA.
Lypopolysaccharide, LPS.
M
Messenger RNA, mRNA.
Macrophage inflammatory protein 1α, MIP-1α.
Major histocompatibility complex, MHC.
Mitogen activated protein, MAP
MHC class I chain related genes, MIC.
Mixed lymphocyte culture, MLC.
Mixed lymphocyte reaction, MLR.
N
Nuclear factor of activated T cells, NF-AT.
Natural killer, NK.
Nitric oxide, NO.
O
Ovalbumin, OVA.
P
Peripheral blood mononuclear cells, PBMC.
Phosphate buffer saline, PBS.
Phorbol-12 myristrate-13 acetate, PMA.
Phospholipase-C, PLC.
Phytohemagglutinin, PHA
PI3 kinase, PI3-K.
Polymerase chain reaction, PCR.
Prostaglandin E2, PGE2.
Protein kinase B, PKB.
Protein kinases C, PKC.
R
Recombination-activating genes, Rag.
Reference strand mediated conformational analysis, RSCA.
Regulated on activation, normal T cell expressed and secreted protein, RANTES.
S
Sequence specific primers, SSP.
SH2-domain-containing leukocyte protein of 76 kDa, SLP-76.
Signal transducer and activator of transcription, STAT.
Staphylococcus enterotoxin B, SEB.
T
T cell receptor, TCR.
Terminal deoxynucleotyl transferase, TdT.
Tetanus toxoid, TT.
T cytotoxic, Tc.
T helper, Th
Th precursor, Thp.
Transporter associated with Ag processing, TAP.
Toxic shock syndrome toxin 1, TSST-1.
Tumour necrosis factor, TNF.
Tumour growth factor, TGF.
U
Ultraviolet, UV.
Unrelated BMT, uBMT.

V

Very late activation Ag, VLA.
1. Chapter 1. Introduction

To remove invading pathogens and abnormal cells, such as cancer cells, organisms have developed the immune system. The immune system originated from a system without specific recognition of antigen (Ag). This limited non-Ag specific response, evolved into a highly Ag specific system. Both mechanisms co-exist in the vertebrate immune system and are known as the innate and adaptive systems, respectively (Ezekowitz, Williams et al. 1991; Kang, Liu et al. 1998; Ehlers 2000).

In the adaptive immune response, cells expressing Ag specific receptors are stimulated at encounter with Ag. They become activated, then divide to generate a clonal response and clear the pathogen by, for example, production of soluble factors and/or cell mediated cytotoxicity. The cells directly involved in specific Ag recognition and responsible for Ag-specific responses are the B (Gorman, van der Stoep et al. 1996; Loffert, Ehlich et al. 1996) and T (Doyle and Strominger 1987; König, Huang et al. 1992) lymphocytes. Both cells recognise Ag through Ag specific receptors. In the B lymphocyte, this receptor is the membrane immunoglobulin (Ig) molecule, which binds to specific proteins, nucleic acids or polysaccharides by recognition of the Ag in its native conformation (Reviewed by (Davies and Cohen 1996). The region recognised by the Ig is called the epitope. The epitope usually comprises segments of the proteins or polysaccharides chains, that are usually in proximity only in the tridimensional conformation of the molecule, which means the Ag does not need to be processed to be recognised by a B cell (Braden and Poljak 1995; Davies and Cohen 1996).

In contrast to B lymphocytes, T cells recognise fragments of antigenic proteins, called peptides (Shimonkevitz, Colon et al. 1984; Townsend, Gotch et al. 1985). The peptide can be of cellular or of extracellular origin and in both cases the peptides are presented to the T cell within specialised molecules (Zinkernagel and Doherty 1974; Watts, A.A. et al. 1989). These molecules are the major histocompatibility complex (MHC) class I and class II molecules (Shevach, Paul et al. 1972; Zinkernagel and Doherty 1974). The requirement for partial degradation or processing of Ag and the phenomenon of MHC restricted presentation of antigenic peptides to T cells is described below.
1.1 Molecules involved in Ag presentation

The class I and class II molecules that present peptides to T cells are encoded by
genes located in the MHC region (Trowsdale 1995). These genes are present in all
jawed vertebrates (Hashimoto, Nakanishi et al. 1992). In addition to the class I and
class II gene, other proteins, including many involved in immune responses, are
encoded within the MHC (MHC sequencing consortium 1999). As it is discussed in
detail in the following sections, the class I and class II molecules bind intracellular and
environmental peptides during their biosynthesis. Once they reach the cell surface,
the class I or class II molecule provide a updated display of peptides that can be
recognised by T cells as antigens.

1.1.1 The MHC genes

At the genomic level, the human MHC is divided into class I, class II and class III
regions and each region encodes for different types of proteins. The class I region of
genes encodes the proteins known as the "classical class I Ag", i.e. the human
leukocyte Ag (HLA)-A, -B and -C. This region also contains the genes for "non-
classical class I molecules" HLA-E, -F and -G (MHC sequencing consortium 1999).
The pseudogenes HLA-L, -J, -K, and H (MHC sequencing consortium 1999) and the
MHC class I chain related (MIC) genes (Bahram, Bresnahan et al. 1994; Leelayuwat,
Townend et al. 1994) are encoded also in this region. The class II region contains the
genes encoding for HLA-DR, -DP,-DQ, DM, and -DO, molecules (MHC sequencing
consortium 1999). The class III region, encodes for soluble factors such as heat shock
proteins, cytokines such as tumour necrosis factor (TNF) and some components of
the complement system, i.e. C2, C4 and factor B (Campbell and Trowsdale 1997).

1.1.2 Structure of the MHC products class I and class II molecules

The MHC or HLA class I molecules are heterodimers of an α and a β polypeptide
chains. The α chain forms a peptide-binding cleft in its membrane-distal domain
where the walls consist of two α-helices and the floor is a β-pleated sheet (Bjorkman,
Saper et al. 1987). The class II molecules are also heterodimers of an α and a β
polypeptide chain, but here both the α and a β chains contribute to the peptide-
binding cleft which is similarly organised as in class I molecules (Stern, Brown et al.
1994). Peptides are mainly bound in the cleft by hydrogen bonds between main-chain
atoms along the peptide and HLA residues in the peptide-binding cleft (Thorsby
1999). Both their structures are described below.
Class I

Figure 1.1 shows a schematic representation of the extracellular domain of a class I molecule. The class I molecule comprises of a transmembrane 45 KD protein which is non-covalently associated with the non polymorphic protein β2-microglobulin (β2-M) (Grey, Kubo et al. 1973; Solheim and Thorsby 1974). The 45 KD protein is called the heavy or α chain and in their tertiary protein structure, the heavy chain α 1 and α 2 domains constitute a β-pleated sheet with two α-helices on top forming a groove (Bjorkman, Saper et al. 1987). The pockets of the groove accommodate the side chains of a peptide that sits in the groove (Saper, Bjorkman et al. 1991; Matsumura, Fremont et al. 1992). These peptides are derived through the endogenous pathway of antigen processing (explained in section 1.1.3).

Class II

The class II molecules are heterodimers of an α-chain and a β-chain (Figure 1.2). Both chains are membrane bound and have two extracellular domains, known as α1 and 2 and β1 and 2. The α1 and β1 domains form a β-pleated sheet with two α-helices (Brown, Jardetzky et al. 1993; Stern, Brown et al. 1994) that bind a peptide processed through the exogenous pathway of Ag processing.

Whereas HLA class I molecules are expressed on every nucleated cell of the organism with few exceptions (Dreizen, Whitsett et al. 1988; Sobel and Ames 1988; Meunier, Vian et al. 1996; Hammer, Hutter et al. 1997), HLA class II molecules are constitutively expressed only on haematopoietic cells and in thymic epithelium (Berrih, Arenzana-Seisdedos et al. 1985). However, class II molecules expression can be induced in some cells types by stimulation, in hemopoietic progenitors and precursors from human embryonic foetal liver by cytokines (Gabbianelli, Boccoli et al. 1990) and can be upregulated in professional antigen presenting cells (APC) (Mommaas, Wijsman et al. 1992) and T lymphocytes with stimulation (Hurme and Sihvola 1989).
Figure 1.1. Three-dimensional structure of the extracellular domain of the MHC class I and β2- microglobulin (β2-M) molecules. Ribbon diagram of the MHC class I and β2-M molecules. Strands of the β-pleated sheets are shown as flattened arrows with arrowhead pointing from the amino-terminus to the carboxyl terminus. The α chain of the class I molecule folds into three extracellular domains (α1, α2 and α3): the α1 and α2 domains consist in two α-helices (shown in magenta) linked by a β-sheet (shown in cyan). This structure forms the groove where endogenous peptide (displayed here in ball and stick) is bound by a process represented in Figure 1.3. The non covalently associated β2-M is shown in green. The α3 domain and β2M are not involved in Ag binding.
Figure 1.2. Three-dimensional representation of the extracellular domain of the MHC class II molecule. The polypeptide backbones of the domains of the $\alpha$ (magenta) and $\beta$ (cyan) chains are depicted as ribbons as explained in Figure 1.1. Both chains fold into two extracellular domains ($\alpha_1$, $\alpha_2$ and $\beta_1$, and $\beta_2$, respectively). Both the $\alpha_1$ and $\beta_1$ domains consist of $\alpha$-helices and a $\beta$-sheet that form the groove where exogenous peptide (displayed here in ball and stick) binds by a process represented in Figure 1.4. The $\alpha_2$ and $\beta_2$ domains are not involved in Ag binding.
Figure 1.3. Three-dimensional structure of an TCR. Backbone ribbon representation of the TCR. The α chain is shown in magenta (residues 1-213), and the β chain is shown in cyan (residues 3-247) (Garcia, Degano et al. 1996).
1.1.3 Ag processing and presentation by class I and class II molecules

Class I proteins present peptides derived from intracellular sources such as cytosolic or nuclear proteins (Chicz, Urban et al. 1992), as well as viral and bacterial products (Shimonkevitz, Colon et al. 1984; Townsend, Rothbard et al. 1986). The peptides are generated by the partial degradation of proteins in the cytosol by a proteolytic complex called the proteasome (Michalek, Grant et al. 1993).

Class I molecules assemble and bind peptide in the endoplasmic reticulum (ER) lumen. The heavy chain and β2-M are synthesised on ribosomes, translocated to the ER lumen and stabilised by a temporary association with the ER-resident chaperonin calnexin (Sugita and Brenner 1994; Nössner and Parham 1995). Peptides of cytosolic origin enter the ER via a transporter associated with Ag processing (TAP) protein (Spies, Cerundolo et al. 1992) and then bind the peptide. Only when a stable complex of properly fold heavy chain, β2-M and peptide is formed can the molecule leave the ER, pass through the Golgi, where it becomes fully glycosylated and then transits to the cell membrane (see Figure 1.4).

Class II molecules present peptides derived from extracellular sources (Demotz, Grey et al. 1989; Nelson, Roof et al. 1992). These proteins are endocytosed and transported through endosomes and lysosomes, where they are subject to degradation to become peptides by the cathepsin proteases (Brodsky, Lem et al. 1996). Class II molecules are synthesised on ribosomes on the rough ER and then translocated into the ER, where the α and β chains assemble and associate with the invariant chain (Ii) protein (Cresswell 1994; Brodsky, Lem et al. 1996). The class II molecules leaves the ER to the endosomes, where it bind exogenously-derived peptide (Deussing, Roth et al. 1998). The invariant chain is degraded leaving only a fragment of Ii, called class II-associated invariant chain peptide (CLIP), that occupies the class II peptide binding site (Denzin and Cresswell 1995; Sloan, Cameron et al. 1995). The complex of class II and bound peptide is then transported to the cell surface.
Figure 1.4. Representation of the pathway of processing and presentation of endogenous peptides by class I molecules. Cytosolic proteins are degraded into peptides in the cytosol by proteasomes. These peptides are transported to the ER by TAP. The class I α chain and the β2-M are synthesised by ribosomes and translocated to the ER, where they are assembled. Then, they can bind the endogenous peptides transported by TAP (a). The class I-peptide complex leaves the ER to the Golgi apparatus, where it is glycosylated (b). Then, it is transported in vesicles (c) to the cell surface.
Figure 1.5. Representation of the pathway of processing and presentation of exogenous peptides by class II molecules. The class II α, β and invariant (ⅱ) chains are synthesised by ribosomes and translocated to the ER, where they are assembled. In this way, they can be prevented to bind endogenous peptides that have been transported by TAP. Heterotrimers of these complexes leave the ER (only one class II-ⅱ complex is shown here) to the Golgi apparatus. Then, the complexes enter MHC class II compartment (a), where ⅱ is degraded in to CLIP by proteases. For the class II molecule to bind peptides derived from extracellular Ag (c), CLIP must be removed, a process catalysed by HLA-DM (c). The class II molecules are then transported to the cell surface.
1.1.4 Polymorphism in MHC genes

The discovery of MHC molecules arose from the study of alloreactivity (the immune response to foreign tissue). In 1936, Peter Gorer found that the rejection of allografts could be related to blood group (Gorer 1936). These seminal studies eventually lead to the discovery that disparities between the MHC class I and/or class II proteins of donor and recipient are responsible of the rapid rejection of transplants.

The MHC genes are the most polymorphic human genes (Lawlor, Zemmour et al. 1990). This applies not only to genes coding for HLA class I and class II molecules, but also MHC encoded genes such as TAP (Carrington, Colonna et al. 1993; Pearce, Trigler et al. 1993), which is involved in Ag presentation and the tumour necrosis factor (TNF) (Nedospasov, Udalova et al. 1991), involved in the regulation of immune responses and the MIC genes (Komatsu-Wakui, Tokunaga et al. 1999; Petersdorf, Shuler et al. 1999). Numbers of alleles for each of the human HLA gene loci are shown in Table 1.1. There is no single very common allele at any locus. Instead, the wide range of alleles within the population indicates a selective evolutionary pressure in to maintain diversity. This polymorphism most likely evolved so that the immune system could mount an effective response to the diverse range of potential pathogens in the environment (Lawlor, Zemmour et al. 1990).

Most of the polymorphism arises from nucleotide substitution in exons 2 and 3 of the genes (Robinson, Malik et al. 2000). These exons encode the α1 and α2 domains of class I and α1 and β1 domains of class II that form the peptide-binding grooves of the molecules. Polymorphism in this region influences both TCR recognition and peptide-binding (Babbitt, Allen et al. 1985). Each allele product binds a distinct set of peptides (Matsumura, Fremont et al. 1992; Zhang, Anderson et al. 1998). The peptide binding preferences are determined by the combinations of polymorphic residues that line the peptide binding groove.

For class I molecules, only the heavy chain is polymorphic, whereas the polymorphism of HLA class II molecules can derive from both the α and β chain, for HLA-DQ and -DP genes. However in the HLA-DR locus, the α chain is monomorphic, and only the β chain genes generate polymorphism. Nevertheless, this is the most polymorphic of the class II genes, with 221 alleles currently identified in the HLA-DRB1 locus (Marsh, Parham et al. 2000). HLA polymorphism is of relevance to organ transplantation, particularly to bone marrow transplant.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Number of alleles</th>
<th>Locus</th>
<th>Number of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>124</td>
<td>HLA-DPA1</td>
<td>15</td>
</tr>
<tr>
<td>HLA-B</td>
<td>258</td>
<td>HLA-DPB1</td>
<td>84</td>
</tr>
<tr>
<td>HLA-C</td>
<td>74</td>
<td>HLA-DQA1</td>
<td>19</td>
</tr>
<tr>
<td>HLA-E</td>
<td>5</td>
<td>HLA-DQB1</td>
<td>39</td>
</tr>
<tr>
<td>HLA-F</td>
<td>1</td>
<td>HLA-DRA</td>
<td>2</td>
</tr>
<tr>
<td>HLA-G</td>
<td>14</td>
<td>HLA-DRB1</td>
<td>221</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>4</td>
<td>HLA-DRB3</td>
<td>19</td>
</tr>
<tr>
<td>HLA-DMB</td>
<td>5</td>
<td>HLA-DRB4</td>
<td>9</td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>8</td>
<td>HLA-DRB5</td>
<td>14</td>
</tr>
<tr>
<td>HLA-DOB</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Polymorphism of HLA class I and class II alleles. Pseudogenes are omitted from this table (From Marsh, Parham et al. 2000).

1.2 T cell specific recognition of Ag

The recognition of Ag by T cells is specific due to the expression of the T cell receptor (TCR) on the cell surface. The TCR recognises peptides of self or non-self origin presented by the MHC molecules. The T cells also require the engagement of co-receptors such as the cluster of differentiation (CD) 3, CD4 or CD8 and co-stimulatory molecules, such as CD2, CD5 or CD28. The engagement is by their ligands which are expressed on APC. These requirements are discussed in the following sections.

1.2.1 The T cell receptor

The TCR comprises an heterogeneous pair of chains. Two out of four chains associate to make an αβ or an γδ heteroduplex. All the chains are membrane-bound glycoproteins, each one consisting of a relatively short cytoplasmic tail, a single membrane spanning domain and an amino terminus extracellular portion. The extracellular portion consist of two domains: the N-terminal variable domain, exposed to encounter Ag and a membrane proximal constant region, anchored on the cell membrane (Clevers, Alarcon et al. 1988) (Figure 1.3). Most αβ heterodimers are
covalently linked, whilst the γδ pairs are not. The genetic organisation of the genes that encode these four glycoproteins resembles the immunoglobulin (Ig) superfamily genes (Rowen, Koop et al. 1996).

Whereas most cells present in the periphery are of the αβ TCR type, T cells expressing the γδ TCR chains (Saito, Kranz et al. 1984) are a minority and considered an ancestral population of lymphocytes.

1.2.2 The structure of the TCR binding site

Co-crystallisation studies of class I molecules and T-cell receptors (Garboczi, Ghosh et al. 1996; Garcia, Degano et al. 1996) showed the variable regions of the α and β chains contacting both the helical sections of the class I molecule and the bound peptide. The TCR docks in a diagonal orientation that is parallel to the strands of β-pleated sheet that form the floor of the peptide-binding groove (Garcia, Degano et al. 1996). The variable regions of the α and β chains contain three hyper-variable loops termed complementary determining regions (CDR) 1, 2 and 3. The α chain CDR1 and 2 loops lie over the amino terminal portion of the peptide, the β chain CDR1 and 2 loops interacts with the carboxyl terminal portion of the peptide and the CDR3 loops of both chains interact with the central portion of the peptide.

The T cell repertoire contains a great variety of TCR that allows recognition of a large number of different ligands. This diversity of TCR specificity is achieved during T cell development in the thymus. Also in the thymus, T cell education takes place. During this process, T cells are positively selected if their TCR recognises the specific Ag (peptide) in the context of the MHC, but cells that react too strongly to self peptides are negatively selected. The generation of TCR diversity and thymic education are explained in the following sections.

1.2.3 T cell development

The cells of the immune system, such as B and T lymphocytes, macrophages and dendritic cells, originate from haematopoietic stem cells which have the capacity to develop into all cells of the haematopoietic lineage (Bondurant and Koury 1993). In the foetus, the haematopoietic stem cells are produced in the foetal liver (Haynes, Martin et al. 1988) and in the adult in the bone marrow (Spangrude, Heimfeld et al. 1988). Pluripotent stem cells and hematopoietic progenitor cells (already committed to a particular hematopoietic lineage), express the molecule CD34 on their surface and can

Some of the CD34⁺ cells in the bone marrow migrate to the thymus and are known as thymic precursors. The CD34⁺ thymic precursors also express the CD45RA and CD7 molecules (Res, Martinez-Caceres et al. 1996). The mechanisms of migration of CD34⁺CD45RA⁺CD7⁺ to the thymus are unknown. Cells that migrate to the thymic cortex are known as thymocytes and they express CD1a (Galy, Verma et al. 1993), a molecule associated with the T cell lineage. These thymocytes do not express the CD4 or CD8 molecules and remain progenitor cells because of their ability to home to and repopulate the thymus of irradiated recipients (Fawkes, Edison et al. 1985). As these cells do not express a TCR, they are known as triple negative (TN) cells. These TN cells express CD44, but not the α chain of the interleukin (IL) -2 receptor, the CD25 (CD44⁺CD25⁻). In the next developmental stage, CD25 is expressed (pro-T cell, CD44⁻CD25⁺) (Malek, Schmidt et al. 1985). The CD44 antigen expression is then down-regulated (CD44⁺CD25⁻) and the thymocytes (known as pre-T cell) mature onto CD44⁻CD25⁻, which are not longer considered precursors (Shortman 1992). These thymocytes mature into T cells by the expression of the TCR and the acquisition of surface molecules that will permit the interaction with the MHC, i.e. the CD4 and CD8 co-receptor molecules and the CD3 complex.

1.2.4 Expression of the TCR

The αβ and γδ chains of the TCR are encoded by the A and B, or G and G genes respectively. These genes are located in three loci: the TCR-A/D, the TCR-B and the TCR-G loci. The TCR-A and TCR-G genes are contained in distinct segments: the V (variable), J (join), and C (constant) segments. The TCR-B and TCR-D genes possess in addition the D (diversity) segment. These segments each contain several genes (see Table 1.2 and Figure 1.6 panel B) and only one gene from each segment is selected to form a TCR chain. To achieve this, the selected genes are brought together in a process known as TCR rearrangement. This occurs through recombination signal sequences (RSS), that flank the genes on the V, J, C and D segments (see Figure 1.7 panel A).
<table>
<thead>
<tr>
<th>Locus</th>
<th>V</th>
<th>D</th>
<th>J</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR-A</td>
<td>45/100</td>
<td>0</td>
<td>61/50</td>
<td>1/1</td>
</tr>
<tr>
<td>TCR-B</td>
<td>75/25</td>
<td>2/2</td>
<td>13/12</td>
<td>2/2</td>
</tr>
<tr>
<td>TCR-G</td>
<td>4/10</td>
<td>3/2</td>
<td>4/2</td>
<td>1/1</td>
</tr>
<tr>
<td>TCR-D</td>
<td>14/70</td>
<td>0</td>
<td>5/3</td>
<td>2/4</td>
</tr>
</tbody>
</table>

Table 1.2. Number of genes in the V, D, J and C segments in the TCR genes.
Figure 1.6. Schematic representation of the genomic organisation of the TCR-A, B, G and D genes. The TCR-G genes are inserted into the middle of the TCR-A genes (panel A). The B and G genes possess V, D, J and C segments, whereas the A and D genes have only V, J and C segments (panel A). These segments contain several genes, as exemplified in a schematic representation of the TCR-B gene shown in panel B. Groups of these genes have been arranged in families according to their similarity (for example, JB1 and JB2 families).
Proteins attach to the RSS and bring two gene segments adjacent to each other to initiate recombination (Figure 1.7 panel B). Recombination is mainly catalyzed by enzymes encoded by the recombination-activating genes (Rag) -1 and Rag-2 (Schatz, Oettinger et al. 1989; Oettinger, Schatz et al. 1990). RAG-1 and RAG-2 splices out the RSS sequences in each segment by (Roth, Nakajima et al. 1992; McBlane, van Gent et al. 1995) nucleotides are added randomly by the terminal deoxynucleotyl transferase (TdT) enzyme (Komori, Okada et al. 1993). There is not consensus about which signals induce a cell to rearrange the αβ or the γδ genes, but only one of these pairs are rearranged per cell. One theory suggests that thymocytes are precommitted to an αβ or γδ lineage, silencing the genes from the opposite lineage (de Villartay, Hockett et al. 1988; Winoto and Baltimore 1989). Another theory proposes that the choice of lineage is random and that any thymocyte could develop into an αβ or γδ T cell (Pardoll, Fowlkes et al. 1987). It has been estimated that the recombination process results in a repertoire of $10^{12}$ different αβ-TCR in peripheral T cells (Arstila, Casrouge et al. 1999).
Figure 1.7. TCR recombination events. A) A simplified scheme of the TCR V, D and J gene segments (dark boxes) is shown. These genes are flanked by recombination signals sequences (RSS) (groups of three white boxes). These RSS, shared by the genes of each segment (V, C, D or J), are flanked by a constant heptamer and nonamer sequences. In addition to these sequences, the RSS posses a 23 bp spacer for the V genes, 12 and 23 bp spacers for the D genes and a 12 bp spacer for the J genes (white boxes). B) A cartoon of the recombination process for the V gene. In the recombination process, RAG-1 and RAG-2 splices out the RSS sequences and join the strands, forming a "harpin", which is subsequently cleaved. Nucleotides are then added randomly by TdT to join this segment with a adjacent segment (J) that has undergone the same recombination process.
1.2.5 Thymic education: positive and negative selection

The TN CD44^-CD25^ state coincides with the beginning of TCRβ rearrangements (Rodewald, Awad et al. 1993) and with the subsequent expression of the TCRβ chain in the thymocyte cytoplasm (Ramiro, Trigueros et al. 1996). At this point, some of these cells can transport a pre-TCR complex to the T cell surface, comprising the TCRβ chain in association with the invariant pre-T cell alpha (pTα) protein, and the CD3 complex. The pre-TCR complex has the capacity to transmit intracellular signals and to induce proliferation (Dudley, Petrie et al. 1994; Fehling, Krotkova et al. 1995), in a process termed β-selection. This signalling also induces the expression of the CD4 co-receptor molecule (Hori, Cupp et al. 1991; Galy, Verma et al. 1993; Kraft, Weissman et al. 1993), followed by the expression of the α (but not the β) chain of the CD8 molecule (Hori, Cupp et al. 1991). These thymocytes are denoted the early double positive (DP) subset. The rearrangement of the TCR α chain occurs at this point and leads to the expression of an αβ TCR. These thymocytes (CD4^-CD8^-TCR^-) are known as triple positive (TP) thymocytes. They interact with the thymic stroma, where they can recognise MHC-peptide complexes via their TCR. Recognition of a MHC-peptide complex delivers survival signals to the TP cells (Surh and Sprent 1994), in a process known as positive selection. Cells that do not receive these signals die by apoptosis (Egerton, Scollay et al. 1990) in a process known as death by neglect. Ligands responsible for positive selection of T cells carrying a γδ TCR, have not been identified although positive selection of these cells in the thymus has been reported (Wells, Tatsumi et al. 1993). Positive selection is based on signalling through CD4 and CD8 (Ramsdell and Fowlkes 1989; Zuniga-Pflucker, McCarthy et al. 1989) and weak interactions between the TCR and MHC-peptide complexes. Only low-affinity MHC-peptide complexes are positively selecting ligands (Alam, Travers et al. 1996). High affinity MHC-peptide complexes induce negative selection during which thymocytes reacting too strongly to peptides presented in by self MHC are deleted by apoptotic cell death. Thus only CD4^-CD8^-TCR^- cells with low affinity to antigens present in the thymus (self-peptide presented in the context of self-MHC molecules) will not apoptose and will progress to become single positive CD4^+ or CD8^+ T cells (Surh and Sprent 1994; Alam, Travers et al. 1996). Positive and negative selection imposes a large reduction in the number of thymocytes.

The positively selected immature CD4^+8^- thymocytes recognise MHC molecules in association with peptides, present on thymic epithelial cells. In contrast, negative
selection (self-tolerance induction) deletes cells that recognise self antigens, which once mature and released into periphery, have the capacity to initiate autoimmune reactions.

1.2.6 The CD4 and CD8 lineage choice

Down-regulation of one of the CD4 or CD8 molecules in the TP thymocyte surface results in the cell’s recognition of ligand only in the context of MHC class II or class I molecules, respectively. CD4 or CD8 commitment occurs after TCR rearrangement and expression (Mombaerts, Clarke et al. 1992; Philpott, Viney et al. 1992) and requires the recognition of the MHC molecule (Zijlstra, Bix et al. 1990), since lack of either TCR or MHC results in a developmental arrest in the TP state.

Various theories for CD4/CD8 lineage commitment have been proposed. The stochastic theory suggests that the TP thymocytes chose a CD4 or CD8 phenotype randomly (Chan, Cosgrove et al. 1993; van Meerwijk and Germain 1993). According to this theory, after engagement with MHC molecules, one of the coreceptors is down-regulated, regardless of the TCR specificity for MHC class I or II molecules (Crompton, Lees et al. 1993; Davis, Killeen et al. 1993). A second theory, known as the instructional theory, proposes that lineage commitment is controlled directly by TCR specificity for MHC and therefore by the signals received by the thymocyte. Data supporting this theory indicates that, whereas TP cells can develop into CD4+ single positive cells in a variety of stimulatory conditions (Takahama, Suzuki et al. 1994; Cibotti, Punt et al. 1997), only weak signalling through the CD3-TCR complex can induce TP thymocytes to develop into CD8+ single positive cells(Ohoka, Kuwata et al. 1996; Takahama and Nakauchi 1996; Basson, Bommhardt et al. 1998). Conversely, strong TCR signals induce CD4 T cell development (Yasutomo, Doyle et al. 2000). Thus, it has been proposed that the strength of the signal transmitted by the CD4 or the CD8 α chain, associated with intracellular kinases such as Lck, instruct a DP thymocyte to become a CD4+ or CD8+ single positive T cell (Wiest, Yuan et al. 1993). Supporting this theory, a preferential recruitment and activation of tyrosine kinases in thymocytes developing to the CD4+ SP state has been found (Sharp, Schwarz et al. 1997; Bommhardt, Scheuring et al. 2000; Legname, Seddon et al. 2000) and those kinases are not necessary for transition into CD8+ thymocytes. Although that an instructive signal delivered by Notch have been suggested induce CD8 commitment (Robey, Chang et al. 1996), recent results indicate that Notch is only necessary for sustained CD8 development but not for initial commitment (Yasutomo, Doyle et al. 2000).
1.2.7 CD4 and CD8 single positive T cells

The CD4 and CD8 molecules are members of the Ig superfamily (see Figure 1.8) and are exclusively expressed on the surface of mature T cells. These two molecules are physically associated with the TCR (Veillette, Bookman et al. 1988; Dianzani, Shaw et al. 1992), contributing to the TCR-MHC binding. Their own binding to the MHC molecules depends on TCR-MHC interactions.

CD8 is an homodimer of two α chains, or a heterodimer of an α and a β chain. Both forms bind to MHC class I molecules (Norment, Salter et al. 1988). Polymorphism in the α3 domain of class I can affect CD8 binding (Ottenhoff, Elferink et al. 1985; Potter, Rajan et al. 1989) and mutagenesis analysis has demonstrated that CD8 molecules interact with conserved determinants in the α3 domain of the MHC class I molecule (Salter, Benjamin et al. 1990; Gao, Dormo et al. 1997). CD8+ cells are known as cytotoxic T cells (Tc) because of their capacity to induce lysis of target cells (Bach, Inouye et al. 1979) and their absence significantly impairs cytotoxic immunity (Fung-Leung, Schilham et al. 1991).

CD4 is a monomer and binds MHC class II molecules (Doyle and Strominger 1987). Allelic polymorphism at the COOH terminus of the β chain of the HLA-DR, leads to different capacities to bind CD4 molecules and ability to activate T cells (Fleury, Thibodeau et al. 1995). CD4+ T cells are classically known as helper cells (Th), for their capacity to secrete soluble factors, cytokines and stimulate other cells to become activated (Jeffries, Green et al. 1985; Berzins, Vargas-Cortes et al. 1988). Mutants of the CD4 molecule do not develop a normal cellular or humoral immunity (Rahemtulla, Fung-Leung et al. 1991). The CD4 molecule is also expressed on other cells of the immune system (Hume, Allan et al. 1987; Senaldi, Mieli-Vergani et al. 1991).

1.2.8 TCR and its association with the CD3

The TCR is the only receptor on the T cell that has contact with the Ag. However, the intracellular signalling following Ag binding to the TCR relies on the CD3 complex of proteins. The TCR is found in association with the CD3 complex at the cell surface. This is because the CD3 chains contain residues susceptible to tyrosine phosphorylation, i.e. the immunoreceptor tyrosine-based activation motifs (ITAM) (Reth 1989) absent in the TCR chains. Thus, CD3 is required for signal transduction (Love, Shores et al. 1993; Ohno, Aoe et al. 1993).

CD3 is composed of the γ, δ, and ε chains (all belong to the Ig superfamily) and the ζ homodimer (which does not belong to the Ig superfamily) (Figure 1.9). Each ζ chain
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of the CD3 has 3 ITAM, whereas the γ, δ, and ε have one each. In addition, CD3 is necessary for TCR surface expression and the assembly of TCR and CD3 chains occurs co-ordinately in the ER (Carson, Kuestner et al. 1991).
Figure 1.8. Schematic representation of the CD4 and CD8 molecules on the cell surface. Mature T cells express either the single chain CD4 or the CD8 (here represented as an αβ heterodimer) co-receptor which engage class II or class I molecules respectively upon TCR engagement. Ligand binding promotes the association of CD4 and CD8 with the PKC Lck.
Figure 1.9. Schematic representation of the TCR-CD3 complex. The TCR chains (α and β in this diagram) are non-covalently associated with the CD3 chains (ε, δ, γ) and the ζ chain. These chains have ITAM domains (represented as a black bar) in their intracellular portion. The ITAM enables them to signal intracellularly, by binding of the PKC Fyn. The TCR recognises Ag presented by MHC molecules.
1.2.9 Signalling through the TCR-CD3 complex

T cell stimulation through the TCR-CD3 complex leads to the clustering of several receptor associated tyrosine kinases (Kolanus, Romeo et al. 1993): Fyn, associated with the TCR-CD3 (Samelson and Klausner 1992), Lck associated with CD4 or CD8 (Rudd, Trevillyan et al. 1988; Veillette, Bookman et al. 1988; Barber, Dasgupta et al. 1989; Abraham, Miceli et al. 1991; Iwashima, Irving et al. 1994) and the cytosolic protein ZAP-70 (Chan, Iwashima et al. 1992; Iwashima, Irving et al. 1994). Upon T cell stimulation, the transmembrane phosphatase CD45 (Tonks, Diltz et al. 1990) activates the cytoplasmic tyrosine kinases Lck and Fyn (Mustelin, Coggeshall et al. 1989; Ostergaard, Shackelford et al. 1989; Altin and Sloan 1997), which then catalyse the tyrosine phosphorylation of the CD3-ζ chain (Sancho, Franco et al. 1993; Weiss 1993). This results in the generation of differentially phosphorylated ζ chain isoforms, p21 and p23 (Kersh, Shaw et al. 1998). The activation of the CD3 allows ZAP-70 to bind to the TCR-CD3 complex, to get phosphorylated (Chan, Iwashima et al. 1992; Iwashima, Irving et al. 1994) and to activate the linker of activation in T cells (LAT) (Zhang, Sloan-Lancaster et al. 1998). Once activated, LAT propagates the signal through the cell membrane and transmits the signal intracellularly. It stimulates phospholipase-Cγ1 (PLCγ1) and the guanine exchange factors SOS (Finco, Kadlecik et al. 1998; Salojin, Zhang et al. 2000). The small GTP binding protein Ras is then recruited in the signalling cascade. The GTP-bound form of Ras activates Raf, a mitogen activated protein (MAP) kinase (Jelinek, Dent et al. 1996). Activated Raf activates the MAP kinase kinase Mek1 and Mek2 (Franklin, Tordai et al. 1994), which then activate the extracellularly regulated kinase (Erk) 1 and Erk2 (Zheng and Guan 1993). This signalling pathway ultimately induce gene transcription by activation of transcription factors (Jacinto, Werlen et al. 1998; Yoshida, Nishina et al. 1998).

Additionally to the Ras pathway of activation, there are two main pathways of T cell activation. This is because PLCγ targets phosphatidylinositol (PI) lipids in the membrane and produces diacylglycerol (DAG) and inositol triphosphate (IP3) (Weber, Bell et al. 1992). DAG activates protein kinase C (PKC) (Nishizuka 1984). PKC is a cytoplasmic calcium activated phospholipid-dependent kinase, belonging to the family of serine/threonine protein kinases. Various isoforms have been described and gathered in three groups : conventional PKC (α, βI, βII), novel PKC δ, ε, Z, η/L, μ and the atypical PKC (λ, τ, ζ). These groups differ in their activation requirements, (i.e. calcium and type of charge in the phospholipids). The different isoforms have different tissue distribution and phosphorylate different substrates (Hofmann 1997),
however all of them are the intracellular receptor for phorbol esters (Niedel, Khun et al. 1983). This pathway leads to the activation of transcription factors (Hardy, Manger et al. 1987; Sung, Bjorndahl et al. 1988)

Other signalling cascade begins with the release of IP3 increases intracellular calcium concentrations and thereby activates the serine-threonine phosphatase calcineurin (Timmerman, Clipstone et al. 1996). Calcineurine activates the cytosolic nuclear factor of activated T cells (NF-ATp) (McCaffrey, Perrino et al. 1993) which can then enter the nucleus. In the nucleus, in combination with AT-1 transcription factor (formed by a dimer of Fos and Jun (Jain, McCaffrey et al. 1992), it acts as a transcriptional regulatory protein. In T lymphocytes, Fos and Jun are activated by the MAP kinases Jnk, which is activated by the Ras pathway (Su, Jacinto et al. 1994).

1.2.10 T cell activation via the TCR

The low number of TCR and MHC on the surfaces of T cells and APC respectively and the low affinity of TCR-MHC interactions are potentially an impediment to T cell activation. Although the CD4 (Doyle and Strominger 1987) and CD8 molecules stabilise the TCR-MHC interaction (Garcia, Scott et al. 1996) and a few seconds of TCR-MHC interaction is enough to trigger tyrosine phosphorylation cascades (Kersh, Shaw et al. 1998), a sustained interaction is necessary to induce an increase in Ca\(^{2+}\) levels and kinase activation and subsequent promotion of T cell proliferation (Nel, Dirienzo et al. 1986). A model to explain T cell activation proposes that a single MHC-peptide complex can sequentially engage and trigger several TCRs (Valitutti, Muller et al. 1995). Thus, a small number of peptide-MHC complexes can activate a T cell when the threshold number of commulative TCR engagements is reached (Viola and Lanzavecchia 1996).

A recent model of T cell activation proposes that as a result of TCR-MHC ligation, the T cell stops migration (Dustin, Bromley et al. 1997) and a transient structure known as the immunological synapse is formed (Monks, Freiberg et al. 1998). The interaction of the T cells and the APC involves adhesion between these cells which can overcome the many barriers to TCR-MHC engagement, such as the large size of the CD43 and CD45 molecules (Shaw and Dustin 1997), the low number of MHC-Ag complexes on an APC (Wang, Gulden et al. 1997) and the low TCR/MHC-peptide affinity (Alam, Travers et al. 1996; Lyons, Lieberman et al. 1996; Kersh, Kersh et al. 1998). Initial adhesion between the T cell and the APC is mediated by adhesion molecules: integrins such as the lymphocyte-associated antigen (LFA)-1 and non integrins (CD2-CD58) (Wild, Cambiaggi et al. 1999) or the intercellular adhesion
molecule (ICAM)-1 (Staunton, Dustin et al. 1990), which also initiates activation of the actin cytoskeleton.

TCR engagement can transmit different signals to the cells, not always activating the T cell. This has been shown when altered peptide ligands (APL) are used instead of peptide ligand to stimulate the T cell. Although the APL and the antigenic peptide differ only in few amino acids, they induce a differential pattern of phosphorylated tyrosine kinases (Sloan-Lancaster, Shaw et al. 1994). Furthermore, stimulation with APL can induce anergy, inhibiting proliferation with or without impairing cytokine production (Evavold and Allen 1991; Sloan-Lancaster, Evavold et al. 1994).

1.3 Co-stimulatory molecules

TCR signalling causes partial activation, anergy, and eventual apoptosis (Jenkins, Chen et al. 1990). Co-stimulation of T cells is required for optimal T cell responses (Harding, McArthur et al. 1992). Co-stimulation can be provided by CD26 (Fleischer 1987; Dang, Hafler et al. 1990; Bednarczyk, Carroll et al. 1991), CD2 (Dang, Hafler et al. 1990), CD43 (Axelsson, Youseffi-Etemad et al. 1988), CD44 (Shimizu, Van Seventer et al. 1989), very late activation Ag (VLA)-4 and VLA-5 integrins (Davis, Oppenheimer-Marks et al. 1990; Shimizu, van Seventer et al. 1990), which all enhance CD3 mediated proliferation. The CD28 co-stimulatory molecule is more effective in inducing cytokine production than other co-stimulatory molecules: whereas T cells’ stimulation by CD3 plus CD2, CD9, CD5, CD44, CD29 or CD11a co-stimulation sustains only short term proliferation, low IL-2 production and leads to apoptosis in naïve T cells, CD28 co-stimulation produces sustained IL-2 secretion (Yashiro, Tai et al. 1998). Similarly, although LFA-3 ligation can promote T cell proliferation levels similar to those mediated by CD28 ligation, it does not induce IL-2 production and produces an state of anergy after initial T cell activation (Boussiotis, Freeman et al. 1993). CD28 triggering promotes T cell proliferation through the induction of production of the T cell growth factor IL-2 in memory, peripheral and naïve T cells (Harding, McArthur et al. 1992; Yashiro, Tai et al. 1998). CD28-induced IL-2 production prevents induction of anergy in T cell clones (Lindstein, June et al. 1989). Costimulation is not only necessary for effective lymphocyte activation, but also enhance the immune response. Furthermore, T cells may become unresponsive to stimulation if they lack appropriate co-stimulation.
1.3.1 The CD28 co-stimulatory molecule

CD28 is a member of the Ig superfamily. It exists as a disulphide linked homodimer or a monomer (Reviewed in (Lenschow, Walunas et al. 1996; Ward 1996) and is expressed constitutively on the surface of 100% of CD4<sup>+</sup> cells, about 50% of CD8<sup>+</sup> cells and on developing thymocytes (Gross, St. John et al. 1990; Gross, Callas et al. 1992). Its expression is dynamic with levels increasing on T cells following activation (Linsley, Greene et al. 1992). CTLA-4, which is a non-constitutive protein, is expressed on T cell activation (Linsley, Greene et al. 1992) and is functionally and structurally related to CD28 (Brunet, Denizot et al. 1987; Azuma, Ito et al. 1993; Freeman, Gribben et al. 1993). The ligands of CD28 and CTLA-4 are proteins of the B cell activation family: B7.1 (or CD80) (Yokochi, Holly et al. 1982) and B7-2 (or CD86) (Azuma, Ito et al. 1993; Freeman, Gribben et al. 1993). These proteins are expressed on B cells (Yokochi, Holly et al. 1982; Freeman, Gray et al. 1991), a variety of APC (Hathcock, Laszlo et al. 1994) and activated T cells (Azuma, Ito et al. 1993).

T cell co-stimulation through CD28 lowers the Ag concentration required for activation of naïve and memory T cells without affecting the rate of TCR engagement and down-regulation (Lezzi, Karjalainen et al. 1998). CD28 mediated co-stimulation enhances the amplitude or duration of the TCR triggered signal (Lezzi, Karjalainen et al. 1998). This allows the T cell to achieve a threshold to activate downstream signalling cascades, which may provide signals similar to those provided by the TCR (Ward 1996).

1.3.2 Signalling through CD28

The TCR-CD3 pathway of intracellular signalling has a strict dependency on calcium and is insensitive to cyclosporin A (CsA) and FK506. However, CD28 signalling is calcium independent (June, Ledbetter et al. 1987). These observations helped to initially differentiate TCR signalling from the CD28 signalling pathway. However, CsA-sensitive CD28 signalling has been observed (using crosslinked anti-CD28 antibodies) suggesting that CD28 mediate signalling can raise Ca<sup>2+</sup>i levels (Ledbetter, Imboden et al. 1990; Ledbetter and Linsley 1992).

Upon ligation, CD28 is phosphorylated by p56 Lck and p59 Fyn, enabling PI3 kinase (PI3-K), ITK and GRB-2 recruitment to the co-receptor (August, Gibson et al. 1994; Raab, Cai et al. 1995). ZAP-70 does not phosphorylate CD28 (Raab, Cai et al. 1995) and is not involved in CD28 signalling. Nevertheless, CD28 affects CD3 early
signalling, increasing TCR-induced phosphorylation of ZAP-70 and the CD3-ζ chain (Tuosto and Acuto 1998).


1.4 Integration of CD3 and CD28 signals

CD28 is implicated in the early events of CD3 mediated signalling (Tuosto and Acuto 1998). CD28 signalling overlaps at some points with the CD3 signalling pathway: activation of p21 ras, MAPK, JNK, Vav and generation of D3 phosphoinositides. The integration of signals from CD3 and CD28 has been proposed to occur at level of the MAP Kinases JNK (JNK1 and JNK2), whose activation requires CD3 stimulation or phorbol esters and is enhanced with CD28 co-stimulation or calcium ionophores (Su, Jacinto et al. 1994). This leads to induction of c-jun and c-fos (Su, Jacinto et al. 1994; Tuosto and Acuto 1998) and activation of the transcription factors AP-1 (Su, Jacinto et al. 1994) and NF-AT (Tuosto and Acuto 1998). However, other reports propose that the integration of the CD3 and CD28 signals occurs at the level of ZAP-70 activation, as it regulates the coupling of TCR and CD28 to Rac-1 (a small GTPase), PAK-1 and p38 MAPK effector molecules (Salojin, Zhang et al. 1999). It has also been reported that TCR and CD28 signalling merge at the level of Vav (Salojin, Zhang et al. 1999), synergistically stimulating the activity of effectors such as JNK and NF-kB (Su, Jacinto et al. 1994; Jung, S, A. Yaron et al. 1995).

1.5 Cytokine production by T cells

Cytokines are a group of low molecular weight proteins, polypeptides or glycoproteins (usually ≤ 30 KD) that are produced by many different cell types
Vilcek, 1991 #429. Cytokine producing cells belong to the immune system (such as T and B lymphocytes, natural killer (NK) cells and monocytes) or are non immune cells (such as fibroblast and epithelial cells). These molecules are important non-Ag-specific effector molecules. They act as cellular mediators, with effects on growth, division, differentiation and activation and are involved in the pathophysiology of a range of diseases. In addition they may have therapeutic potential in immunological disorders (Vilcek and Le 1991).

The first observation of the influence of a soluble factor in cellular functions was done in the 1920’s, when leukocytes were found to release soluble substances that regulated cell wall vessel function (Zinsser and Tamiya 1926). The interferons where identified in the 1950’s as agents capable of interfering with viral replication (Issacs and Lindermann 1957; Wheelock 1965). The colony stimulating factors were identified in the 1960’s when soluble agents produced by tissues like spleen, uterus or lung where found to support the growth of hematopoietic cells in semi-solid culture (Bradley and Metcalf 1966).

In the 1970’s the concept of soluble factors as important regulators of immune cells was accepted: in 1978 the term Interleukin was first introduced in the Second International Lymphokine Workshop, meaning soluble molecules mediating signalling between leukocytes and belonging to the cytokines (Araden, Brunner et al. 1979).

In the immune system, T cells are important producers of cytokines, examples are interleukin (IL) -2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-16, IL-17, IL-18, lymphotactin, TNF α and β, interferon (IFN) γ, and the regulated on activation, normal T cell expressed and secreted protein, RANTES (Marini and Cohen 2000). CD4⁺ and CD8⁺ T can become activated to produce cytokines as a consequence of TCR occupancy. The T cell production of cytokine as a consequence of stimulation through the TCR or T cell co-receptors are discussed in the following sections.

1.5.1 Cytokine gene expression

Cytokine gene expression requires the binding of specific transcription factors to the promoter region of the genes. NF-AT is a T cell transcription factor expressed after stimulation through the TCR (Shaw, Utz et al. 1988). The transcription of several cytokine genes in activated T cells is regulated by proteins of the NF-AT family (Northrop, Ho et al. 1994). NFAT1/NFATp is the cytosolic phosphoprotein component that confers DNA-binding specificity to NF-AT. NFATc (Hoey, Sun et al.
1995) (also known as AP-1), is the nuclear component of the NF-AT, composed of c-fos and c-jun (Jain, McCaffrey et al. 1992) and it is activated upon PKC stimulation (Jain, McCaffrey et al. 1992). Other members of the family are NFATx (Masuda, Naito et al. 1995) and NFAT3 (Hoey, Sun et al. 1995).

The NF-AT proteins control not only the induction, but also the termination of cytokine gene transcription. Although cells from NF-AT1-/- mice have full capacity to produce inflammatory cytokines (such as IL-2, IFNγ) upon CD3 stimulation, their cells differentiate preferentially into IL-4 and IL-5 secretors, due to their failure to down-regulate IL-4 and IL-5 messenger RNA (mRNA) expression (Kiani, Viola et al. 1998). In mice prone to IFNγ/IL-2 production (such as the B210.D2 and 129/SvJ strains), the absence of NF-AT1 and development of a profile of IL-4/IL-5 production renders them susceptible to Leishmania major (L. major) infection (when the pro-inflammatory IL-2/IFNγ producing phenotype is advantageous to combat this parasite)(Kiani, Viola et al. 1998). Next, the expression of the IL-2, IFNγ and IL-4 genes is described for their relevance to this work.

**IL-2 gene expression**

The regulatory elements in the IL-2 gene promoter include OCT-1, NF-AT/AP-1, NF-kB, NF-IL-2A, NF-IL-2b, NF-IL-2D (Williams, Moolten et al. 1991) and the CD28 responder element (CD28RE) (Fraser, Irving et al. 1991). NF-AT binds to a NF-AT-specific site in the murine promoter of IL-2 (Jain, McCaffrey et al. 1992; Boise, Petryniak et al. 1993; McCaffrey, Perrino et al. 1993). Transfection assays indicate that each of the four NF-AT proteins can activate the IL-2 promoter in T cells (Hoey, Sun et al. 1995). The transfection of NF-ATc activates the IL-2 promoter in non-T lymphocytes (Northrop, Ho et al. 1994). Only the whole complex (NF-ATp and fos-jun) binds to the IL-2 enhancer (Jain, McCaffrey et al. 1992).

**IL-4 gene expression**

The IL-4 promoter possesses several motifs known as P elements (P0, P1, P2, P3 and P4) that are critical for activation of IL-4 transcription (Bruhn, Nelms et al. 1993; Chuvpilo, Schomberg et al. 1993; Li-Weber, Krafft et al. 1993; Szabo, Gold et al. 1993; Todd, Grusby et al. 1993). The P elements contain sequences for the binding of factors belonging to the NF-AT family. The activation of the AP-1 family members Jun-B, c-Jun and Jun-D (Li-Weber, Giasi et al. 1998; Li, Tournier et al. 1999), NF-AT (Rooney, Hoey et al. 1995) and the NF-kB subunits c-Rel and pp65 (Li-Weber, Giasi et al. 1998) induces IL-4 gene transcription.
The regulatory sites in the IL-4 promoter region contain binding sites for the NF-AT proteins NF-Y (Szabo, Gold et al. 1993; Li-Weber, Davydov et al. 1994) and NF-ATc (Szabo, Gold et al. 1993), c-Maf (Rooney, Hoey et al. 1995), the high mobility group protein HMG I(Y) (Chuvpilo, Schomberg et al. 1993) and the nuclear protein and transcriptional repressor of the IFN genes, IFN regulatory transcription factor (IRF)-2 (Li-Weber, Davydov et al. 1994). Stimulation through CD3 or to calcium ionophores (but not PMA) induces the binding of an inducible NF-AT protein to the IL-4 gene (Rooney, Hodge et al. 1994). The binding site for the NF-AT/AP-1 dimer (P1) in the IL-4 gene confers Th2 restricted expression of IL-4 mRNA during T cell differentiation (Wenner, Szabo et al. 1997). Although it has been proposed that both NF-AT and AP-1 are necessary for the activity of the NF-AT/AP-1 composite site in the IL-4 promoter (Rooney, Hoey et al. 1995), it has also been found that the activity of NF-AT, regardless of AP-1, is sufficient to confer IL-4 expression in naïve and effector cells (Aune and Flavell 1997). Thus, in T cells at any developmental stage, the NF-AT binding to the IL-4 promoter is comparable (Rincón, Dérijad et al. 1997). Indeed, the AP-1 and NF-AT binding to DNA in naïve cells occurs within hours of antigenic activation (Rincón, Dérijad et al. 1997) and decreases during cytokine-driven Th cell differentiation (Rincón, Dérijad et al. 1997). The NF-AT transcriptional activity exist only in effector and not in naïve cells (Aune and Flavell 1997), where it can be induced by CD3 and CD28 co-stimulation (Aune and Flavell 1997).

Although NF-AT is shared by the IL-2 and the IL-4 genes, there are differences in its promotional activity. Using a NF-AT reporter-transgenic mice, it was found that the NF-AT transcriptional activity is enhanced in differentiated Th2 cells, compared to Th1 cells (Rincón, Dérijad et al. 1997). This may be because this effector T cell subset differ in the expression of Fos-Jun family members (Rincón, Dérijad et al. 1997), which co-operate with NF-AT proteins to regulate the expression of IL-4 (Rooney, Hoey et al. 1995). All NF-AT-DNA interactions depend on calcium (Jain, McCaffrey et al. 1992; Boise, Petryniak et al. 1993; McCaffrey, Perrino et al. 1993; Rooney, Hodge et al. 1994).

**IFNγ gene expression**

IFNγ gene expression is regulated by transcription factors that bind to the enhancer in the 5′ region of the gene (Hardy, Manger et al. 1987; Young and Ortaldo 1987) where specificity for the expression of the IFNγ gene is conferred (Penix, Weaver et al. 1993). The c-rel proto-oncogene product c-Rel, that can be induced by phorbol esters, binds to the IFNγ enhancer region and can induce IFNγ expression (Sica, Tan et al. 1992; Penix, Weaver et al. 1993). Binding sites for the AP-1/CRE binding-protein...
(CREB) (Cippitelli, Sica et al. 1995), OCT-1 (Cippitelli, Sica et al. 1995) and NF-kB (Sica, Dorman et al. 1997) have been described in the IFNγ promoter. Recently, Rac2 (a small guanosine triphosphatase protein or GTP) has been found to enhance IFNγ gene expression (Li, Yu et al. 2000).

DNA methylation has been described as a mechanism of regulation for IFNγ gene expression: site specific hypomethylation of in the IFNγ promoter is found in differentiated Th2 cells, correlating with gene expression, whereas the same site in Th1 clones is methylated (Young, Ghosh et al. 1994). Methylation of the IFNγ promoter is also observed in resting T cells (Melvin, McGurn et al. 1995) thymocytes, neonatal and adult naïve T cells (Young, Ghosh et al. 1994).

1.5.2 Cytokine production is enhanced by CD28 co-stimulation

Although signalling through TCR-CD3 induces gene transcription and T cell proliferation in resting cells, stimulation through CD28 enhances production of cytokines. In resting peripheral T cells, gene transcription is enhanced by CD28 signalling (Thompson, Lindsten et al. 1989; Fraser, Irving et al. 1991). It also enhances gene expression and prolongs the survival of IL-2 mRNA (Lindstein, June et al. 1989; Fraser, Irving et al. 1991) by stabilising it. This is because of the CD28 responsive element (CD28RE) present in the IL-2 gene enhancer (Fraser, Irving et al. 1991). However, CD28 stimulation can also destabilise IL-2 mRNA since a second CD28RE in IL-2 mRNA confers late instability (Powell, Ragheb et al. 1998), thereby regulating IL-2 production.

In naïve and primed cells, CD28 co-stimulation via CD28 enhances IL-4 expression (King, Stupi et al. 1995) because after CD3/CD28 croslinking, Vav associates with the PKC isoform θ and the adaptor protein SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa), activating the IL-4 promoter and IL-4 mRNA production (Hehner, Li-Weber et al. 2000). Furthermore, the IL-4 promoter contains a CD28 responder element PRE-I, in addition to the P1 element that binds AP-1 and NF-kB upon CD28 engagement (Li-Weber, Giasi et al. 1998).

The effect of CD28 co-stimulation in IFNγ production is limited to early stages of T cell development. Thus, whereas naïve T cells require CD3 plus CD28 stimulation to produce IFNγ (Nakajima, Watanabe et al. 1997), memory T cells do not require CD28 co-stimulation for IFNγ production (Webb and Feldmann 1995; Nakajima, Watanabe et al. 1997; Rulifson, Sperling et al. 1997). CD28 signalling prolongs the survival of IFNγ mRNA in peripheral T cells (Fraser, Irving et al. 1991) and CD28 ligation by
B7.2 augments IFNγ produced at low Ag concentration (Schweitzer, Borriello et al. 1997). Nevertheless, memory cells do not increase IFNγ production by CD28 co-stimulation (Rulifson, Sperling et al. 1997).

1.6 T cell cytokine compartmentalisation in the immune system

Clinical studies show that, upon infection with the leprae bacteria, humans can develop two diseases: lepromatous leprosy (an aggressive disease) or tuberculoid leprosy (a milder disease) (Turk and Bryceson 1971). These two diseases differ in both their aggressiveness and the type of immune response developed by the infected individual. This phenomenon is now known to be due to differences in cytokine production by T cells.

Th1 and Th2 CD4+ T cells

In 1986, Mosmann and colleagues showed for the first time a dichotomised cytokine production by established murine CD4+ T cell clones (Mosmann, Cherwinksi et al. 1986). These clones, called T helper (Th) 1 and Th2, are currently defined in the mouse by their production of IFNγ, TNFβ plus IL-2 for Th1 or IL-4, IL-5, IL-6 plus IL-13 for Th2 (Abbas, Kenet et al. 1996; Mosmann and Sad 1996). The relationship between these two groups of cytokine producing cells and human disease was formulated later. Th1 cells promote phagocyte-dependent host responses to parasites. Their secretion of IFNγ, IL-2, and TNFβ induces the production of the opsonizing IgG2a antibody and mediates antibody dependent cell cytotoxicity (ADCC), as well as delayed type hypersensitivity (DTH) reactions (Toellner, Luther et al. 1998). Th2 cells provide help for humoral responses, promote isotype switching, mucosal immunity mediated by mast cell help for B cells in IgE production (Maggi, Del Prete et al. 1988; Maggi, Del Prete et al. 1988) and eosinophil growth and differentiation (Mosmann and Coffman 1989). Although these functionally distinct T cell subsets were originally described in the mouse, human T cell clones were subsequently found to display similar, but not identical, restricted cytokine profiles. Ag specific clones that display a Th1-like cytokine pattern have been derived from patients who developed the protective reaction to leprae (tuberculosis leprae), characterised by delayed type hypersensitivity (Haanen, de Wall et al. 1991). Allergic reactions involving Th2 cells and Th2 clones have been derived from atopic individuals which are specific for allergens (van-Reijesen, Bruijnzeel-Koomen et al. 1992). These discoveries have influenced the interpretation of many immune phenomena both in vitro and in vivo (Abbas, Kenet et al. 1996; Mosmann and Sad 1996).
Clones producing both Th1 and Th2 cytokines were also found and classified as Th0 (Paliard, de Wall et al. 1988; Firestein, Roeder et al. 1989; Kelso 1990; Street, Schumacher et al. 1990; Mosmann, Schumacher et al. 1991). It has been proposed that the Th1 and Th2 committed cells are derived from a Th0 precursor (Kamogawa, Minasi et al. 1993), which in turn derives from an ontogenically more primitive Th precursor (Thp), which produces only IL-2 (Sad and Mosmann 1994). This precursor arises from the naïve CD4⁺ cells that mature in the thymus and are abundant in secondary immune organs and in immunologically naïve animals. From the common pool of precursor cells, Thp cells differentiate into Th0 and Th1 or Th2 depending on the factors present during their differentiation. This is discussed in the following sections of this introduction.

Tc1 and Tc2 CD8⁺ T cells.

Although activated CD8⁺ T cells are classically considered IFNγ producers (Lewis and Wilson 1990), it has been shown that, depending on the cytokines present during primary stimulation, CD8⁺ T cells can also be polarised into type 1 and type 2 cells, producing IFNγ plus IL-2 or IL-4 plus IL-5, IL-6 or IL-10, (T cytotoxic (Tc) 1 and Tc2 respectively) (Seder, Boulay et al. 1992; Croft, Carter et al. 1994; Maggi, Giudizi et al. 1994; Sad, Marcotte et al. 1995) without losing their cytotoxicity capacity (Carter and Dutton 1995; Sad, Marcotte et al. 1995; Cerwenka, Carter et al. 1998). Tc2 cells provide help to B cells (Erard, Wild et al. 1993). The Tc1 and Tc2 cells can generate memory populations, indicating that the phenotypes are stable (Cerwenka, Carter et al. 1998). The acquisition of a polarised phenotype correlates with a differential chemotactic migration pattern (Cerwenka, Morgan et al. 1999).

This work has included the CD4⁺ and CD8⁺ T cell contribution to the cytokines profile within a CD3⁺ population. The response dominated by products of Th1/Tc2 cells is thereby called type 1, and type 2 when it is dominated by the reciprocal group of lymphocytes, unless the contribution of CD4⁺ or CD8⁺ T cells to the cytokine profile was determined.

1.6.1 Cross-regulation of the type 1 and type 2 immune responses

Th1 or Th2 responses dominate different diseases due to their ability to regulate each other. IFNγ, a Th1 product, inhibits the development of Th2 responses in mice, by abrogating Th2 cell proliferation (Gajewski and Fitch 1988; Gajewski, Goldwasser et al. 1988; Fernandez-Botran, Sanders et al. 1989). Conversely, IL-4 inhibits the development of naïve cells into Th1 effectors (Hsieh, Heimberger et al. 1992; Seder,
Paul et al. 1992) and IFNγ production (but not proliferation) by established Th1 clones (Powrie, Menon et al. 1993). Thus, both of IFNγ and IL-4 have opposite regulatory effects for the development of Th1 and Th2 clones (Parronchi, De Carli et al. 1992). IL-10, a Th2 cytokine in mouse, inhibits IFNγ secretion by Th1 clones (Fiorentino, Zlotnik et al. 1991).

1.7 Phenotypic differences in type 1 and type 2 cells

1.7.1 Cytokine receptors

Polarising cytokines down-regulate the expression of features on the opposite phenotype, thereby stabilising the emerging phenotype. There is a divergent expression of cytokine receptors, partially controlled by the cytokines that control T cell polarisation. Thus, the IFNγ receptor (R)-β subunit, which mediates inhibition of proliferation, is present on Th2 cells (Pernis, Gupta et al. 1995) but is lost in Th1 cells (Bach, Szabo et al. 1995). IFNγ up-regulates the expression of the IL-12R-β2 chain, whereas IL-4 down-regulates it (Szabo, Dighe et al. 1997). Similarly, IL-4 up-regulates the expression of the IL-4R-α chain (Kotanides and Reich 1996).

As a result of the differential expression of some cytokine receptors during murine T cell differentiation, it was found that developing murine Th2 cells were resistant to reversing to a Th1 phenotype (Perez, Lederer et al. 1995; Szabo, Jacobson et al. 1995). It was proposed that this was due to their inability to respond to IL-12, as it was subsequently found that the IL-12R-β2 chain is not expressed by naïve cells but it is up-regulated on Th1 cells and inhibited by IL-4 (Szabo, Dighe et al. 1997). Thus, Th2 polarising conditions could be the reason for the extinction of the IL-12 signalling pathway during early Th2 development (Szabo, Dighe et al. 1997). However, human Th2 clones can retain IL-12 responsiveness (Manetti, Gerosa et al. 1994; Yssel, Fasler et al. 1994) and produce IFNγ (Sornasse, Larenas et al. 1996). This is due to a basal level of IL-12R-β2 chain expression on human lymphocytes, which can induce IL-12 signalling on both Th1 or Th2 human T cell lines (Rogge, Barberis-Maino et al. 1997). The observed differential IL-12 responsiveness by Th1 cells is due to a transient up-regulation of the IL-12R-β2 level only in Th1 cells upon stimulation (Rogge, Barberis-Maino et al. 1997). The discrepancy in the results of IL-12 responsiveness by Th2 cells derived from mouse and humans may be due to the use of Th2-prone mice strains (such as BALB/c) in the murine studies. Thus, using mice strains other that BALB/c, IFNγ was able to up-regulate IL-12R-β2 expression in cells cultured in Th2 conditions (Szabo, Dighe et al. 1997). In comparison with the murine system, IFNα
induces the expression of IL-12R-β2 and responsiveness to IL-12 by human Th2 cells, which makes the presence of IFNγ irrelevant (Rogge, Barberis-Maino et al. 1997).

### 1.7.2 Extracellular markers

In the last few years, surface molecules which preferentially associate with human and murine Th1 or Th2 cells have been described. Th1-related molecules include the murine ganglioside GD1α (Ebel, Schmitt et al. 1992), surface IFNγ in murine and human cells (Assenmacher, Scheffold et al. 1996), and the human CD26 (Scheel-Toellner, Richter et al. 1995; Seitzer, Scheel-Toellner et al. 1997; Seitzer, Scheel-Toellner et al. 1998), IL-12 receptor (IL-12R) β1 chain (Rogge, Barberis-Maino et al. 1997; Szabo, Dighe et al. 1997), IL-18R (Xu, Chan et al. 1998), lymphocyte activation gene (LAG) -3 (Annunziato, Manetti et al. 1996; Annunziato, Cosmi et al. 1999), the chemokine receptors CCR5 and CXCR3 (Sallusto, Lenig et al. 1998; Hariharan, Douglas et al. 1999) and Chandra, a murine four-transmembrane domain protein of unknown function (Venkataraman, Schaefer et al. 2000). CD30 (Manetti, Annunziato et al. 1994; Del Prete, de Carli et al. 1995; Chilosì, Facchetti et al. 1996), eotaxin receptors CCR3 (Sallusto, Mackay et al. 1997), CCR8 (Zingoni, Soto et al. 1998) and monocyte-derived chemokine receptor CCR4 (Sallusto, Lenig et al. 1998) in humans and IFNγR-β chain (Pernis, Gupta et al. 1995; Groux, Sornasse et al. 1997), CD62 ligand (CD62L) (Kanegane, Kasahara et al. 1996), ST2L (Xu, Chan et al. 1998) and ganglioside GD1α (Ebel, Schmitt et al. 1992) in mouse, preferentially associate with Th2 or Tc2 cells during certain phases of their differentiation/activation process. It has been proposed that the presence or absence of these molecules may influence the differentiation of T cell subsets because of the signalling capacity of molecules as CD30 (Del Prete, De Carli et al. 1995) or the IL-12R-β1 (Szabo, Jacobson et al. 1995) and IFNγR-β receptors (Bach, Szabo et al. 1995). The expression of these markers is limited to memory T cells (Sallusto, Kremmer et al. 1999) and some of them have been found on human T cells derived from patients with diseases related with Th1 or Th2 cytokine profiles (Manetti, Annunziato et al. 1994; Maggi, Manetti et al. 1995; Chilosì, Facchetti et al. 1996; Balashov, Rottman et al. 1999).

These receptors induce a differential recruitment of polarised T cells to chemoattractant gradients in vitro (Bonecchi, Bianchi et al. 1998; Sallusto, Lenig et al. 1998) and to sites of inflammation in vivo (Borges, Tietz et al. 1997; Cerwenka, Morgan et al. 1999; Loyd, Delaney et al. 2000). For example the expression of CCR3 selectively induces Th2 responsiveness to eotaxin and to thymus and activation regulated chemokine, TARC (Sallusto, Lenig et al. 1998) whereas IP-10, the ligand for the chemokine CCRX5, favours the migration of Th1 and Th0 cells (Sallusto, Lenig et
al. 1998). Although the P selecting receptor PSGL-1 is expressed on Th1 and Th2 cells, it induces selectin-binding only in Th1 cells and mediates the migration of Th1 cells into skin undergoing DTH reactions (Borges, Tietz et al. 1997). The preferential migration of Th1 or Th2 cells induced by different chemoattractants may be due differences in the level of receptors expressed by each cell type because expression of the receptors is normally not exclusive to either Th1 or Th2 cells (Sallusto, Lenig et al. 1998).

However there is controversy over the association of some these markers with a polarised cytokine phenotype. For example, some authors have not found differences in CD30 expression on polarised T cells derived from cord blood (Spinozzi, Agea et al. 1997). It has been found that stimulation of T cells with anti-CD3 Ab (Alzona, Jack et al. 1994; Bengtsson, Johansson et al. 1995) or Ag (Hamann, Hilkens et al. 1996; Martinez, Villanueva et al. 1998) induces the simultaneous secretion of IFNγ, IL-5 and/or IL-10 regardless of the expression of CD30. Similarly, there was no correlation found on CD30 expression by T cells derived from patients with either of the two forms of leprosy (Seitzer, Flad et al. 1996; Seitzer, Scheel-Toellner et al. 1997). However, it has also been reported that whereas Th1 cells can express CD30 after stimulation, only Th2 cells express it in a sustainable manner (Bengtsson, Johansson et al. 1995). Limitations in the detection technique (Dummer, Rose et al. 1998), receptor shedding (Chilosi, Facchetti et al. 1996; De Pita, Frezzolini et al. 1997) or down-regulation (Sallusto, Mackay et al. 1997) of these molecules account for these varying results. A clear correlation of expression of these molecules and cytokine phenotype may be limited to some subsets of memory T cells obtained after cell sorting. Although none of these molecules can be considered as a truly selective marker of human Th1 or Th2 cells, their combined detection could help to characterise the type of immune response both in vitro and in vivo.

1.7.3 Transducers of cytokine signalling and intracellular signalling

Several cytokines signal through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) family of transduction molecules (Darnell, Kerr et al. 1994; Hou, Schindler et al. 1994; Zhong, Wen et al. 1994; Jacobson, Szabo et al. 1995). Occupancy of the cytokine receptor by its ligand induces recruitment and tyrosine phosphorylation of the STAT proteins, followed by STAT dimerization, nuclear translocation and binding to specific DNA sequences (cytokine responsive-target genes) (Darnell, Kerr et al. 1994; Hou, Schindler et al. 1994).
The expression of specific STAT proteins, at early time points in T cell development, induces the expression of Th1 or Th2 cytokines. In differentiating Th1 cells, exogenous STAT6 can induce the expression of c-Maf and GATA-3 mRNA and IL-4 and IL-10, but not in committed Th1 lymphocytes (Kurata, Lee et al. 1999). STAT6 is implicated in IL-4 signalling and Th2 cell development (Kaplan, Schindler et al. 1996; Takeda, Tanaka et al. 1996), although Th2 development can be partially STAT6 independent (Ouyang, Lohning et al. 2000). IL-4 activates STAT6 (Kotanides and Reich 1996), inducing STAT6 dimerization and binding to the IL-4 receptor (IL-4R) (Hou, Schindler et al. 1994). In STAT6−/− negative mice, the B and T cell response (expression of cell activation markers and cell proliferation) to IL-4 is impaired, as well as in vivo development of a Th2 response (Takeda, Tanaka et al. 1996). In non committed CD4+ cells, the expression of STAT6 inhibits IFNγ production via impairment of IL-12p40 expression in cells developing under Th1 conditions (Kurata, Lee et al. 1999). However, these effects are limited to early stages of development (Kurata, Lee et al. 1999). Th1 committed cells may express a factor that inhibits the effects of STAT6, although the inhibitor has not yet been identified.

STAT4 induces the development of Th1 responses in vivo (Kaplan, Sun et al. 1996). STAT4 is activated by IL-12 (Cho, Bacon et al. 1996; Kaplan, Sun et al. 1996) and IFNγ, by induction of tyrosine-serine phosphorylation and DNA binding of STAT4 (Cho, Bacon et al. 1996). This confers IL-12 the ability to induce Th1 differentiation and IFNγ the ability to enhance established Th1 phenotypes (Cho, Bacon et al. 1996). However, STAT4 is not enough to induce Th1 responses, since it can be activated by IFNα and β in Th1 and Th2 cells without inducing IFNγ secretion (Rogge, D'Ambrosio et al. 1998). STAT4 induction of Th1 responses to IL-12 in lymphocytes is by the direct interaction of STAT4 with the IL-12R (Naeger, McKinney et al. 1999; Yao, Niu et al. 1999). Thus, STAT4−/− mice have reduced production of IFNγ, NK function and development of Th1 cells (Kaplan, Sun et al. 1996; Thierfelder, van Deursen et al. 1996) in response to either IL-12 or Listeria monocytogenes (L. monocytogenes) (Kaplan, Sun et al. 1996) or L. major infection (Stamm, Satoskar et al. 1999). Furthermore, STAT4−/− mice tend to develop Th2 cells (Kaplan, Sun et al. 1996; Stamm, Satoskar et al. 1999). In contrast, mice transfected with STAT4 gene develope Th1 immune responses to dinitrophenyl-keyhole limpet haemocyanin(KLH) (Wirtz, Finotto et al. 1999). Nevertheless, STAT4 is not exclusively expressed in Th1 cells (Jacobson, Szabo et al. 1995; Thierfelder, van Deursen et al. 1996; Ouyang, Jacobson et al. 1999).

Antigen stimulation induces the JNK MAP kinase pathway in Th1 but not in Th2 effector cells (Yang, Conze et al. 1998). This signalling is necessary for Th1
development, as JNK2-deficient mice have an impaired Th1 immune response (Yang, Conze et al. 1998). Similarly, the p38 MAP kinase pathway induces IFNγ expression in differentiated Th1 cells and is necessary for the development of Th1 responses, as demonstrated by the impairment of Th1 responses in dominant-negative p38 MAP kinase transgenic mice (Rincon, Enslen et al. 1998). The GTP binding protein Rac2 induces IFNγ gene expression by activation of the p38 and NF-κB pathways and is necessary for Th1 cell development, conferring the capacity of IFNγ production on transfection in Jurkat cells (Li, Yu et al. 2000). Thus, Rac2, JNK and p38 signalling pathway has been found selectively activated in effector Th1 cells.

1.7.4 Transcription factors

Th1 transcription factors.

During polarisation of the Th1 and Th2 phenotypes, regulatory proteins (i.e. transcription factors) are expressed that influence the differentiation process. For example, the IFN regulatory transcription factors IRF (Miyamoto, Fujita et al. 1988) and the ezrin, radixin and moesin (ERM) transcription factors are expressed in Th1 cells. Although ten IRF transcription factors have been described (IRF-1 to 10) that are expressed in lymphocytes after viral infection, only IRF-1 is related to Th1 immunity. IRF-1 can be induced in naïve CD4⁺ T cells after stimulation through the TCR (Coccia, Passini et al. 1999) and its expression is elevated upon mitogenic stimulation on resting lymphocytes (Miyamoto, Fujita et al. 1988). IRF-1 is necessary to induce Th1 differentiation (Taki, Sato et al. 1997), IFNγ production and development of Th1 response in vivo (Lohoff, Ferrick et al. 1997). IL-12 induces mRNA up-regulation and expression of IRF-1 via STAT4 binding to the IRF-1 promoter region exclusively in Th1 cells (Coccia, Passini et al. 1999). However, no significant differences of IRF-1 expression exist in established Th1 and Th2 cells (Coccia, Passini et al. 1999). Furthermore, the expression of IRF-1 in Th2 cells is upregulated with IFNγ culture due to their expression of the IFNγR-β chain (Groux, Sornasse et al. 1997).

The Ets family of transcription factors comprises several members involved in the regulation of gene transcription. ERM-binding membrane protein, a member of the Ets family, was originally characterised as a regulator of cell adhesion and cortical morphogenesis (de Launoit, Audette et al. 1998; Yonemura, Hirao et al. 1998; Hayashi, Yonemura et al. 1999; Mangeat, Roy et al. 1999; Yonemura and Tsukita 1999). ERM has been described as a transcription factor in Th1 cells (Ouyang, Jacobson et al. 1999). ERM is inducible by IL-12-culture and on its own or with c-Jun
can stimulate IFN\(\gamma\) transcription (Ouyang, Jacobson et al. 1999). However, it does not up-regulate IFN\(\gamma\) secretion (Ouyang, Jacobson et al. 1999).

More recently, the transcription factor T box-expressed in T-cells (T-bet), was found to be exclusively expressed in Th1 and NK cells (Szabo, Kim et al. 2000). T-bet is over-expressed in Th1 cells on stimulation and can transactivate IFN\(\gamma\) expression along with repressing IL-4 and IL-5 production in developing and committed Th2 cells (Szabo, Kim et al. 2000). This repression may be mediated by down-regulation of Th2-transcription factors and not through IFN\(\gamma\) mediated signalling.

### Th2 transcription factors.

The oncogene c-Maf and the regulator of cellular differentiation, GATA-3, are transcription factors exclusively expressed by Th2 cells and not in Th1 cells (Ho, Hodge et al. 1996; Zheng and Flavell 1997). The expression of both c-Maf and GATA-3 increases with Th2 differentiation (Ho, Hodge et al. 1996; Zheng and Flavell 1997). Expression of c-Maf, either native or due to transfection, is sufficient to induce IL-4 expression (Ho, Hodge et al. 1996; Hodge, Chun et al. 1996) and is necessary for IL-4 production in vivo (Kim, Ho et al. 1999). GATA-3 requires PKC and calcium signalling to promote IL-4 activity (Ho, Hodge et al. 1996; Zheng and Flavell 1997) and can activate other Th2 cytokine enhancers, such as IL-10 and IL-5 (Zheng and Flavell 1997). Similarly, STAT6 is a transcription factor that influences chromatin remodelling of various Th2 cytokines (IL-4 and IL-13) in developing Th2 cells (Agarwal and Rao 1998).

#### 1.8 Factors that influence the development of a type 1 and type 2 phenotype in lymphocytes

Several factors have been described which influence the development of the T cell cytokine dichotomy. These are discussed below.

##### 1.8.1 Cytokines as polarising factors for the type 1 and type 2 phenotype

Cytokines were the first identified factors to polarize the lymphocyte cytokine phenotype and the type of immune response.

IL-12, produced by stimulated macrophages and dendritic cells, is a major cytokine involved in Th1 cell development and IFN\(\gamma\) production (Hsieh, Macatonia et al. 1993; Manetti, Parronchi et al. 1993; Seder, Gazzinelli et al. 1993; Manetti, Gerosa et al. 1994). This cytokine influences the cytokine secretion pattern of developing CD4\(^+\)
cells, inducing transient production of IFN\(\gamma\) by allergen specific Th2 clones (Yssel, Fasler et al. 1994), and stabilising the IFN\(\gamma\) production by Th1 clones (Manetti, Gerosa et al. 1994). IFN\(\gamma\) reinforces Th1 commitment by up-regulation of the IL-12R and inhibiting growth of Th2 cells (O'Garra 1998). In the absence of IL-12, IFN\(\gamma\) is sufficient to generate type 1 cells from bulk cultures (Manetti, Gerosa et al. 1994).

Recently, IL-18 was found to be an inducer of IFN\(\gamma\) production (Okamura, Tsutsi et al. 1995) and it is correlated with the development of type 1 immunity in mice (Novick, Kim et al. 1999). It has been reported that costimulation with IL-12 and IL-18 induces IFN\(\gamma\) secretion by murine macrophages (Munder, Mallo et al. 1998) and B cells (Yoshimoto, Okamura et al. 1997), suggesting that an ongoing Th1 response can be reinforced by the presence of IL-12 and IL-18 in the milieu.

IL-4 is the major cytokine involved in driving a Th2 response. IL-4 was chronologically the first described factor that differentiated non-committed CD4\(^+\) T cells into polarised T helper cells (Le Gros, Ben-Sasson et al. 1990; Swain, Weinberg et al. 1990; Chatelain, Varkila et al. 1992). IL-4 can inhibit Th1 development (Hsieh, Heimberger et al. 1992), regulating the production of IFN\(\gamma\) (Maggi, Parronchi et al. 1992). Furthermore, a developing Th2 response can be inhibited by the administration of anti-IL-4 antibodies to an immunised animal (Chen, Inobe et al. 1996). This inhibition induces the development of Th1 cells (Heinzel, Sadick et al. 1989; Chatelain, Varkila et al. 1992). The origin of the IL-4 for the development of Th2 cells has been controversial, since IL-4 is mainly produced by type 2 αβ CD4\(^+\) and CD8\(^+\) primed T cells (Bendelac and Schwartz 1991; Fischer, MacNeil et al. 1991; Seder, Boulay et al. 1992; Yamamura, Wang et al. 1992). However, IL-4 could also come from non-T cells such as basophils (Brunner, Heusser et al. 1993; Aoki, Kinzer et al. 1995) eosinophils (Moqbel, Ying et al. 1995), or mitogen-stimulated mast/basilophil cells lines (Plaut, Pierce et al. 1989). Eosinophils produce IL-4 early in response to \textit{S. mansoni} eggs, which can initiate Th2 differentiation at the sites of infection (Sabin, Kopf et al. 1996). Several different types of lymphoid cells have been suggested as the primary source of IL-4. For example, a subset of T cells with NK function (NK1.1 cells) make high levels of IL-4 upon primary stimulation and have been proposed as the initial source of IL-4 (Arase, Arase et al. 1993; Brunner, Heusser et al. 1993; Yoshimoto and Paul 1994). Nonetheless, type 2 immunity can develop independently of NK1.1 cells in chronic parasitic infections (Brown, Fowell et al. 1996; von der Weid, Beebe et al. 1996) and the IL-4 produced by NK1.1 cells after repeated polyclonal stimulation seems to be more involved in inducing IgE secretion (Yoshimoto, Bendelac et al. 1995; Yoshimoto, Bendelac et al. 1995; Bendelac, Hunziker et al. 1996). Subsets of immature TCR\(^{\text{DN}}\) thymocytes
and splenic lymphocytes (Zlotnik, Godfrey et al. 1992) and naïve T cells (Gollob and Coffman 1994), that produce IL-4 with CD3 cross-linking has been described. These findings suggest that the IL-4 originate from Ag specific naïve T cells. It was subsequently found that IL-6 from APC origin stimulates IL-4 production in naïve cells which can direct differentiation toward the Th2 phenotype (Rincon, Anguita et al. 1997). However, IL-6 deficient mice can still develop a Th2 response (Romani, Mencacci et al. 1996) indicating that IL-6 is only partially responsible of the development of type 2 immunity. The expression of the chemokine monocyte chemoattractant protein 1 (MCP-1) has also been related with polarised Th2 responses (Chensue, Warmington et al. 1995; Lu, Rutledge et al. 1998) MCP-1 is able to up-regulate IL-4 secretion (Karpus, Lukacs et al. 1997; Karpus, Kennedy et al. 1998). It has been reported that cells from MCP-1 deficient mice are unable to secrete IL-4 and IL-5 in vitro, provide Th1 type help to B cells and are more resistant to L. major infection than wild type mice (Gu, Tseng et al. 2000).

1.8.2 TCR and MHC affinity for the antigen

Affinity of the Ag has been shown to influence Th differentiation. In BALB/c mice, high affinity peptides analogues induced Th1 development, whereas low affinity peptides induced development of Th2 cells and intermediate affinity peptide induced both Th1 and Th2 cells (Chaturvedi, Yu et al. 1996). In agreement with this finding, it has been reported that I-A\(^d\) binds to a human collagen peptide more avidly than I-A\(^b\) and presentation of this Ag in the I-A\(^b\) context favours the development of Th1 cells (Pfeiffer, Stein et al. 1995). Albumin presented in the context of I-A\(^b\) induced a Th1 response only when a high dose of Ag was used (Pfeiffer, Stein et al. 1995). Similarly, it has been reported that TCRs differing by only one amino acid have different affinity for a peptide, and lymphocytes expressing the high affinity TCR develop into Th1, whereas lymphocytes expressing the low affinity TCR develop into Th2 cells (Blander, Sant’Angelo et al. 2000). Different cytokines were secreted by naïve cells of these mice after stimulation with a wild type (WT) or an altered peptide (R2G) which has a weaker interaction with the TCR. Whereas the WT peptide induced only IL-4 secretion at medium and high doses, the R2G peptide induced IL-4 secretion only at low doses and IFNγ secretion at high doses (Tao, Grant et al. 1997; Blander, Sant’Angelo et al. 2000). The differences in antigen affinity correlate with strong or week TCR signaling. Strong TCR signaling inhibits development of IL-4 producing cells even when costimulation through CD28 is provided (Tao, Constant et al. 1997).
1.8.3 Co-stimulatory molecules.

T cell contact with APC occurs through the TCR and through co-stimulatory molecules and these molecules deliver different signals to the cells, as discussed in Section 1.3. Expression of co-stimulatory molecules at particular time points during cell activation has been shown to influence the polarisation of CD4\(^+\) T cells to Th1 or Th2.

Engagement of CD4 have been found to facilitate Th2 differentiation (Brown, Moskowitz et al. 1997; Fowell, Magram et al. 1997) probably by lowering the threshold for activation (Leitenberg, Boutin et al. 1998). Similarly, OX40 signalling facilities Th2 differentiation (Flynn, Toellner et al. 1998; Tanaka, Demeure et al. 2000). Signalling via the CD30 molecule, expressed by some CD4\(^+\) and CD8\(^+\) T cells, can increase production of IL-4, whereas blocking this molecule in bulk cultures induces the cells to differentiate towards type 1 (Del Prete, De Carli et al. 1995). Abrogation of signalling through LFA-1 and its ligand, ICAM-1, affects the production of cytokines in Th2 clones but does not have a marked inhibitory effect in Th1 clones, indicating that Th2 cells require LFA-1 signalling whereas Th1 cells do not. (Faith, Higgins et al. 1995). Blocking of the CD40 ligand (CD40L) can lead to down-regulation of IFN\(_\gamma\) production and increased IL-4 secretion (Kennedy, Picha et al. 1996; Stuber, Strober et al. 1996). However, a study showed both production of Th1 and Th2 cytokines upon CD40-CD40L interaction, probably due to the use of mitogenic stimulation and not APC to induce cytokine production (Peng, Kasran et al. 1996). The interaction of LFA-1 with ICAM in naïve cells promotes Th1 differentiation by enhancing CD40L expression and stimulation provided by dendritic cells, whereas its blockade augments Th2 development (Salomon and Bluestone 1998; Ruedl, Bachmann et al. 2000). Th1 polarisation can also be induced by CTLA-4 signalling when it binds CD80, competing with CD28 for the same ligand (Kato and Nariuchi 2000).

CD28 stimulation also plays a role in development of Th1 cells, because naïve T cells require CD3 plus CD28 stimulation to produce IFN\(_\gamma\) (Nakajima, Watanabe et al. 1997). However, CD28 is not required for the maintenance of a type 1 response because IFN\(_\gamma\) secretion by primed cells does not require co-stimulation (Nakajima, Watanabe et al. 1997; Rulifson, Sperling et al. 1997). Naïve cells require CD28 co-stimulation to produce IL-2 (Dubey, Croft et al. 1996; Yashiro, Tai et al. 1998). The absence of the CD28 ligands reduces the production of IL-2 in peripheral cells (Schwartz 1992), whereas cross-linking of CD28 in resting cells induces activation of
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the IL-2 transcription factors NF-kB and NF-AT (Crabtree 1989; Siefken, Klein-Hessling et al. 1998).

Th2 cell differentiation is dependent on CD28/B7 interactions. Disruption of CD28/B7 interactions inhibits the priming of Th2 cells, whereas CD28 cross-linking induces Th2 priming. Stimulation through CD28 is necessary for the development of Th2 cells (Webb and Feldmann 1995). Studies using transgenic mice have shown that B7.1 and B7.2 can influence Th2 differentiation because IL-4 secretion by naïve cells is dependent on B7.1 whereas IL-5 is dependent on B7.2 co-stimulation (Manickasingham, Anderton et al. 1998). However, a similar study found B7.2 and not B7.1 was necessary for the development of IL-4 producing cells (Schweitzer, Borriello et al. 1997). Using a transgenic mice model, it has been reported that CD28 co-stimulation can induce low levels of IL-4 production in naïve CD4+ cells from neonates, which is enough to support the development of these cells into high IL-4/IL-5 producers by autocrine stimulation (Delespesse, Yang et al. 1998). Although early results of CD4+ cell stimulation showed influence on CD28 co-stimulation for proliferation and cytokine production by Th1 but not Th2 clones (McKnight, Perez et al. 1994), it has been shown that CD28 signalling induces the production of Th2 cytokines by resting cells. IL-4 and IL-5 secretion is poor in primary cells (Rulifson, Sperling et al. 1997) and T cell clones (Rulifson, Sperling et al. 1997; Stack, Thompson et al. 1998) derived from CD28− mice. In peripheral CD4+ lymphocytes, B7.2 co-stimulation and to a lesser extent B7.1, induce IL-4 production (Nakajima, Watanabe et al. 1997) and in CD4+ and CD8+ T cell lines the IL-4 and IL-5 production induced upon CD3 stimulation is increased with CD28 engagement (Rulifson, Sperling et al. 1997). Thus, in the absence of CD28 signalling, naïve T cells are biased towards the Th1 phenotype and whereas CD28 is involved in enhancement of Th1 cytokine production by naïve cells, it is required for Th2 cytokine production by naïve and differentiated cells.

The CD80 and CD86 molecules are differentially expressed on various populations of APC (Azuma, Ito et al. 1993; Caux, Vanbervliet et al. 1994; Hathcock, Laszlo et al. 1994) and has been proposed that their interaction with the coreceptors CD28 and CTLA-4 respectively, can influence the development of a Th1 or Th2 dominated reaction in vivo (Kuchroo, Das et al. 1995). However, in vitro studies using transgenic T cells and APC that express either CD80 or CD86 have shown that these molecules do not induce the development of a particular T helper profile (Schweitzer, Borriello et al. 1997).
1.8.4 Type of Ag Presenting Cell

As described in section 1.1.3, Ag presentation to Th and Tc cells occurs in the context of MHC molecules expressed on the surface of cells. In specialised APC, the expression of co-stimulatory molecules, such as CD80, CD86 and CD40, enhances and directs the T cell's response to Ag.

Different Ag-presenting APC can induce Th1 or Th2 differentiation of naïve cells. This influence on Th commitment is due to the secretion of cytokines by the APC upon contact with naïve T cells. Naïve T cells induce production of IL-4 by Ag presenting B cells which in turn induce Th2 differentiation (Stockinger, Zal et al. 1996). Dendritic cells prime naïve cells in the lymph nodes (Toellner, Luther et al. 1998). IL-12 secretion by dendritic cells is induced upon CD40 ligation by CD40 ligand (CD40L), present on naïve T cells (Cella, Scheidegger et al. 1996; Peng, Kasran et al. 1996). IL-12 can also be found in the membrane of dendritic cells that have been in contact with intracellular parasites (Quinones, Ahuja et al. 2000). Thus, dendritic cells have been considered as Th1-inducing APC. However, the capacity of dendritic cells to induce Th1 or Th2 differentiation depends on the conditions of dendritic cell culture (Vieira, de Jong et al. 2000). Dendritic cells derived from the respiratory tract induce Th2 differentiation in naïve cells, but they also induce Th1 when they are cultured in GM-CSF (Stumbles, Thomas et al. 1998). Myeloid dendritic cells (DC1), which secrete IL-12, IL-1α and IL-6 upon CD40 stimulation and lymphoid dendritic cells (DC2), which secrete low levels of those cytokines, have been described (Rissoan, Soumelis et al. 1999). Although both DC1 and DC2 secrete comparable levels of IL-18, DC1 induce Th1 commitment in naïve T cells and DC2 induce Th2 differentiation (Rissoan, Soumelis et al. 1999). As the type of response induced by certain APC can be modified with exogenous cytokines (Finkelman 1995), soluble cytokines remain an important polarising factor. Once the T cell phenotype is set, distinct APC have different stimulation capabilities to activate the T cells. For example, compared with B cells, dendritic cells and macrophages have a better capacity to stimulate allergen reactive T cell clones (Kahlert, Grage-Griegenow et al. 2000) and macrophage presentation of super antigen to resting T cells induces the secretion of IFNγ, whereas B cells induced only IL-2 secretion (Schmitz, Assenmacher et al. 1993).

1.8.5 Route of immunisation

Different routes of immunisation of Ag can induce the development of different cytokine responses. Inhalation of ovalbumin (OVA) by mice induces the production
of IgE (characteristic of a type 2 response) whereas peritoneal injection of OVA promotes the development of airway inflammation (type 1 response) (Renz, Smith et al. 1992). Similarly, oral immunisation with tetanus toxoid (TT) increases the production of IL-4 in mucosa associated tissues and spleen (Marinaro, Staats et al. 1995), whereas systemic TT immunisation induces Ag-specific IgG and IgM, but not IgA antibodies in serum and a Th0 cytokine profile in CD4⁺ cells in mice (Xu-Amano, Kiyono et al. 1993). These findings may reflect the presence of different APC associated with the facilitation of Th1 or Th2 responses.

1.8.6 Antigen dose

The dose of Ag also influences the profile of T cell immunity developed. Using naïve cells from a transgenic mouse, it was shown that high doses and very low doses of Ag induces the development of Th2 cells, whereas medium doses induces Th1 cells (Constant, Pfeiffer et al. 1995; Hosken, Shibuya et al. 1995). However, it has been also found that high Ag dose induce Th1 differentiation (Ruedl, Bachmann et al. 2000; Tanaka, Demeure et al. 2000) when IL-2 is present (Rogers, Huston et al. 1998) and that high doses of Ag presented by dendritic cells induce the rapid expression of CD40L in naïve T cells, inducing the development IFNγ producing cells (Ruedl, Bachmann et al. 2000). In vivo, susceptibility to L. major observed in BALB/c mice could be reversed by injection of low-doses of parasites, inducing a protective IFNγ-dominated memory response (Bretscher, Wei et al. 1992). Similarly, oral low-dose feeding of protein induces secretion of type 2 cytokines (IL-4, IL-10, tumor growth factor, TGF) in mice, whereas minimal secretion of these proteins was observed with high doses-feeding in mice (Chen, Inobe et al. 1996). It has been recently reported that continuous administration of low doses of soluble hen egg lysozyme (HEL) correlates with a down-regulation of existing Th1 cells that shared specific CDR3 motifs in their TCR’s (Foucras, Gapin et al. 2000), suggesting that the expression of TCR repertoire in polarised Th cell subsets is strongly influenced by the mode of Ag administration (Foucras, Gapin et al. 2000).

1.8.7 Genetic background.

The genetic background influences the cytokine phenotype of a T cell. This has been shown in murine studies where T cells from C57BL-6, 129-SvJ and B10.D2 mice strains tend to differentiate in vitro into Th1 cells under non-polarising conditions, whilst the BALB/c strain favours Th2 cell development (Hsieh, Macatonia et al. 1995), which results in BALB/c mice being incapable of controlling L. major infection (Hsieh, Macatonia et al. 1995). This may be because primed T cells from BALB/c
become unresponsive to IL-12 (Güler, Gorham et al. 1996) and require IL-1α and TNFα to develop into Th1 cells (Shibuya, Robinson et al. 1998).

The genetic background also influences the function of other cells of the immune system, such as macrophages (Mills, Kincaid et al. 2000). Macrophages derived from Th1-prone murine strains (M1) produce high levels of nitric oxide (NO) under LPS stimulation, compared with macrophages from Th2-prone strains which produce more TGFβ1. Although M1 macrophages induces production of IL-4 and IFNγ by T cells, M2 macrophages induce the production of IL-4 and TGFβ1 but not IFNγ (Mills, Kincaid et al. 2000).

The hereditary differences in these mice strains may be due to a specific locus. Thus, cells from B10.D2 mice can be responsive to IL-12 (i.e. to make IFNγ) (Güler, Gorham et al. 1996). IL-4 has been considered a candidate regulatory gene for this locus due to its ability to drive T helper cell differentiation along the Th2 pathway. The locus is on chromosome 11 which also contains a cluster of other cytokine genes, including GM-CSF, IL-3, IL-5 and IL-13 (Gorham, Güler et al. 1996).

1.8.8 Stability of committed helper T cells clones.

Although it has been shown that murine Th1 and Th2 clones have stable patterns of cytokine secretion after repeated passages in vitro (Bucy, Panoskala-H-Mortari et al. 1994; Murphy, K et al. 1996), the stability of the Th1 and Th2 phenotypes has been questioned in several studies.

In mice, in vitro experiments using transgenic cells have shown that polarising cells can be reversed in the first days of polarisation, but long term clones do not reverse their cytokine profile (Perez, Lederer et al. 1995; Szabo, Jacobson et al. 1995; Murphy, K et al. 1996). However, in vivo, differentiated murine Th1 cells can be reversed to the Th2 phenotype and can retain this phenotype when they are adoptively transferred to severe combined immunodeficient (SCID) mice infected with L. major (Mocci and Coffman 1995). In a murine model of arthritis dominated by IFNγ production, immunisation with a collagen peptide, progressively induced a Th2 dominant phenotype (Doncarli, Stasiuk et al. 1997). In contrast, murine Th2 cells are resistant to reversion to Th1 cells when they are challenged with cytokines alone (Perez, Lederer et al. 1995). The administration of IL-12 alone to virus-infected mice, which develop a non-protective Th2 response, induced a Th1 like response by increasing the IFNγ production by T cells and serum IgG2a whilst decreasing IgG1, IgG2b, IgG3 and the number of eosinophils in lungs and broncheous (Hussell, Khan et
al. 1997). However, a systemic protection to the virus was not observed (Hussell, Khan et al. 1997). In another approach, an in vivo shift of a Th2 developing response to a Th1 response was induced when IL-12 and antiparasite drugs were administrated within the first week of L. major infection, maybe by decreasing antigen levels (Nabors, Afonso et al. 1995).

Changes in ligand density can also modulate the cytokine profile of a immune response. Using mice strains where low ligand density primed Th2 effectors and high-ligand density induced type 1 immunity, Schwantz et al attempted to switch the cytokine phenotype of differentiated T helper cells. The authors found that only the Th1 cells were subject to regulation of their cytokine profile, by lowering the ligand density, whereas increasing Ag did not induced a change in the cytokine profile of Th2 cells (Schwantz, Kasselman et al. 1996). It has been recently reported that a shift in T helper phenotype can be induced using Ag analogue of different affinities to re-stimulate short and long term Th2 lines in vitro: using peptides analogues, stabilised murine Th2 cells were re-challenged with a peptide of high affinity, which resulted in a shift toward a Th1-type cytokine profile. However, Th1 cells did not changed their cytokine profile when re-stimulated with a low affinity peptide (Chaturvedi, Yu et al. 1996). Other study have shown that after six days of in vivo peptide analogue therapy, cells from mice lung lymph nodes were obtained and stimulated with Ag. A reduction in IL-4 and IL-5 secretion was observed compared with non-treated mice (Janssen, van Oosterhout et al. 2000).

It has been reported that whereas Th1 cells are not responsive to IL-4, Th2 lines are responsive to IL-12 and can change their cytokine profile (Sornasse, Larenas et al. 1996). Thus, there is some accessibility to the IFN\(\gamma\) genes in some human Th2 cells. Similarly, it has been found that human Th2 cells stimulated by B cells retain their cytokine phenotype, but upon re-stimulation with dendritic cells the cytokine profile was reversed to Th0-Th1 like (Kalinski, Smits et al. 2000). This was due to the dendritic cell secretion of the IL-12 p70 subunit induced by both Th1 and Th2 cells, that is not down-regulated by IL-4 (Kalinski, Smits et al. 2000). However, there are Th2 human cells derived from patients with atopic disease that are resistant to IL-12 mediated phenotype reversal (Hilkens, Messer et al. 1996). Dendritic cells that do not produce IL-12 have been isolated from gut and airway-associated lymphoid tissues (Stumbles, Thomas et al. 1998; Iwasaki and Kelsall 1999). Thus, the type of APC may be able to reinforce the Th phenotype.

In humans, modulation of the T helper response in the clinic could be theoretically applied to the management of disease, as several diseases are characterised by a
particular T cell cytokine phenotype (discussed in detail in section 1.8). For example, \textit{in vitro} culture with imidazoquinines induce a predominant secretion of Th1 cytokines by PBMC from healthy donors (Wagner, Ahonen et al. 1999). The reversal of a cytokine phenotype in humans has been observed in response to therapeutic treatments. In lepromatous leprosy patients, administration of IFN\(\gamma\) causes a systemic reversal toward a Th1 dominated phenotype (Sampaio, Moreira et al. 1992) whereas a decrease in IL-10 in response to antiparasitic drugs has been observed in patients (Karp, el-Safi et al. 1993). Recently, altered peptides have been successfully used to vaccine patients with autoimmune diseases (Th1 dominated) to induce bystander suppression of the type 1 immunity by inducing a type 2 response. The re-stimulation of autoreactive Th0 cell lines derived from patients with an altered peptide ligand from myelin basic protein, is able to induce a shift to a Th2 phenotype of cytokine secretion, by reducing the secretion of IFN\(\gamma\) (Ausubel, Krieger et al. 1997). In multiple sclerosis patients, one year of daily injections of altered peptides from myelin basic protein induced a replacement of the dominant IFN\(\gamma\) secretion by PBMC to a Th0 or Th2 profile (Duda, Schmied et al. 2000).

These results encourage pursuit of vaccines which the aim to modulate the cytokine profile of a disease.

\textbf{1.9 Type 1 and type 2 T cells in disease}

Upon encounter with Ag, un-primed T cells can develop into type 1 or type 2 cytokine producing cells, depending on the environmental conditions. This dichotomy has been found both \textit{in vitro} and \textit{in vivo} and a large range of physiological conditions have been associated with particular sets of cytokines deriving from either type 1 or type 2 T cells. Some of these conditions are pathologies such as infectious diseases, allergy or autoimmune diseases, whereas others are a consequence of changes in physiological circumstances, such as pregnancy. In addition, and of particular relevance to this thesis, is the cytokine profile that develops following organ transplantation between individuals from the same species (allogeneic transplantation), a condition not found in nature. This is discussed in detail below.

\textbf{Infectious diseases.}

The development of particular T cell-cytokine profiles occurs in bacterial infections. The CD4+ type 1 T cell immunity generated in leprae infections is protective, however a type 2 response can develop, which exacerbates disease (Haanen, de Wall et al.
1991). In vitro, peripheral blood mononuclear cells (PBMC) stimulation with *Mycobacterium bovis* induces production of IL-1, IL-6, TNFα, and IL-1 receptor antagonist characteristic of a macrophage dominated response induced by Th1 cells. Similarly, *L. monocytogenes* infection in mice activates macrophages, induces IL-12 production and leads to development of CD4+ Th1 cells (Hsieh, Macatonia et al. 1993). The development of a Th2 response in infections by helminths can confer protection (Else, Finkelman et al. 1994; Finkelman, Madden et al. 1994; Urban, Maliszewski et al. 1995). The response to virus in an immuno-suppressed individual is dominated by type 2 lymphocytes (Gazzinelli, Makino et al. 1992). CD8+ T cells derived from HIV+ individuals, produce IL-4 and IL-5 but not IFNγ, and provide help to B cells for antibody secretion as a typical Tc2 response (Maggi, Giudizi et al. 1994; Maggi, Manetti et al. 1997).

**Autoimmune diseases**

Autoimmunity has a characteristic cytokine phenotype. These diseases are associated with production of type 1 cytokines. For example, in insulin dependent diabetes mielitus, progression of disease is correlated with an increase of type 1, over type 2 cytokines (Rapoport, Mor et al. 1998) and in rheumatoid arthritis a correlation has been made with Th1 CD4+ T cells derived from the arthritic synovium, although Th2 and Th0 cells have also been found (Miltenburg, van Laar et al. 1992; Quayle, Chomarat et al. 1993).

**Allergic reactions**

Allergic reactions are dominated by type 2 cytokines (Del Prete, De Carli et al. 1991; Robinson, Hamid et al. 1993). Examples are atopic asthma (Kay, Ying et al. 1991; Robinson, Qutayba et al. 1992) and dermatitis (van-Reijsen, Bruijnzeel-Koomen et al. 1992).

**Pregnancy**

A micro-environment dominated by type 2 cytokines has been associated with a successful pregnancy (Wegmann, Lin et al. 1993; Delassus, Coutinho et al. 1994), whereas a maternal environment dominated by type 1 cytokines is associated with increased risk of miscarriage (Raghupathy 1997; Raghupathy 1997). The skewing toward type 2 cytokines may be due to changes in hormones present pregnancy (Piccinni, Giudizi et al. 1995).

**Transplantation**

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In solid organ transplantation, the development of a Th1 response in the recipient has been correlated with organ rejection (Cunningham, Dunn et al. 1994; Thai, Fu et al. 1995). In rat heart transplant, abrogation of the Th1 response reduces transplant rejection. There is a reduction in IL-2 and IFNγ production and an increase in the levels of IL-4 (Heidecke, Hancock et al. 1996). However, in humans no correlation has been found in the level of these cytokines and heart transplant rejection episodes (Van Hoffen, Van Wichen et al. 1996).

Stem cell transplantation (where bone marrow is commonly the source of cells) has a particular pathology related to the production of cytokines post-transplant. This is caused by an immune reaction from the graft against the host explained in detail in the following sections.

1.10 Allorecognition

T cell allorecognition involves recognition of foreign MHC class I and class II molecules by T cells from histo-incompatible individuals of the same species. Characteristically, alloreactivity is a very strong response involving a large number of T cells. This is because there is a high frequency of T cells that can recognise the foreign allogeneic MHC molecules (Ford and Burger 1983) both within the naïve and memory populations of T cells (Skinner and Marbrook 1976). Estimates indicate that the T lymphocyte pool is capable of recognising allogeneic MHC molecules at frequencies that are 10-1000 fold higher than the frequencies of T cells that recognise self MHC restricted antigen (Ford and Atkins 1973; Lindahl and Wilson 1977; Ford and Burger 1983). Two forms of T cell allorecognition can be distinguished (reviewed by Sherman and Chattopadhyay 1993)). The direct recognition of allogeneic MHC molecules occurs when T cells recognise the complex of allogeneic MHC molecule and its bound peptide on the foreign cells. Some alloreactive T cells recognise specific components of both the allogeneic MHC molecule and its bound peptide (Heath, Hurd et al. 1989; Panina-Bordignon, Corradin et al. 1991) whilst others appears to recognise only the polymorphic regions of the allogeneic MHC molecule and are not influenced by the nature of the bound peptide (Elliott and Eisen 1990; Smith, Brunmark et al. 1997). The second mechanism is called indirect allorecognition. In this case, T cells recognise peptides derived from polymorphic proteins of the foreign cells that are presented by self MHC molecules. As MHC molecules are highly polymorphic, these peptides are often derived from the foreign MHC molecules (Clayberger, Parham et al. 1987; Olson, Williams et al. 1989; Liu, Braunstein et al. 1992).
1.10.1 Histocompatibility in transplantation

Foreign MHC molecules trigger a strong alloreactive T cell response that in the context of a transplant can lead to graft rejection. Therefore it is clearly beneficial to match the MHC class I and class II types of the donor and patient as close as possible in order to reduce the incidence and severity of detrimental alloresponses. Correlation between HLA matching and success of a transplant have been reported for both solid organ transplants of kidney (Opelz 1991), pancreas (So, Moudry-Munns et al. 1991), heart (Smith, Danskine et al. 1992) and cornea (Völker-Dieben, D’Amaro et al. 1989) and for bone marrow stem cell transplants.

Histocompatibility in BMT

The best approach to preventing GvHD is to match the HLA type of the donor and recipient as close as possible (Howard, Hows et al. 1990; Petersdorf, Longton et al. 1995; Spencer, Szydlo et al. 1995; Speiser, Tiercy et al. 1996; Petersdorf, Longton et al. 1997). HLA matching has been shown to be the single most important factor influencing the outcome of an unrelated BMT (reviewed by Madrigal (Madrigal, Scott et al. 1997)). HLA typing of donors and patients was until recently performed by serology. However, this method achieves only low resolution typing. Retrospective studies have subsequently shown that most transplants performed with serologically matched donor and patients were actually not HLA identical (Scott, O’Shea et al. 1998). Better matching has been achieved with the development of high resolution typing techniques for the HLA alleles (Speiser, Tiercy et al. 1996). Typing for both HLA class I and class II is now routinely done using high resolution molecular methods. Assays of T cell function are also used to evaluate the alloreactive potential of determined donor-patient pairs.

Cellular methods to measure T cell alloreactivity.

The mixed lymphocyte culture (MLC) and mixed lymphocyte reaction (MLR) (Bach and Hirschhorn 1964), are cellular assay were PBMC from two individuals are cultured together at a specific ratio. The PBMC from one individual acts as stimulators and PBMC from the other as responders. In a MLR, differences in HLA activate the responder T cells (CD4+), leading to IL-2 production and proliferation after 5-6 days of in vitro culture. The amount of proliferation depends on the frequency of alloreactive T cells and can be measured by H3-thymidine incorporation (Bach and Hirschhorn 1964; Bach and Voynow 1966). Proliferation of the stimulator cells is prevented by a variety of methods, for example exposure to by sub-lethal
doses of radiation. This assay was developed to determine differences in class II alleles: HLA-DR, DQ and to a lesser extent, DP alleles. However a low correlation between the proliferative reaction and the probability of developing acute GvHD has been found in DR mismatched (Mickelson, Guthrie et al. 1994) and matched (Mickelson, Longton et al. 1996) donor-patients pairs. Modifications of this technique, such as introducing cytokines to boost proliferation, have achieved better typing results (Bishara, Brautbar et al. 1994). However, the capacity of the MLR to accurately predict the BMT outcome is controversial. For example, the MLR can not reliably detect HLA-DP or minor Ag disparities between siblings.

The MLC has been adapted as an in vitro model for the study of alloreactivity. Initial studies described cells in a MLC undergoing "blastic formation" (Janis and Back 1970) with "rapid" (2-3 days) production of IL-2 in the presence of allogeneic leukocytes (Janis and Back 1970; Bishara, Kedar et al. 1991; Bishara, Kedar et al. 1993). The effects of the allogeneic stimulus can be measured by monitoring the production of various cytokines, for example IL-4, IL-5, IL-6, IFNγ, and TNFα, by different types of cells (Akbar, Salmon et al. 1991; Toungouz, Denys et al. 1994; Tanaka, Imamura et al. 1995; Ohshima and Delespesse 1997).

The cytotoxic T lymphocyte precursor-frequency (CTLP) assay quantifies the presence of activated cytotoxic (CD8+) cells in an allogeneic culture. As in the MLR, in the CTLP there are responder and stimulator PBMC. The frequency of responder cells is determined when the patient's blasting cells are labelled with 51Cr and then used as targets for different dilutions of donor cells. The degree of alloreactivity is proportional to the amount of 51Cr released in the assay (Kaminski, Sharrock et al. 1988). High CTLP frequencies correlate with HLA class I disparities both in sibling and unrelated donor and patient pairs and are correlated with higher probability to develop GvHD after BMT (Kaminski, Sharrock et al. 1988; Kaminski, Hows et al. 1989). The helper lymphocyte T cell precursor (HLTp) assay determines the frequency of IL-2 producing cells in a MLC and a high frequency of HLTp correlates with differences in HLA-DR between unrelated individuals, to GvHD development (Nierle, Bunjes et al. 1993; Bunjes, Theobald et al. 1995) and poor survival after BMT (Schwarer, Jiang et al. 1994). High HLTp frequencies have also been correlated with differences in minor histocompatibility antigens in identical siblings (Schwarer, Jiang et al. 1993).
1.11 Graft versus host disease as a consequence of a type 1 and type 2 immune response

Haematological disorders such as leukaemias and anemias, can lead inexorably to death unless medically treated (Goldman 1989). A principal treatment for these disorders is stem cell transplantation. The most common treatment to restore a normal hematopoiesis is the transplant of bone marrow. Due to the low availability of histocompatible siblings, unrelated bone marrow transplantation (uBMT) using an histocompatible donor takes place in most cases of stem cell replacement therapy (Madrigal, Scott et al. 1997). In recent years, cord blood has begun to be used an alternative source of haematological pluripotent cells (Gluckman, Broxmeyer et al. 1989), mainly for children and young adults.

Bone marrow transplantation and graft versus host disease

Bone marrow transplant between a donor and a recipient who are not HLA identical can generate a pathological condition known as graft versus host disease (GvHD). This is a complication of allogeneic BMT caused by donor T lymphocytes reactive to differences in histocompatibility antigens of the recipient (Thiele, Eigenbrodt et al. 1989; Martin, Schoch et al. 1990). In the absence of immunosuppression, GvHD can cause death of the patient (Sullivan, Deeg et al. 1986). This is the most significant transplant-related complication which can adversely affect the outcome. GvHD can progress in two consecutively phases, the acute and chronic phases. The acute phase (aGvHD), occurs in the first 100 days after the transplant. The chronic phase of GvHD often occurs after aGvHD has been resolved and is less life-threatening than the acute disease (Ringden and Deeg 1997). These two phases differ in time of presentation, severity of the disease, the organs affected and the profile of cytokines produced (Waldmann, Cobbold et al. 1994).

The activity of donor T cells plays a major role in the development of GvHD. Soon after the transplant takes place, there is an expansion of donor T cells due to the activation of alloreactive CD4+ (Hakim, Sharrow et al. 1991). This donor-derived CD4+ expansion is followed by a large donor CD8+ expansion (Hakim, Sharrow et al. 1991). The GvHD reaction starts when CD4+ cells recognise host alloantigen and produce IL-2, activating both CD4+ and CD8+ T cells (Rus, Svetic et al. 1995). Murine models of GvHD have shown that both CD4+ and CD8+ T cells participate in the recognition of alloantigen: CD8+ T cells are able to recognise class I mismatches (Sprent, Schaefer et al. 1986) and CD4+ T cells are reactive to class II mismatches (Korngold and Sprent 1985).
Murine models and clinical studies of BMT patient's sera, biopsies and cell cloning have characterised the acute phase as dominated by type 1 cytokines, whereas the chronic phase has been related with the expression of type 2 cytokines (Fowler and E. 1997). In murine models of GvHD, the acute disease is strongly dependent on the presence of CD8+ T cells that both secrete and promote the secretion of IFNγ by CD4+ T cells (Rus, Svetic et al. 1995). The IFNγ-production rather than the cytotoxicity potential of these CD8+ T cells, may play a major role in development of acute GvHD (aGvHD) (Rus, Svetic et al. 1995). Thus, the cytokines produced by alloreactive T cells appear to be responsible for the onset of aGvHD. Based in these findings, a model of GvHD, called the cytokine storm theory, has been proposed (Antin and Ferrara 1992; Krenger and Ferrara 1996). This model proposes that alloreactive Th1 cells release IFNγ and IL-2 upon encounter with alloantigen, stimulating macrophage and NK cells from both the host and the donor. Indeed, both murine models and clinical studies show evidence of a type 1 cytokine profile in the acute disease, characterised by increased levels of IFNγ (Niederwieser, Herold et al. 1990; Allen, Staley et al. 1993) and IL-2 (Imamura, Hashino et al. 1994; Bunjes, Theobald et al. 1995). In contrast, in chronic GvHD (cGvHD), the levels of IL-4 increase (Allen, Staley et al. 1993; De Wit, Van Mechelen et al. 1993). Furthermore administration of Th2 cells prevents lethality in a murine model of acute disease (Fowler, Kurasawa et al. 1993; Fowler 1994), by reduction of CD8+ engraftment and levels of IFNγ and TNFα production (Fowler 1994).

1.12 Stem cell transplant using cord blood.

In 1988, the first cord blood transplant (CBT) was performed to treat a haematological disease (Gluckman, Broxmeyer et al. 1989). Since then, the use of cord blood as an alternative source of stem cells for transplantation has increased with more than 1000 CBT performed to date (Gluckman, Rocha et al. 1998). CBT’s are associated with reduced incidents and severity of GvHD compared with BMT (Gluckman, Rocha et al. 1997; Rocha, Wagner et al. 2000). It has been proposed that the lower alloreactive potential of cord blood derived lymphocytes may reflect their naïve state. Between 22-84% of T cells from cord blood express the CD45RA molecule indicative of unprimed naïve cells, whereas the percentage in adult T cells is lower (14-53%) with a corresponding increase in the percentage bearing the CD45RO marker (25-68%) (Harris, Schumacher et al. 1992; Rabian-Herzog, Lesage et al. 1993; Han, Hodge et al. 1995; Mills, TG et al. 1996), indicative of memory T cells (Akbar, Terry et al. 1988; Sanders, Makgoba et al. 1988; Wallace and Beverley 1990; Pinto, Covas et al. 1991; Yamada, Kaneyuki et al. 1992). Some studies indicate that on
response to stimulus, cord blood T lymphocytes have lower levels of proliferation and produce lower levels of IL-2, IFNγ, TNFα and IL-4 compared to adult cells (Nair, Schwartz et al. 1985; Ueno, Miyawaki et al. 1985; Weatherstone and Rich 1989; Watson, Oen et al. 1991; Nonoyama, Penix et al. 1995; Lee, Suen et al. 1996; Qian, Lee et al. 1997) (see Table 1.3). Furthermore, some cytokines are present in lower levels in CB serum compared with adult serum (such as TGFα, IFNγ, IL-10, IL-6 and erythropoietin (EPO) (Westgren, Ek et al. 1995; Hata, Kawamura et al. 1997). The different properties of the CB and adult blood serum has been considered as another reason of the decreased lymphocyte responsiveness of CB samples, as only adult and not CB serum enhance both mitogen and IL-2 specific T cell growth (Cohen, Morgan et al. 2000). However, the proposal that cord blood lymphocytes have reduced functional potential remains controversial because some studies have observed normal B cell (Pastorelli, Rousset et al. 1990; Peleman and Delespesse 1990), macrophage (Glover, Brownstein et al. 1987; Weatherstone and Rich 1989) and dendritic cell (de Saint-Vis, Fugier-Vivier et al. 1998) activity. Similarly, some cytokines such as M-CSF and GM-CSF have been found only in CB serum (Westgren, Ek et al. 1995; Cohen, Woolley et al. 2000).
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cytokine</th>
<th>AD compared to CB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>IL-1</td>
<td>AD=CB</td>
<td>(Glover, Brownstein et al. 1987; Weatherstone and Rich 1989; Sautois, Fillet et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>IL-8</td>
<td>AD&gt;CB</td>
<td>(Chang, Suen et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>AD&gt;CB</td>
<td>(Lee, Suen et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>IL-15</td>
<td>AD&gt;CB</td>
<td>(Qian, Lee et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>AD&gt;CB</td>
<td>(Weatherstone and Rich 1989)</td>
</tr>
<tr>
<td></td>
<td>TGFβ1</td>
<td>AD&gt;CB</td>
<td>(Chang, Suen et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>AD&gt;CB</td>
<td>(Buzby, Lee et al. 1996)</td>
</tr>
<tr>
<td>T cells</td>
<td>IL-2</td>
<td>AD&gt;CB</td>
<td>(Watson, Oen et al. 1991; Kruse, Neustock et al. 1993; Takahashi, Imanishi et al. 1995; Chalmers, Janossy et al. 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AD=CB</td>
<td>(Miyawaki, Seki et al. 1985; Anderson, Anderson et al. 1990)</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>AD&gt;CB</td>
<td>(Anderson, Anderson et al. 1990; Lewis, Yu et al. 1991; Takahashi, Imanishi et al. 1995; Chalmers, Janossy et al. 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AD=CB</td>
<td>(Trivedi, HayGlass et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>AD&gt;CB</td>
<td>(Anderson, Anderson et al. 1990; Chalmers, Janossy et al. 1998)</td>
</tr>
</tbody>
</table>

Table 1.3. A comparison of cytokine production by adult (AD) and cord blood (CB) APC and T cells under various stimuli.

AD=CB; levels of cytokine derived from adult and cord blood cells were equal
AD>CB; levels of cytokine were greater when derived from adult compared to cord blood.
TGFβ1, Tumour growth factor β1
GM-CSF, granulocyte macrophage colony stimulating factor.

Prevention of GvHD.

Bone marrow transplants performed across an HLA mismatch are likely to lead to GvHD. The administration of immunosuppressive drugs to the recipient decreases the incidence of cGvHD (Sullivan, Shulman et al. 1981; Sullivan, Deeg et al. 1986).
Another preventive approach used to reduce the risk of GvHD involves *in vitro* depletion of T cells from the donor bone marrow prior to transplant (Howard, Hows et al. 1990; Drobsky, Ash et al. 1994). Removing the potential alloreactive T cell does limits GvHD (Howard, Hows et al. 1990; Blazar, Taylor et al. 1993; Hale and Waldmann 1994) however T cell-depleted donor marrow takes longer to engraft and can result in graft failure (Mitsuyasu, Champlin et al. 1986; Delain, Cahn et al. 1993), leaving the recipient in an immuno-compromise state for longer and therefore at increased risk form opportunistic infections such as cytomegalovirus.

Because the development of aGvHD is related with the presence of alloreactive type 1 T cells whereas the presence of type 2 cells after BMT has been correlated with a less aggressive disease (Niederwieser, Herold et al. 1990; Tanaka, Imamura et al. 1994; Imami, Brookes et al. 1998), the preferential development of type 1 or type 2 cells within a donor-patient pair may be related with the development of aggressive or milder GvHD. Thus, the better understanding of the development of these Th phenotypes may be a strategy for the management of the GvHD developed with non-T cell depleted grafts. Additionally, CB has been used as an alternative from BMT as a source of stem cells: the incidence and severity of GvHD is reduced using CB cells compared with bone marrow (Rocha, Wagner et al. 2000), which has been explained because the naïveté of the cells from CB, that allows the use of more HLA mismatches in unrelated pairs. However, T cells from CB could respond to stimulation to adult levels if their threshold of stimulation is reached: it has been observed that CB derived T cells have a threshold of stimulation of 6 HLA mismatches (Wang, Sviland et al. 1998).
Aims of this thesis.

The aim of this thesis was to determine the frequencies of type 1 and type 2 alloreactive cytokine producing T cells developed with certain HLA mismatches.

To do this, the following objectives were developed:

To standardise a method for the intracellular detection of the cytokines produced by alloreactive T cells. The negative control cells for cytokine production were naïve (CD45RA⁺) T cells obtained from CB and stimulated with various mitogens. Positive controls for cytokine production were resting T cells from adult blood (a mixture of CD45RA⁺ and CD45RO⁺ cells) and memory CD45RO⁺ T cell obtained from T cell lines.

To detect frequencies of cytokine producing alloreactive T cells. This was performed after mitogenic and alloantigen specific re-stimulation of short term alloreactive T cell lines.

To adapt the sensitive IFNγ ELISPOT assay to detect the frequency of IFNγ producing alloreactive T cells. This was because the frequency was not detected by intracellular cytokine determination due to the strength of the stimulation used to induce cytokine production.

To detect Th1/Th2 extracellular markers as an additional tool to define frequencies of type 1 and type 2 cytokine producing alloreactive T cells.
2. Chapter 2 Material and Methods.

2.1 In vitro culture medium

To wash the cells and immobilise antibodies, RPMI (Biovittaker, Walkersville, Maryland, USA) supplemented with antibiotics (Penicillin-G at 10, IU/ml and streptomycin at 10mg/ml sulphate in saline solution; Gibco, Paisley, UK) (serum free medium) was used. The antibiotics were used to prevent Gram positive and negative bacteria contamination.

To culture cells, serum free medium was complemented with 10% normal AB positive human serum (Biovittaker, Walkersville, Maryland, USA) (complete medium). CTLL-2 medium was RPMI plus 10% heath inactivated foetal calf serum (FCS, Biowittaker, Walkersville, Maryland, USA).

2.2 Obtaining mononuclear cell populations

The lymphocytes of human origin were obtained either from healthy donors from the staff, from commercial buffy coats from healthy individuals, from CB or from selected donors at the panel of Bone Marrow donors from the Anthony Nolan Bone Marrow Trust.

2.2.1 Mononuclear cells from commercial blood (Buffy coats)

Peripheral blood mononuclear cells (PBMC) from adult blood buffy coats were obtained from the North London Blood Transfusion Centre (Edgware, Middlesex, UK). To purify the mononuclear cells, the buffy coats were diluted 1:1 in serum free medium and overlayed on Lymphoprep (Nycomed Pharma, AS, Oslo, Norway) in a 1:1 ratio (diluted buffy coat:medium) to separate the leukocytes by density gradient. After centrifuging at 2400 rpm (998.0 g) for 20 minutes in a Heraeus Megafuge 2.0. (Heraeus Instruments limited, Brentwood UK), the mononuclear cells from the interface were recovered and washed once in serum-free medium at 1800 rpm (748.5 g) for 18 minutes. They were then washed in serum free medium, at 1500 rpm for 15 minutes. The cells were then resuspended in complete medium. An aliquot was taken and diluted 1:1 with trypan blue (0.4% Sigma, Pool, Dorset, UK) to count cell viability by vital-dye exclusion (Hay 1992). A minimum of 100 live cells were counted in a hemocytometer (Improved Neubauer, Weber, UK). Fresh samples always had >90-95% viability. The cells were used immediately for experiments or were frozen, as described in Section 2.6.
2.2.2 Mononuclear cells from healthy adult members of staff at the Anthony Nolan Research Institute

50 ml of blood from members of staff was taken by a qualified doctor and overlayed undiluted on Lymphoprep, in a 1:1 ratio (blood:Lymphoprep), to purify mononuclear cells as described in Section 2.2.1. The cells obtained were used immediately or frozen as described in Section 2.6.

2.2.3 Mononuclear cells from the Anthony Nolan panel of bone marrow donors

Donors from the Anthony Nolan Bone Marrow trust were selected on bases of their HLA type. This is described in detail in Chapter 7 of this thesis.

To obtain these bloods, 5 tubes with lithium-heparin-coated beads (Sarstedt Ltd, Leicester, UK) were sent by post to the donors with an explanatory letter of the project, a consent form and instructions on how to donate the blood. Donors were asked to be bled by their General Practitioner, whose address was recorded in our registries. The donor's samples were send back by post in a postage prepaid self addressed parcel. The parcel normally arrived within one to two days, although some samples arrived later than expected. The latest was after 10 days (with an average of 2 days). All this information was registered. The mononuclear cells were recovered by Lymphoprep immediately on receiving the sample (viability >95%) and frozen immediately. Between 1-9x10^7 cells per donor/donation were obtained.

2.2.4 CB mononuclear cells

Umbilical CB mononuclear cells were obtained from normal and caesarean full-term deliveries, by puncturing the umbilical vein (Domínguez de Ortega, Lowdell et al. 1996). Blood was deposited in a heparinised tube and mononuclear cells were separated by density gradient, as described for PBMC in Section 2.2.2. Normally 50ml of blood per CB were obtained, with 1x10^6 PBMC/ml.

2.3 Obtaining pure T cells populations

2.3.1 CB CD3⁺CD45RA⁺ pure cells

CD45RA⁺ T cells were prepared by depletion of monocytes (CD14⁺), NK cells (CD56⁺/CD16⁺), B-cells (CD19⁺) and memory T-cells (CD45RO⁺) from the CB mononuclear cell preparation. This was achieved by magnetic separation using
sheep anti-mouse antibody (Ab)-coated Dynabeads® (Dynal A.S. Oslo, Norway). The beads were labelled for 30 minutes at room temperature with mouse anti-human anti-CD14, CD16, CD19, CD56 or anti-CD45RO antibodies (see Table 2.1) at 0.4 mg of Ab/10⁷ beads. Beads were then washed twice with serum-free medium and incubated with CB mononuclear cells at room temperature for 30 minutes at a bead:target ratio of 10:1. These cells were then washed and used immediately.

<table>
<thead>
<tr>
<th>Ab type</th>
<th>Ab epitope</th>
<th>Sp epitope</th>
<th>Ab isotype</th>
<th>Used for depletion of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>CD14</td>
<td>Human</td>
<td>Mur IgG2a</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Mo</td>
<td>CD16</td>
<td>Human</td>
<td>Mur IgG1</td>
<td>NK cells</td>
</tr>
<tr>
<td>Mo</td>
<td>CD19</td>
<td>Human</td>
<td>Mur IgG1</td>
<td>B cells</td>
</tr>
<tr>
<td>Mo</td>
<td>CD56</td>
<td>Human</td>
<td>Mur IgG1</td>
<td>NK cells</td>
</tr>
<tr>
<td>Mo</td>
<td>CD45RO</td>
<td>Human</td>
<td>Mur IgG2a</td>
<td>Memory cells</td>
</tr>
</tbody>
</table>

Table 2.1. Antibodies used for CB CD45RA⁺ T cell purification by magnetic negative depletion. Abbreviations: Sp epitope, specie for the epitope. Mo, monoclonal Ab. Mur, Ab of murine origin. All Ab were from Becton and Dickinson (B & D, San Jose, CA, USA).

2.3.2 T cell lines

Generation of non-specific T cell lines

Non specific T cell lines were generated by polyclonal stimulation by anti-CD3 Ab or PHA stimulation. For PHA lines, 1X10⁶ PBMC/ml from healthy individuals were placed in 2ml/well in 24 well plates (Falcon plates, B &D, Franklin Lakes, NJ, USA), in complete medium with a predetermined optimal concentration of PHA (determined as described in Appendix Section10.2). 20ng/ml of human recombinant (hr)interleukin (IL)-2 was added at day 7, and at day 14 the cells were transferred to fresh complete medium containing IL-2, PHA and PBMC feeders. To obtain these feeders, allogeneic PBMC were irradiated with 10,000 Rad in a Cesium-γ-irradiator for 6.5 minutes (Haematology department, the Royal Free Hospital, Hampstead). They were used at a ratio of 2:1, irradiated PBMC:T cells. These cells where cultured in complete medium supplemented with IL-2 after another 7 days. All lines were maintained on this 14 day cycle of allogeneic feeders plus PHA and IL-2 with a supplement of IL-2 at day 7. To determine if contaminant irradiated PBMC remain in the culture at day 6, the flow cytometric phenotype of these cells was determined before and after irradiation. An aliquot of cells was taken before irradiation and at days 1 (Day 1) and 4 (Day 4) after irradiation, was fixed in 0.1% paraformaldehyde for 20 minutes at 4°C and analysed in a flow cytometer as described in Section
2.7.1. At this time point all irradiated cells had disintegrated (Figure 2.1). At day 4, most of the cells had disintegrated and disappeared from the live gate. At least two cycles were completed before T cell lines were used in experiments. At this point the phenotype of the lines was always >99% CD3⁺ (Figure 2.2).
**Figure 2.1.** Forward and size scatter of irradiated lymphocytes. Flow cytometry dot plots were obtained before (Day 0) and after irradiation of PBMC cultured in complete medium. The cells were fixed and analysed for flow cytometry as described in Section 2.7.1. This figure represents the size and granularity of cells before and after irradiation, measured by forward (FSC) and side (SSC) light scatter, as described in Section 2.7.1. Live cells are only those included in the gate 1 (R1). The live gate represent 17% (Day 0), 2.2% (Day 4) and 0.5% (Day 4) of the total of events for each time point.

**Figure 2.2.** Typical phenotype of non Ag-specific T cell lines. T cell lines were generated as described in Section 2.3.2. To assess their purity, after two cycles of culture, the cells were stained extracellularly with antibodies for CD3 (PerCp) as explained in detail in Section 2.7.1. A) Gate of live lymphocytes defined as explained in Figure 2.1 (gate 1) B) Histograms gated on gate 1. The non-stained cells (grey histogram, M1 region) histogram was used as a negative control to set the positive region (M2, which showed cells stained with anti-CD3-PerCp Ab).

To generate T cells lines with anti-CD3 Ab stimulation, PBMC were plated at $10^6$/ml of complete medium plus 20ng/ml IL-2 and 35ng/ml anti-CD3 monoclonal antibody (OKT3 culture supernatant) for 7 days. Cells were then supplemented with fresh medium and 20ng/ml IL-2 for another 7 days. At the end of this 14 day cycle the
resulting T cells were placed with irradiated PBMC plus anti-CD3 and IL-2 for 7
days then supplemented with fresh medium and 20ng/ml IL-2 for another 7 days.
Cells were maintained on this 14 day cycle. T cells were always used in assays at
day 14 of the cycle, when the T cell population was always >90%.

**Generation of type 1 and type 2 T cell lines**

Type 1 and type 2 PHA-stimulated cell lines were generated under polarising
conditions previously described (Swain, Weinberg et al. 1990; Hsieh, Macatonia et
al. 1993; Manetti, Parronchi et al. 1993; Rogge, Barberis-Maino et al. 1997; Sallusto,
Mackay et al. 1997). 2x10⁶ PBMC/ml were cultured at a predetermined optimal
concentration of PHA for one week in polarising conditions. These conditions
consisted of complete medium supplemented with hrIL-2 for both cell types. For
type 1 cells, hrIL-12 was added and for type 2 cells, hrIL-4 plus anti-IL-12
neutralising Ab was added (Table 2.2). After this first cycle, the cells were
harvested, washed twice in serum-free medium, and stimulated again in their
polarising milieu.

<table>
<thead>
<tr>
<th>Used for</th>
<th>Factor</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell growth</td>
<td>hrIL-2</td>
<td>20ng/ml</td>
<td>First Link Ltd (West Midlands, UK)</td>
</tr>
<tr>
<td>Polarising cytokine for type 1 T cells</td>
<td>hrIL-12</td>
<td>2 ng/ml</td>
<td>R and D systems (Abingdon, UK)</td>
</tr>
<tr>
<td>Polarising cytokine for type 2 T cells.</td>
<td>hrIL-4</td>
<td>200 U/ml</td>
<td>R and D systems</td>
</tr>
<tr>
<td>Neutralising the type 1 polarising factor IL-12.</td>
<td>IgG1 mouse anti-human-IL-12 Ab</td>
<td>2 μg/ml</td>
<td>R and D systems</td>
</tr>
</tbody>
</table>

Table 2.2. Polarising factors to obtain type 1 and type 2 T cell lines. Type 1 or type 2 T polarising cytokines and anti-cytokine Ab were used to develop type 1 or type 2 non-specific T cell lines *in vitro*.

**Allospecific T cell lines**

PBMC were obtained as described in Section 2.2.1 and 2.2.3 and typed for HLA
class I and II as described in Chapter 2 Section 2.10. PBMC pairs were selected on
basis of their tissue type, to be cultured in a one way mixed lymphocyte culture
(MLC). The cells were used fresh or frozen. Fresh cells were used after purification
by Ficoll Hypaque as described in Section 2.2.2. For frozen PBMC (used as the
allogeneic stimulators) the cells were thawed by washing twice in serum-free medium
at 37°C, counted and irradiated (10,000 Rad in a Cesium γ-irradiation source). The
cells were resuspended in complete medium with PBMC from a different donor (responder cells, thawed in the same way). All allogeneic cultures were done in a 1:1 responder:stimulator ratio, at 2x10^6 total cells/ml in 6-24 well plates, for 6 days. At day 6, the responder cells were recovered, washed twice in serum-free medium and used.

2.4 Cell proliferation assessed by ^3^H thymidine incorporation assay

2.4.1 Proliferation of T cells obtained from PBMC

T cell activation, growth and division can be sensitively measured as proliferation by ^3^H-Thymidine incorporation (Reinsmoen 1993). To assess T cell proliferation to a stimulus in a PBMC environment, 1x10^5 PBMC/well in 200μl complete medium, were cultured with the stimulus, in 96 well plates (Falcon microtest, Franklin Lakes, NJ, USA) in triplicates. Cells were incubated for three days in a humidified atmosphere at 37°C and CO₂ 1μ Curie (Ci) of [^3]H thymidine in 20μl of serum free medium, were added to each well 18 hours before harvesting the wells on a glass-fibre filter (Wallac, Turku, Finland) in an automated harvester (Tomec, Orange, Connecticut). The glass-fibre filter was deposited in a sample bag (Wallac, Turku, Finland) with 5 ml of scintillation liquid (Wallace, Loughborough, UK) and sealed (Heat Sealer 1295-012 Wallac, Turku, Finland). Radioactivity was counted immediately in a liquid scintillation counter (Wallac 1450 microbeta, Turku, Finland) and recorded in the program UTMAC 1.2 (Wallac, Turku, Finland). Results are expressed as mean of triplicate counts per minute (cpm) ± SD.

2.4.2 Proliferation of CTLL-2 cell line

The proliferation of cells of the human IL-2 and IL-15 responsive CTLL-2 T cell line (ATCC, Maryland, USA) was done plating 1x10^4 viable CTLL-2 cells/well in CTLL-2 medium with hrIL-2 or hrIL-15. The cells were grown in CTLL-2 medium + 20 ng/ml hrIL-2. Before the assay, the cells were washed twice in serum free medium and rested for 24 hrs in the absence of IL-2. 5X10^3 cells/well were then plated in 96 round bottom well plates and hrIL-2 (R and D), hrIL-15 (R and D), mouse antihuman-IL-2 Ab, mouse anti human-IL-15 Ab or control antibodies (murine IgG1) were added in triplicate per well (Cohen, Clayton et al. 1995) (Figure 2.3). These cultures were maintained for 24 hours at 37°C in 5% CO₂ and pulsed with 1 μCi /well of ^3^H-thymidine 8 hours before harvesting onto glass fibre filters as described in Section 2.4.1. Scintillation counting was performed immediately.
Figure 2.3. Proliferation of the murine line CTLL-2 to hrIL-2 and hrIL-15. The CTLL-2 cells were washed in serum free medium and cultured without IL-2 for 24 hr before the assay. Then, 5X10^3 CTTL-2 cells/well were plated in 96 round bottom well plates plus different concentrations of IL-2, IL-15, and IL-4 and IL-12 as a negative control (Panel A), 20 ng/ml hr IL-15 ± anti-IL-15 Ab or control Ab (panel B) or dilutions of hrIL-2 ± anti-IL-2 Ab (panel C) or control Ab (panel D). The cells were then cultured for 24 hours at 370C in 5% CO2 and pulsed with 1 μCi /well of 3H-thymidine 8 hours before harvesting as described in Section 2.4.1. All the cytokines and Ab were from R and D systems. Results represent the mean amount of radioactivity incorporated in the triplicates ± SD.
2.5 Mitogenic stimulation of T cells using antibodies for CD3, CD28, phorbol-12 misystrate-13 acetate (PMA), ionomycin or specific stimulation with alloantigen.

Mitogenic stimulation.

When stimulation was mitogenic and non-antigen specific, it consisted in stimulation through the T cell receptor associated polypeptide CD3 (Kasahara, Djeu et al. 1983; Leo, Foo et al. 1987) or the T cell co-stimulatory molecule CD28 (Thompson, Lindsten et al. 1989), phorbol esters (Nishizuka 1984) or ionomycin (Bennet, Cockcroft et al. 1979) in different combinations.

PMA (Sigma, Dorset, UK) was used at 0.5, 5 or 50 ng/ml. Ionomycin (Calbiochem, Nottingham, UK) was used at 0.1, 1 and 10 μM. PMA, ionomycin and monensin (Calbiochem, Nottingham, UK) stocks were prepared and for each experiment, a new aliquot was taken: the PMA stock was 1mg/ml in DMSO. The ionomycin stock was 1mg/352μl DMSO. The monensin stock solution was 12mM in ethanol. All the stocks were stored at -20°C in the dark. The stocks were prepared in small aliquots and used only once after thawing.

The anti CD3 Ab (OKT3) was either purified from the supernatant of a hybridoma (ATCC Maryland, USA) as described in Appendix Section10.3, or bought from commercial sources (see Table 2.3). The anti-CD28 Ab was obtained from commercial sources (see Table 2.3). Plate immobilised or soluble OKT3 Ab was titrated as described in Appendix Section10.4.1.

The anti-CD3 (0, 5, 50, 200 μg/ml) ± anti-CD28 (0, 1, 10, 30 μg/ml) Ab, were immobilised overnight in serum-free medium in 24 or 96 flat-bottom well plates, as previously described (Katsikis, Cohen et al. 1994). The plates were then washed with serum-free medium and incubated in complete medium for 30 minutes at 37°C. The medium was removed and cells were added to the plates for stimulation.

For expression and intracellular detection of cytokines, the cells were stimulated with plate immobilised anti-CD3 ± anti-CD28, plate immobilised anti-CD3 ± PMA or PMA ± ionomycin, always in the presence of monensin at a final concentration of 3μM (Jung, Schauer et al. 1993) for the last 6 hrs of stimulation. Monensin is used to prevent protein secretion because it disrupts transport from the Golgi apparatus to the milieu (Tartakoff 1983).
Allogeneic stimulation

For allogeneic specific T cell stimulation, PBMC from different individuals were chosen as responders or stimulators to be cultured in a MLR. Irradiated stimulators and responders were cultured in complete medium at a 1:1 responder:stimulator ratio, with 1x10^6 responder cells /ml, as described in Section 2.3.2.

To evaluate cytokine production of allospecific T cell lines, the cells were cultured for one week in a MLC. Then, the cells were either mitogenically stimulated (with 50μg/ml anti-CD3 Ab plus 5ng/ml of PMA) to induce cytokine production, or the cells were rechallenged with the same stimulator. The cytokine production of these cells was evaluated by ICS or by ELISPOT (Chapter 2 Sections 2.7.5 and 2.9). To do the ELISPOT, the responder cells were harvested, washed and rechallenged at a 1:1 responder:stimulator ratio and cultured overnight in 200μl/well in 98 "u" bottom plates. The re-stimulated cells where then transferred to a ELISPOT plate to detect cytokine secretion, as explained in Section 2.9.

<table>
<thead>
<tr>
<th>Ab type</th>
<th>Ab Epitope</th>
<th>Sp epitope</th>
<th>Ab isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>CD3</td>
<td>Human</td>
<td>Mur IgG2a</td>
<td>ATCC</td>
</tr>
<tr>
<td>Mo</td>
<td>CD3</td>
<td>Human</td>
<td>Mur IgG2a</td>
<td>OrthoDiagnostics (Raritan, New Jersey, USA)</td>
</tr>
<tr>
<td>Mo</td>
<td>CD28</td>
<td>Human</td>
<td>Mur IgG1</td>
<td>Pharmingen</td>
</tr>
</tbody>
</table>

Table 2.3. Antibodies used for T cell stimulation. The anti-CD3 Ab was purified from a hybridoma (from the American Tissue Culture Catalogue, ATCC), as described in Appendix Section10.3, or bought from OrthoDiagnostics (to induce T cell proliferation and cytokine expression). The anti-CD28 Ab was obtained from Pharmingen.

2.6 T cell cryopreservation

PBMC or T cell lines were frozen following standard procedures (Hay 1992). The cells were washed twice in serum free medium and counted before being resuspended in ice cold-freezing mixture (FCS plus 10% dimethyl sulphoxide (DMSO, BDH Laboratories, Pool, Dorset, UK). A minimum of 5x10^6 cells/ml aliquot was transferred in ice-cold-cryotube vials (Nunc, Denmark) in 1ml/vial and transferred to a -70°C freezer in polystyrene boxes. The vials were transferred to liquid nitrogen (N₂) the next day.

2.7 Identification of T cell subsets by flow cytometry.
Chapter 2

The flow cytometer is an optical apparatus that detects particles as they move in a liquid stream and disturb a laser ray. As the particle (for example a cell) passes through the laser beam, light is scattered in all directions. Light scattered in the forward (FSC) direction is generally proportional to the size of the molecule. Light enters the particle and is reflected and refracted by the nuclear and cytoplasmic contents. Thus, the granularity and morphology of the cell is proportional to light scattering (SSC) (Macey 1994). The laser ray excites fluorescent proteins (fluorochromes). In this study, the fluorochromes were covalently bound to Ab. The fluorochromes fluorescein isothiocyanate (FITC), phycoeritrin (Pe), R-phycoerythrin (PerCp) and (Allophycocyanin (APC) were used, with emissions detected in the FL-1, FL-2, FL-3 and FL-4 channels.

2.7.1 Staining for extracellular markers using fluorescence conjugated or non-conjugated Ab

12 x 75 mm polystyrene tubes (Falcon, Franklin Lakes, NJ, USA) or round bottom 96-well plates (Falcon, Franklin Lakes, NJ, USA) were used to perform the staining with 1x10⁶ cells/tube or 0.2-0.5 x10⁶ cells well. Before staining, the cells were washed twice with staining buffer (1% heat inactivated FCS, 0.1% sodium azide (BDH Chemicals, Ltd), in PBS pH 7.4-7.6). The cells were then resuspended in 100µl (per tube) or 50µl (per well) of staining buffer with a dilution of non-conjugated or fluorochrome-conjugated monoclonal Ab specific for a cell surface antigen (Table 2.4) for 30 minutes at 4°C in the dark. The cells were then washed twice with staining buffer and pelleted by centrifugation at 250g for 3 minutes at 4°C. When the primary Ab was not conjugated with a fluorochrome, secondary fluorochrome-conjugated Ab was added after washing the cells in staining buffer and the cells were incubated for further 30 minutes at 4°C in the dark.

The stained cells were fixed by adding 250µl/tube or 100µl/well of a formaldehyde fixing solution (Cell-FIX solution, Becton and Dickinson), or 0.1% formaldehyde (Sigma, Pool, Dorset, UK) in PBS for 20 minutes at 4°C in the dark.

Cells were then resuspended in staining buffer and acquired by flow cytometry, where a minimum of 5,000 events were acquired per sample. For acquisition, for FSC and SSC settings for PBMC were used. All cells analysed were gated and in some cases, a second gate was set for CD3⁺ and CD8⁺ cells (Figure 2.4). The negative region of the dot plots and histograms was set according with fluorescence obtained with non stained cells or cells stained with isotype matched control Ab (Ctr Ab). All the results obtained are expressed as the frequency of events (in the FSC, SSC or
fluorescence channels FL-1, FL-2, FL-3 or FL-4) and are represented as histograms, contour plots or dot plots for these channels.

Figure 2.4. Lymphocyte gate. A) A FSC and SSC plot was used to set a gate of live lymphocytes (R1) B) Dot plot for FSC and FL-3 channel (CD3 staining) gated in R1 was used to define CD3⁺ lymphocytes (R2).
Table 2.4. Antibodies used for flow cytometry. Abbreviations: FC, flow cytometry. ICS, intracellular cytokine staining. Pharm, Pharmingen. Serotec is based in Oxford, UK and Harlan Sera-Lab Ltd in Loughborough, UK.

2.7.2 Dilutions of fluorescent antibodies used to stain extracellular markers.

Based on the manufacturers instructions, optimal dilutions of fluorescent Ab were used to stain the cells. An Ab dilution was used when a population of cells was clearly differentiated in a histogram as a separated population after staining (For an example see Figure 2.5).

CD45RA and CD45RO double positive cells were observed at low proportions in T cells from periphery (6.0-7.8%, mean 6.8%) and T cells from CB (0.1-4.7%, mean 3.2 %), but not in T cell lines.
Figure 2.5. Histogram of the titration of anti-CD3-PerCp Ab. PBMC were stained with serial dilutions of the anti-CD3-PerCp Ab as described in Section 2.7.1. The cells were acquired in a flow cytometer and a histogram of the fluorescence vs frequency was plotted. The grey histogram represents non stained cells, used to define the positive (M1) region for fluorescence. The dark line represents an Ab dilution of 1:10, the thin line a dilution of 1:20 and the dotted line a dilution of 1:50.

2.7.3 Expression of CD3, CD4 and CD8 Ag in lymphocytes before and after stimulation.

The CD3, CD4 and CD8 Ag were used to define frequencies of different T cell populations by flow cytometry. In some experiments, PMA and ionomycin were used to stimulate the cells and analyse the cytokine production. The expression of CD4 is down-regulated in the presence of PMA (>3ng/ml) and this effect is enhanced with the addition of ionomycin (>0.1μM) (Anderson and Coleclough 1983). This was observed for fresh PBMC and for lines grown with polyclonal stimulation (with plate immobilised anti-CD3 Ab, as explained in Section 2.5) or allogeneic stimulation (as explained in Section 2.3.2). In the presence of these two stimulus, the percentage of lymphocytes expressing CD4 is down-regulated from 50% to 13% after 6 hrs of stimulation, whereas the CD8 antigen is not down-regulated to that extend (Tables 2.5 and 2.6) (Anderson and Coleclough 1983; Picker, Singh et al. 1995). Therefore, the detection of extracellular CD4 was not possible when PMA was used.
Table 2.5. Expression of the CD3, CD4 and CD8 antigens in PBMC before and after PMA and ionomycin stimulation.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No stimulation (%)</th>
<th>PMA plus ionomycin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>86</td>
<td>83</td>
</tr>
<tr>
<td>CD4</td>
<td>50</td>
<td>13</td>
</tr>
<tr>
<td>CD8</td>
<td>36</td>
<td>31</td>
</tr>
</tbody>
</table>

PBMC from a healthy donors were purified by Fycoll Hypaque as explained in Section 2.2.2. Then, the cells were left unstimulated or were stimulated with 5ng/ml PMA, 1μM ionomycin and 3μM monensin, for 6 hrs. Then, the cells were harvested, washed twice in staining buffer and stained with anti-CD3, anti-CD4 or anti-CD8 Ab (as explained in Section 2.7.1). The cells were acquired and analysed by flow cytometry as explained in Section 2.7.1. Here, the percentages of expression of the antigens CD3, CD4 or CD8 in unstimulated and stimulated cells in the lymphocyte gate are compared.

Table 2.6. Expression of the CD3, CD4 and CD8 antigens in a T cell line.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Culture</th>
<th>Stimulation after culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-CD3</td>
<td>Anti-CD3+PMA</td>
</tr>
<tr>
<td>CD3</td>
<td>Anti-CD3</td>
<td>87.9</td>
</tr>
<tr>
<td></td>
<td>Allogeneic</td>
<td>99.6</td>
</tr>
<tr>
<td>CD4</td>
<td>Anti-CD3</td>
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</tr>
<tr>
<td></td>
<td>Allogeneic</td>
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</tr>
<tr>
<td>CD8</td>
<td>Anti-CD3</td>
<td>46.6</td>
</tr>
<tr>
<td></td>
<td>Allogeneic</td>
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</tbody>
</table>

PBMC from members of the staff were purified as explained in Section 2.2.2. The cells were cultured for one week with plate immobilised anti-CD3 (as explained in Section 2.5) or in an allogeneic culture with irradiated PBMC (as explained in Section 2.3.2). After this, the cells were recovered from the cultures and left unstimulated or re-stimulated with plate immobilised anti-CD3 Ab (50μg/ml), anti-CD3 Ab plus 5ng/ml PMA, or PMA plus ionomycin (1μM) in the presence of 3μM monensin for 6 hrs. Then, the cells were harvested, stained, acquired in a flow cytometer and analysed as explained in Table 2.5. Results are expressed as the percentages of expression relative to the unstimulated cells in the lymphocyte gate (for CD3 expression) or CD3⁺ lymphocyte gate (for CD4 and CD8 expression) and are representative for three PBMC.

2.7.4 Detection of intracellular CD4.
As the expression of the CD4 antigen was down-regulated from the cell surface with PMA and ionomycin, it was attempted to detect it intracellularly. PBMC were washed in staining buffer and stained extracellularly for CD4 as described in Section 2.7.1 or permeabilised by washing twice with permeabilisation buffer (staining buffer plus 0.1% saponin (Sigma, Pool, Dorset, UK). The anti-CD4-FITC Ab (normally used to stain extracellularly, see Table 2.4) was added in permeabilisation buffer to stain the internalised CD4. Cells were washed twice in staining buffer and fixed (Figure 2.6). Intracellular staining of CD4, resulted in a lost of CD4⁺ detection within a live lymphocyte gate. As the CD4⁺ stained cells did not represent the frequency of CD4⁺ cells in the sample, CD4⁺ could not be stained intracellularly with the methodology used here.

![Figure 2.6. Comparison of extracellular and intracellular staining of CD4 on PBMC. PBMC were stained with a 1:50 dilution of anti-CD4 FITC Ab with (A) or without (B) permeabilisation (see Section 2.7.3). After incubation for 30 minutes at 4°C in the dark, the cells were washed twice in staining buffer, fixed and acquired in the flow cytometer (10,000 events). Cells were gated on live lymphocytes. A and B are dot plots and represents results of CD4 detection without (A) or with (B) permeabilisation. The horizontal bar represent the negative level set on unstained cells. C represents the histogram of the frequency of extracellular (line) or intracellular (dashed line) CD4 stained cells. The M1 region (or positive region of staining) was set based on unstained cells.](image)
2.7.5 Analysis of the frequencies cytokine producing cells by the intracellular cytokine staining technique.

Cytokines produced by cells on stimulation can be detected with intracellular cytokine staining (ICS), by cell permeabilisation and subsequent introduction of fluorochrome conjugated anti-cytokine Mo Ab to determine the frequency of cytokine producing cells.

The cells were stimulated mitogenically with CD3 ± CD28, CD3 ± PMA or PMA ± ionomycin (as described in Section 2.5) to induce cytokine production, harvested and stained extracellularly with an optimal pre-determined concentration of anti-CD3 PerCP or anti CD8 PerCP and fixed as explained in Section 2.7.1. The cells were then stained with Ab intracellularly for cytokines using flow cytometry tubes or round bottom 96-well plates.

For intracellular staining, the cells were washed twice with permeabilisation buffer using 100μl for wells and 250μl/tube. They were spun down and resuspended in 50μl of a previously determined optimal dilution of a fluorochrome-conjugated anti-cytokine Ab (described in Table 2.4). For all the intracellular cytokine determinations in this work, a duplicate sample was stained with an isotype matched control Ab used at the same dilution as the anti-cytokine Ab.

The cells were resuspended in the Ab suspension and incubated at 4°C for 30 min in the dark. Then, the cells were washed twice with permeabilisation buffer and resuspended in staining buffer. Cells were analysed in the flow cytometer within 24 hr.

For analysis, the cells were gated on live CD3+ or live CD8+ cells.

2.8 Titration of anti-cytokine antibodies.

Titration of anti-cytokine antibodies was performed in stimulated and non-stimulated PBMC from healthy donors, stained extracellularly for CD3. PMA and ionomycin were used to stimulate the cells.

Based on the literature (Sanders, Andersson et al. 1991), the cells were incubated for 6 hrs in PMA (5ng/ml) and ionomycin (1μM), in the presence of 3μM monensin. Conjugated anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-10, anti-tumour necrosis factor (TNF) α and anti-interferon (IFN) γ Mo Ab and their corresponding isotype-matched control Ab were titrated (these Ab are listed in Table 2.4). The lymphocyte gate (R1)
Chapter 2

plus the CD3 gate (R2) (Figure 2.4) were used to define the cytokine production by CD3⁺ lymphocytes. All anti-cytokine and isotype control Ab staining in stimulated and non-stimulated PBMC was restricted to that gate. The anti-CD3 Ab used to define the T lymphocyte gate was FITC, Pe or PerCP conjugated. For intracellular detection of cytokines, a minimum of 40,000 events of the samples were acquired within 24 hrs of staining and were analysed in a flow cytometer (B & D) using Cell Quest software (B & D).
Figure 2.7. Titration of anti-IL-2-Pe conjugated Ab and isotype matched control Ab in CD3⁺ lymphocytes. PBMC before (A) or after (B and C) 6 hrs of stimulation with 5ng/ml and 1μM ionomycin in the presence of 3 μM monensin were stained extracellularly with a 1:50 dilution of CD3-FITC and intracellularly with serial dilutions of (B) anti-IL-2 Ab (Pe conjugated) or (C) isotype matched control Ab. All samples were gated in a CD3⁺ lymphocyte gate (Figure 2.4). Non-stained cell’s (A) were used to define the positive (M1) region for IL-2-Pe (FL-2 channel in the X axis). Only a low dilution of the Ctr Ab produces non specific staining on the cells (Panel C). The results are expressed as frequencies of events (counts) in the FL-2 channel. The thin line is from the Ab dilution 1:2, the dotted line corresponds to the 1:10 dilution, the dark line corresponds to the 1:100 dilution and the dashed line corresponds to the 1:1000 dilution of the antibodies. These histograms are overlayed for comparison.
Figure 2.8. Titration of anti-IFNγ-FITC conjugated Ab and isotype matched control Ab in CD3⁺ lymphocytes. PBMC before (A) or after (B and C) 6 hrs of stimulation with 5ng/ml and 1µM ionomycin in the presence of 3 µM monensin were stained extracellularly as described in Figure 2.7 and intracellularly with serial dilutions of (B) anti-IFNγ-Ab (FITC conjugated) or C) isotype matched control Ab. The positive (M1) region for IFNγ fluorescence (FL-1 channel) was defined as explained in Figure 2.7. The results are expressed as frequencies of events (counts) in the FL-1 channel. The thin line is from the Ab dilution 1:2, the dotted line corresponds to the 1:10 dilution, the dark line corresponds to the 1:100 dilution and the dashed line corresponds to the 1:1000 dilution of the antibodies. These histograms are overlaid for comparison.
Figure 2.9. Titration of anti-TNFα-FITC conjugated Ab and isotype matched control Ab in CD3+ lymphocytes. PBMC before (A) or after (B and C) 6 hrs of stimulation with 5ng/ml and 1μM ionomycin in the presence of 3 μM monensin were stained extracellularly as described in Figure 2.7 and intracellularly with serial dilutions of (B) anti-TNFα-Ab (FITC conjugated) or (C) isotype matched control Ab. The positive (M1) region for TNFα fluorescence (FL-1 channel) was defined as explained in Figure 2.7. The results are expressed as frequencies of events (counts) in the FL-1 channel. The thin line is from the Ab dilution 1:2, the dotted line corresponds to the 1:10 dilution, the dark line corresponds to the 1:100 dilution and the dashed line corresponds to the 1:1000 dilution of the antibodies. These histograms are overlayed for comparison.
Figure 2.10. Titration of anti-IL-4 and isotype matched control Ab in CD3⁺ lymphocytes. PBMC before (A) or after (B and C) 6 hrs of stimulation with 5ng/ml and 1μM ionomycin in the presence of 3 μM monensin were stained extracellularly as described in Figure 2.7 and intracellularly with serial dilutions of (B) anti-IL-4 Ab (Pe conjugated) or C) isotype matched control Ab. The positive (M1) region for TNFα fluorescence (FL-2 channel) was defined as explained in Figure 2.7. The results are expressed as frequencies of events (counts) in the FL-2 channel. The thin line is from the Ab dilution 1:2, the dotted line corresponds to the 1:10 dilution, the dark line corresponds to the 1:100 dilution and the dashed line corresponds to the 1:1000 dilution of the antibodies. These histograms are overlayed for comparison.
Figure 2.11. IL-4 production in stimulated T cells. PBMC were stimulated with PMA and ionomycin as described in Figure 2.7 and stained with anti-IL-4 or isotype control Ab as described in Figure 2.10. A) Histograms of the 1:100 dilution of the anti-IL-4 Ab (thin line) and control Ab (dark line) are shown. A frequency of 4.1% of T cells was found in the positive M1 region.
Figure 2.12. Titration of anti-IL-10 and isotype matched control Ab in CD3+ lymphocytes. PBMC before (A) or after (B and C) 6 hrs of stimulation as described in Figure 2.7 were harvested and stained extracellularly with a 1:50 dilution of CD3-Pe and intracellularly as described in Section 2.8.1 with serial dilutions (1:1, 1:50, 1:100, 1:1000) of (B) anti-IL-10 Ab (FITC conjugated) or C) isotype matched control Ab. Both non-stained cell's (A) and staining with Ctr Ab (C) were used to define the positive (M1) region for IL-10-fluorescence (FL-2 channel) was defined as explained in Figure 2.7. The results are expressed as frequencies of events (counts) in the FL-2 channel. The thin line is from the Ab dilution 1:2, the dotted line corresponds to the 1:10 dilution, the dark line corresponds to the 1:100 dilution and the dashed line corresponds to the 1:1000 dilution of the antibodies. These histograms are overlayed for comparison.

2.8.1 Specific staining of anti-cytokine antibodies.

Cytokines were detected by ICS only in stimulated T cells, stained with anti-cytokine Ab (Figure 2.13 C). No intracellular cytokine was detected when the T cells from PBMC from healthy donors were not stimulated (Figure 2.13 A) or when they were
stained with the appropriate dilution of the isotype control Ab after stimulation (Figure 12 B). This was true for all the cytokines studied.

A 1:100 dilution was chosen as the optimal concentration for IL-2, IL-4, TNFα and IFNγ Ab and their isotype matched controls. No IL-10 production by T cells was satisfactorily detected with any dilution at any time point (Figure 2.12).

![Image](image-url)

**Figure 2.13. Specific staining of anti-cytokine antibodies.** The detection of intracellular cytokines in a CD3+ live lymphocyte gate using ICS, was possible only after stimulation in the presence of monensin and staining with the appropriate anti-cytokine Ab. PBMC were stained extracellularly with anti-CD3 Ab and intracellularly with anti-IFNγ and IL-4 Ab or isotype matched control Ab without (A) or with (B and C) 6 hrs stimulation as described in Figure 2.7. All cells were incubated in the presence of monensin 3μM. The simultaneous expression of IL-4-Pe (Y axis) and IFNγ-FITC (X axis) in a CD3 gate is presented here in a quadrant dot plot. The quadrants were set first according to unstained cells and then according to isotype control Ab (shown in A). A) and C) Dot plot of anti IFNγ and IL-4 Ab staining B) Dot plot isotype matched control Ab staining.

### 2.8.2 Cytokine production by monocytes

As IL-10 could not be detected in stimulated T cells, IL-10 detection was assessed in lyopolyssacharide (LPS) stimulated monocytes (de Waal Malefyt, Abrams et al. 1991; Taimi, Defacque et al. 1993). PBMC from normal donors were purified and cultured in 6 well plates in complete medium to remove non-adherent cells. Total PBMC or adherent cells only were stimulated with 25μg/ml LPS. Cells were scraped with a cell scraper and adherent cells (monocytes) were stained extracellularly with anti-CD14 Ab and intracellularly with 1:100 dilution of anti-TNFα or anti-IL-10 or isotype control antibodies. TNFα was included as a positive control for monocyte activation and production of cytokine. Figure 2.14 shows the production of these cytokines by CD14+ cells in a macrophage-monocyte gate (Figure 2.14 panel A).
Whereas TNFα was detectable in CD14⁺, IL-10 was not detected (Figure 2.14 panel B). The cells were incubated for 17 hr and monensin was added the last 6 hr of incubation. IL-10 was not detected at this time point (Figure 2.15 A) whereas TNFα was detected (Figure 2.15 B). In conclusion, it was not possible to detect IL-10 in any of the PBMC derived from normal donors using this technique.

Figure 2.14. Time course of cytokine production by CD14⁺ monocytes. The frequency of TNFα and IL-10 producing cells in CD14⁺ monocytes was determined over time before and after stimulation with 25μg/ml LPS, as described in the text (Section 2.8.2). Monensin was added 6 hrs before harvesting. Staining for extracellular CD14 (1:50 dilution of FITC or Pe anti-CD14), intracellular TNFα and IL-10 or control Ab (1:100 dilution for all intracellular Ab) was performed as described in Figure 2.7. Intracellular staining was gated on the monocyte region (A) and in CD14⁺ cells. The results in B represent the frequency of TNFα expression (circles) and IL-10 (diamonds) above the control staining (no stained cells and isotype matched controls) during the time of stimulation.

Figure 2.15. Monocyte production of TNFα and IL-10 at 17 hrs of stimulation. PBMC from normal donors were purified and stimulated with 25 μg/ml LPS and cultured for 17 hrs. Monensin was added in the last 6 hrs of culture. Cells were stained as explained in Figure 2.14. Cytokine results are presented as the percentage of positive cells in the double positive (CD14⁺ plus cytokine) quadrant, gated in the
monocyte gate. A) IL-10 production by CD14⁺ monocytes (0.5%) and B) TNFα production by CD14⁺ monocytes (12.6%). 40,000 events were acquired.

2.8.3 Time courses of cytokine production.

Using the optimal anti-cytokine Ab dilution (1:100 as defined in Section 2.8), time courses of expression of each cytokine was performed. Cells from PBMC were stimulated with 5ng/ml PMA plus 1μM ionomycin as explained in Section 2.8 during a time course. For intracellular cytokine detection, monensin (final concentration 3μM) was added 4-6 hrs before harvesting. Cells were then stained with anti-CD3 plus anti-CD69 Ab, anti-CD3 plus anti-cytokine Ab or anti-CD3 plus the appropriate isotype matched control Ab. A minimum of 40,000 cells were then acquired and the frequency of cytokine producing T cells and the amount of fluorescence per cell (mean fluorescence intensity), were determined.

The expression of CD69, an early-activation marker, was up-regulated in CD3⁺ T cells at 6 hrs of stimulation and then its expression decreased (Figure 2.16). In CD3⁺ T cells, detection of all cytokines except IL-10 was optimal at 4-6 hrs. Then, 6 hrs was chosen as the optimal detection point for both frequency and MFI determination of intracellular production of IL-4, IL-2 and IFNγ.

![Figure 2.16. Time course of CD69 expression on T cells. PBMC were stimulated with PMA (5ng/ml) and ionomycin (1μM) and at different time points, the cells were harvested and stained extracellularly with anti-CD3 (Pe) plus anti CD69 (FITC) Ab and acquired (10,000 events). The results, gated on CD3⁺ lymphocytes, represent the frequency of CD69 positive cells (A) and the amount of CD69 expressed (MFI) (B) at different times (hrs).](image-url)
Figure 2.17. Time course of IL-2 expression in T cells. PBMC were stimulated as explained in Figure 2.16 and harvested at different time point to be stained extracellularly with anti-CD3 Ab(PerCp) and intracellularly with a 1:100 dilution of anti-IL-2 or an isotype control Ab (non shown) both FITC conjugated and acquired (40,000 events). 3 μM monensin was added for the last 6 hrs of stimulation before harvesting. The results, gated in CD3+ lymphocytes, represent the frequency of IL-2 positive cells (A) and the amount of IL-2 expressed per cell (MFI) (B) in time (hrs).

Figure 2.18. Time course of IFNγ expression in T cells. PBMC were stimulated as described in Figure 2.17 and stained extracellularly with anti-CD3 Ab(PerCp) and intracellularly with a 1:100 dilution of anti-IFNγ or an isotype control Ab (non shown) both FITC conjugated and acquired (40,000 events). The results, gated in CD3+ lymphocytes, represent the frequency of IFNγ positive cells (A) and the amount of IFNγ expressed per cell (MFI) (B) in time (hrs).
Figure 2.19. **Time course of IL-4 expression in T cells.** PBMC were stimulated as described in Figure 2.17 and stained extracellularly with anti-CD3 Ab(PerCp) and intracellularly with a 1:100 dilution of anti-IL-4 or an isotype control Ab both Pe conjugated and acquired. The results, gated in CD3⁺ lymphocytes, represent the frequency of IL-4 positive cells (A) and the amount of IL-4 expressed per cell (MFI) (B) in time (hrs).

Figure 2.20. **Time course of IL-10 expression in T cells.** PBMC were stimulated and stained extracellularly with anti-CD3 Ab (PerCp) and intracellularly with a 1:100 dilution of anti-IL-10 or an isotype control Ab (non shown) both Pe conjugated, and acquired as described in Figure 2.17. The results, gated in CD3⁺ lymphocytes, represent the frequency of IL-10 positive cells (A) and the amount of IL-10 expressed (MFI) (B) in time (hrs).

**ICS of fresh and frozen PBMC.**

The intracellular staining for IFNγ, IL-2, IL-4, IL-10 and TNFα was assessed in PBMC before and after freezing (freezing and thawing was performed as described in Section 2.6). PBMC from healthy donors were purified as described in Section
2.2.2. One fraction of the sample was frozen and the other fraction was stimulated for cytokine production with PMA plus ionomycin in the presence of monensin as described in Section 2.7.4. ICS was then performed on the fresh sample. Two days later, the frozen sample was thawed by taking it out of the liquid nitrogen, submerging in 37°C water bath until thawed and then washing it twice with serum-free medium. Cells were resuspended in complete medium and counted. Viability was always >90%. These PBMC were then stimulated and stained for the same cytokines as the fresh samples with ICS. The samples were then acquired and the frequencies of cytokine producing cells in the fresh and frozen samples were compared. The frequencies of IL-2, TNFα and IL-10 decreased after freezing, although as mentioned IL-10 was not consistently detected in all the samples.

All samples used in this work were subsequently used fresh except for those used in Chapter 7, where the PBMC were obtained from the A. Nolan panel of donors (Section 2.2.3).

Figure 2.21. The frequency of cytokine producing lymphocytes decreases after freezing. Purified PBMC were frozen in N₂ (as described in Section 2.6) for two days or used fresh for ICS. The samples were stimulated with PMA (5ng/ml) plus ionomycin (1µM) and ICS was performed as described in Section 2.7.4. The staining, stimulation and analysis was performed the same for both samples, with the same number of events acquired (40,000). The results represent the frequency of cells positive for each cytokine (IFNγ, IL-2, TNFα, IL-10 and IL-4) in a lymphocyte gate. The frequencies of IL-2, TNFα and IL-10 positive lymphocytes were reduced after freezing and thawing (cell viability was always >90% after thawing, assessed by
trypan blue exclusion as explained in Section 2.2.1). This figure is representative of two experiments performed with different PBMC.

2.9 Determination of frequencies of cytokine producing lymphocytes by enzyme linked immunospot (ELISPOT)

This assay was originally described to detect Ab secreting cells and adapted latter to detect antigen-secreting cells (McCutcheon, Wehner et al. 1997). Based on the enzyme linked immunosorbent assay (ELISA), the ELISPOT uses plate bound Ab to detect, with high sensitivity, cytokine secreted by antigen specific cells. Thus, the frequency of cytokine secreting cells can be determined.

For the IFNγ ELISPOT, anti-human-IFNγ Ab was diluted at 10μg/ml (determined as the optimal concentration by PhD C. Morte at the Anthony Nolan Research Institute) in sterile 0.1 M NaHCO3 pH 8.2 buffer (see Appendix Section10.1.5). This dilution was then aliquoted at 50μl/well in flat-bottomed 96-well sterile microtiter "capture" plate (NUNC Maxisorp, Denmark) for overnight immobilisation at 4°C.

The next day, the capture plate was blocked with 250 μl/well saline phosphate buffer (PBS) + 5% bovine serum albumin (BSA, Sigma, UK), at room temperature (RT) for 1 h. The plate was subsequently washed before adding 2×10^5 or 5×10^5 cells that had been re-stimulated 24 hrs with alloantigen in a MLC, or 1×10^5 cells from the MLC stimulated with PHA instead of alloantigen. The plate was then washed once with PBS, and twice with serum free-medium. The cells were then transferred from the stimulation plate to the capture plate (100 μl/well) and incubated undisturbed in a humidified atmosphere at 37°C and 5% CO₂ for 20 h.

The cells were then discarded and the plate was washed three times with PBS-Tween buffer (PBST). 100 μl of rabbit anti-human IFNγ polyclonal Ab (1:500 dilution in PBST + 1% BSA) was then added to each well and the plate was incubated for 2 h at RT. After this, the plate was washed three times with PBST and once with Tris-NaCl Buffer (TBST).

100 μl of alkaline phosphatase-conjugated mouse anti-rabbit polyclonal Ab (diluted at 1:2000 in TBST + 1% BSA) was then added to each well and the plate was incubated for 1.5 h at RT. The plate was washed three times with TBST and once with NaCl, MgCl, TRIS-alkaline phosphatase buffer (APB). 50 μl of fresh alkaline phosphatase substrate solution (3.2 μl of BCIP (Gibco) plus 4.4 μl NBT (Gibco) in 1 ml APB) was added/well and the plate was incubated at RT for 30 minutes. The reaction was stopped washing twice with water, the plate was air dried. All the
spots for each well were counted using the NIH image 1.57 Analysis Software for Mackintosh.

The positive control for IFNγ detection by ELISPOT consisted of 1X10⁵ responder cells stimulated overnight with a previously determined optimal dilution of PHA (see Appendix Section 10.2), for each responder:stimulator pair. The negative control for this assay were autologous MLC and irradiated stimulator PBMC on their own. Results for the ELISPOT are expressed as the frequency of IFNγ spots/plated cells.

2.10 Tissue typing

Tissue typing of the blood samples used in Chapter 7 was performed by serology and molecular typing. This was done at the Anthony Nolan Round Table Laboratories (serology and SSO and SSP typing) and by Dr M Perez-Rodriguez (RSCA), Anthony Nolan Research Institute.

Low resolution typing was requested to the Anthony Nolan Round Table Laboratories when broad mismatches between two PBMC were expected. Then, when samples from members of the staff or from buffy coats were randomly selected, class I typing by serology was done. SSO was done for medium resolution typing and SSP was used for high resolution typing. RSCA was used to type class I alleles at high resolution.

PBMC were used for serology typing, whereas DNA extracted from PBMC or from whole blood was used for molecular typing by SSO (Tiercy, Morel et al. 1991) SSP or RSCA. The methodology of this tissue typing techniques was revised in Chapter 1 Section 1.13.4.

2.11 DNA extraction for tissue typing

DNA for tissue typing was extracted from whole blood of PBMC using a DNA extraction kit (TCS biologicals Ltd, Buckingham, UK). Blood was placed in a 15 ml tube and red cells were lysed with red blood cell lysis solution, incubating 10 minutes at RT. Then, the tube was centrifuged for 10 minutes at 2000g to obtain a pellet of lymphocytes. These cells were lysed with cell lysis solution, pipetting up and down and incubating at 37°C if clumps were visible. 25 μl of RNA solution were added to clump-free samples, mixing by inverting the tube several times (25 according to the Kit manufacturer) and incubated at 37°C for 15-30 minutes. It was then cooled down at RT. Once cold, 1 ml of protein precipitation solution was added to the cell lysate and the tube was vortexed vigorously. The tube was then centrifuged at 2000g
for 10 minutes to separate DNA in suspension and protein, transferring the supernatant into a clean 15 ml tube with 3 ml 100% isopropanol. The tube was inverted until the white threads of DNA formed a visible clump and then centrifuged at 2000g for 3 minutes. The supernatant was discarded and the tube was drained on clean absorbent paper before adding 3 ml of 70% ethanol and inverting several times to centrifuge at 2000 g for 1 minute. The ethanol was then carefully poured off and the tube drained on clean absorbent paper to air-dry the sample for 15 minutes. Then, 250 μl of DNA hydration solution were added and the sample was left overnight at RT to re-hydrate.
3. Chapter 3. A high frequency of naïve CD3⁺CD45RA⁺ cells derived from CB is capable of IL-2 production.

3.1 Introduction.

IL-2 was originally described as a product of activated T cells that enhances thymocyte proliferation and supports the growth of T cell lines and is a major growth factor for T cells (Morgan, Ruscetti et al. 1976; Paetkau, Mills et al. 1976). It is produced by CD4⁺ and CD8⁺ T lymphocytes (Mosmann, Schumacher et al. 1991; Salgame, Abrams et al. 1991) and therefore acts in both autocrine and paracrine manner. The engagement of the TCR by CD3⁺ lymphocytes from peripheral blood results in T cell activation which, by four hours, results in production of IL-2, expression of the IL-2R and cell proliferation (Smith 1980). The optimal IL-2 mRNA expression and protein secretion occur when both CD3 and CD28 co-stimulation are provided (Fraser, Irving et al. 1991).

The production of IL-2 by naïve (CD45RA⁺) T cells within CB or the adult peripheral blood is lower than that of primed (CD45RO⁺) T cells in peripheral blood (Jung, Schauer et al. 1993). Thus, unfractioned CB T cells which contain a high proportion of CD45RA⁺ T cells, produce less IL-2 compared with adult cells which have a higher proportion of CD45RO⁺ cells and less CD45RA⁺ (Kawano, Noma et al. 1987; Gerli, Bertotto et al. 1989; Bertotto, Gerli et al. 1990; Watson, Oen et al. 1991; Kruse, Neustock et al. 1993; Takahashi, Imanishi et al. 1995). Nevertheless, some studies have shown that CB lymphocytes can produce levels of IL-2 comparable to those from adult cells under certain stimulation conditions (Hassan and Reen 1997).

In this Chapter, the ability of CD3⁺ CD45RA⁺ lymphocytes, derived from CB, to produce IL-2 was examined. To do this, both the frequency of IL-2 producing cells and the amount of cytokine made was analysed at the single cell level using the ICS technique. The frequency of IL-2 producing T cells at different developmental stages (naïve CD45RA⁺, from CB, memory CD45RO⁺ and resting T cells from PBMC) under different stimulation was compared.

The stimulus used included the most common mitogenic stimulation used for the ICS PMA and ionomycin (Appendix Section 10.6, Table 10.1), a T-cell restricted stimuli (plate-immobilised anti-CD3 plus anti-CD28 Ab (Groux, O'Garra et al. 1997)) and PMA plus anti-CD3 stimulation (Jung, Schauer et al. 1993; Assenmacher, Schmitz et al. 1994).
3.2 Materials and Methods

3.2.1 Cells

CB was obtained from normal and caesarean full-term deliveries by puncturing the umbilical vein (Dominguez, Madrigal et al. 1998). CD45RA+ T cells were obtained from CB by negative depletion using magnetic bead separation as described in Chapter 2, Section 2.3.1. Cells were washed and used immediately after depletion. CD45RO+ T cells were obtained from non-specific T cell lines which were driven with PHA (Chapter 2 Section 2.3.2). Cells were harvested, washed and used immediately. Resting T cells from PBMC were obtained from healthy donors as described in Chapter 2 Section 2.2.2. These cells were used immediately after purification.

3.2.2 Antibodies

Anti-CD3 antibody used to stimulate T cells (OKT3) was made in house as described in Appendix Section 10.3. Anti-CD28 Ab used to stimulate T cells was obtained from Pharmingen (Cambridge Biosciences, Cambridge, UK).

Anti-CD3-PercP, anti CD45RA FITC, anti-CD45RO Pe anti-CD69 Ab FITC were obtained from Becton Dickinson UK Ltd (Oxford, UK). Antibodies used to stain for intracellular IL-2 (anti-IL-2 and it isotype matched control) was obtained from Pharmingen (Cambridge Biosciences).

3.2.3 Stimulation of T cells and ICS

Fresh cells were stimulated with varying concentrations and combinations of immobilised anti-CD3Ab, anti-CD28 Ab, PMA and/or ionomycin. Antibodies were immobilised to plastic plates (as described in Chapter 2 Section 2.5). To measure frequency of IL-2 production, the stimulation was performed for 6 hrs in the presence of 3 µM monensin. The cells response to the different stimulations was measured as cytokine production (by ICS), proliferation (Chapter 2 Section 2.4), and expression of activation markers.

3.2.4 Flow cytometry.

After stimulation, cells were harvested from the plates, washed stained for extracellular antigens (CD3, CD45RA, CD45RO, CD69) as described in Chapter 2 Section 2.7.1. For detection of intracellular cytokines, cells were fixed, permeabilised and stained intracellularly with anti-IL-2 or isotype control Ab (as described in Chapter 2 Section
2.7.5). Cells were analysed within 48 hours of staining by a FACScan flow cytometer (Becton Dickinson) after acquiring >20000 cells. Results are expressed as the percentages of positive cells within the CD3⁺ gate and the median fluorescence intensity.
3.3 Results

3.3.1 Phenotype of resting, memory and naïve T cells

Figure 3.1 shows typical profiles of the three populations of cells used in this study. Resting T cells were T cells within a PBMC microenvironment. In the flow cytometry analysis, the lymphocyte gates contained 65.7-86.0% CD3+ cells, which were a mixture of CD45RA+ and CD45RO+ cells. Memory T cells (CD3+ CD45RO+) had been activated in vitro for at least 3 rounds of mitogenic stimulation and were always >95.0% CD3+CD45RO+ and >60.0% CD4+. Pure naïve T cells, obtained by negative depletion were always 95.0% CD45RA+ within the CD3+ gate. The percentages of double positive CD45RA+ CD45RO+ cells in a CD3 gate varied between T cell populations. In CB before purification, the percentage of CD45RA+ CD45RO+ double positive T cells ranged between 0.1-7% (mean 3.2 %), whereas in resting T cells, 6.0-7.8% were double positive (mean 6.8%). No double positive cells were seen in the T cell lines.

3.3.2 IL-2 production by cells at different stages of differentiation.

Since PMA plus ionomycin is a stimulus commonly used for ICS to stimulate PBMC (Appendix Section 10.6), this population and stimulation protocol were used as positive control for cytokine detection.

The frequencies of IL-2 producing CD45RO+ T cells after stimulation.

The CD45RO+ T cells had the highest frequency of IL-2 producing cells with all stimulations. Stimulation through CD3 and CD28 induced 10.0% of this population to produce detectable levels of IL-2 (Figure 3.2 panel A). The addition of CD28 did not significantly increase the anti-CD3 induced production of IL-2. The frequency of IL-2 producing cells was increased however, with addition of PMA to anti-CD3 stimulation (reaching levels above 10.0%) (Figure 3.2 panel B), although no large differences were noted between different the concentrations of PMA used (0.5-50ng/ml). As little as 0.5 ng/ml PMA had the same effect as 50ng/ml on the differentiated T cells. PMA and ionomycin induced the production of IL-2 by the largest number of cells when provided simultaneously. Indeed even the lowest concentration of ionomycin used (0.1µM) plus the lowest concentration of PMA used (0.5 ng/ml) resulted in a significant detection (>30.0%) of IL-2 producing T cells (Figure 3.2 Panel C). 50ng/ml PMA plus 0.1-1µM of ionomycin induced the highest percentages of IL-2 producing cells in this population.
Figure 3.1. Typical phenotype of memory, resting and naïve T cells. The CD4⁺, CD8⁺, CD45RA⁺ and CD45RO⁺ phenotype of CD3⁺ cells from memory T cells (CD3⁺CD45RO⁺), resting T cells, and naïve T cells (CD3⁺CD45RA⁺) is shown. The naïve T cells were analysed pre-cell depletion and after enrichment for CD45RA⁺ CD3⁺ cells by negative depletion of monocyte/macrophages (CD14⁺), natural killer cells (CD16⁺, CD56⁺), B cells (CD19⁺) and memory cells (CD45RO⁺), using magnetic beads (see Chapter 2 Section 2.3.1). The phenotype of the cell populations was analysed by flow cytometry and results are expressed as the percentage of CD3⁺ cells in the total population (CD3⁺) or the percentage of CD4⁺, CD8⁺ CD45RA⁺ and CD45RO⁺ in the CD3 gated populations (CD3 gated). This figure is representative of three experiments.
Figure 3.2. Frequencies of IL-2 producing memory (CD45RO+) cells after stimulation. Memory T cells were stimulated for 6 hours with varying concentrations of A) immobilised anti-CD3 Ab ± immobilised anti-CD28 Ab, B) immobilised anti-CD3 Ab ± soluble PMA or C) PMA ± ionomycin, as described in Chapter 2 Section 2.5. All stimulation's were performed in the presence of monensin. Cells were then harvested and stained extracellularly for CD3 and intracellularly for IL-2 with and anti-IL-2 Ab or an isotype matched control Ab (not shown) and analysed by flow cytometry, as described in materials and methods. All results were gated in a live lymphocyte CD3+ gate as described in Chapter 2 Section 2.7.5. The horizontal bars were set on the negative staining of both unstained cells and a IL-2 isotype matched control Ab for each stimulus dilution. Results above the horizontal bar of each profile show the percentage of double positive (CD3+/IL-2+) cells in the CD3+ gate. Where no value is given the percentage is 0.0%. These figures show one representative experiment of two.
The frequencies of IL-2 producing resting T cells after stimulation.

Only low frequencies of IL-2 producing CD3+ cells in the resting population (<2.0%) were detected with anti-CD3 plus anti-CD28 Ab (Figure 3.3 panel A) in all the experiments performed, in spite of the variation between samples.

A similar lack of IL-2 production was seen in stimulation with anti-CD3 Ab and PMA stimulation. Although the frequency of IL-2 producing cells was low (<2.0%), the flow cytometry profiles suggested that at these concentrations of stimulation there was a response (Figure 3.3 panel B). The optimal stimulation was PMA and ionomycin for resting T cells. Thus, the highest frequencies for IL-2 producing cells in the resting population where seen with 50ng/ml PMA and 1 μM ionomycin (Figure 3.3 panel C).

The frequencies of IL-2 producing CD45RA+ cells after stimulation.

T cell restricted stimulation (anti-CD3 ± anti CD28) of naïve CD45RA+ T cells produced no IL-2 (Figure 3.4 panel A). This behaviour was observed in all experiments and IL-2 was not detected even at the highest concentration of antibodies used (200μg/ml anti-CD3 plus 30 μg/ml anti-CD28). Addition of PMA to CD3 mediated stimulation did not increase the frequency of IL-2+ naïve T cells (Figure 3.4 panel B) and no more than 0.2% of these cells produced IL-2, even at the highest concentrations of PMA and anti-CD3. Notably, PMA plus ionomycin stimulation of the naïve CD3+CD45RA+ population did produced easily detectable IL-2 producing cells (Figure 3.4 panel C). However, this was only seen with the addition of ionomycin (0.1-10 μM) when a high concentration of PMA (50ng/ml) was used.

3.3.3 CD45RA+ T cells derived from CB require strong stimulation with PMA to produce IL-2 at high frequency.

Memory cells produced IL-2 with all stimulations used, whereas resting cells produced IL-2 at high frequencies with different concentrations of PMA and ionomycin. However, naïve T cells from CB only produced IL-2 when ionomycin plus 50 ng/ml PMA was used to stimulate (Figure 3.5), which indicates a strong requirement for PKC stimulation. Once reached, the stimulated cells can produce IL-2 at high frequency.
Figure 3.3. Frequencies of IL-2 producing resting T cells after stimulation. Resting PBMC from healthy donors were obtained by Ficoll Hypaque purification as described in Chapter 2 Section 2.2.2. The cells were stimulated for 6 hours with varying concentration of A) immobilised anti-CD3 Ab ± immobilised anti-CD28 Ab, B) immobilised anti-CD3 Ab ± soluble PMA or C) PMA ± ionomycin, as described Figure 3.2. All stimulation's were performed in the presence of monensin. Cells were then stained extracellularly for CD3 and intracellularly for IL-2 and analysed by flow cytometry, as described in Figure 3.2. These figures show one representative experiment of three.
Figure 3.4. Frequencies of IL-2 producing CD45RA+ cells after stimulation. Pure naïve (CD45RA+) T cells were obtained by magnetic separation from CB cells as explained in Chapter 2 Section 2.3.1, and stimulated for 6 hours with varying concentration of A) immobilised anti-CD3 Ab ± immobilised anti-CD28 Ab, B) immobilised anti-CD3 Ab ± soluble PMA or C) PMA ± ionomycin, as described Figure 3.2. All stimulations were performed in the presence of monensin. The cells were then stained extracellularly for CD3 and intracellularly for IL-2 and analysed by flow cytometry, as described in Figure 3.2. These figures show one representative experiment of three.
Comparison of different stimulations for intracellular detection of IL-2.

Whereas anti-CD3 plus anti-CD28 Ab induced IL-2 production only in the CD45RO+ T cells in a dose dependant manner (Table 3.1 panel A), PMA plus ionomycin produced the highest levels of IL-2 producing CD3+ cells (Table 3.1 panel C). Optimal concentrations for anti-CD3 and PMA varied between the cells types being analysed (Table 3.1 panel B). The naïve CD3+CD45RA+ population had the highest threshold of stimulation, and only 50ng/ml PMA plus 1μM (or more) ionomycin stimulation, induced a frequency of IL-2 producing cells in the naïve population of 37.2% in one experiment (Table 3.1 panel C).

Whereas anti-CD3 plus anti-CD28 stimulation only enabled the detection of IL-2 producing CD45RO+ T cells (Table 3.1 panel A), anti-CD3 plus PMA stimulation induced detection of IL-2 producing cells in the memory and resting T cells (Table 3.1 panel B). In contrast, PMA plus ionomycin induced detection of IL-2 producing cells in all populations of T cells: from the PBMC environment, naïve and memory T cells (Table 3.1 panel C). As expected the CD45RO+ T cells had the highest frequency of IL-2 producing cells whereas CD45RA+ T cells achieve a high frequency of IL-2 producing cells with PMA plus ionomycin stimulation.

Panel A

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<th>T cell population</th>
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<th>CD28 (μg/ml)</th>
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</thead>
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<td>Resting</td>
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<td>30.0</td>
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<tr>
<td>CD45RA+</td>
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Panel B

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<th>Frequency (%)</th>
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<th>PMA (ng/ml)</th>
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<tr>
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<td>-----</td>
<td>3.4.A</td>
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</table>

Panel C

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<th>Ionomycin (μM)</th>
<th>Figure</th>
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<td>50.0</td>
<td>1.0</td>
<td>3.4.C</td>
</tr>
</tbody>
</table>

Table 3.1. Maximum frequency of IL-2 produced in different cell populations. Different stimulations induced different frequencies of IL-2 in the cell populations analysed. Here, the frequency of IL-2 with optimal stimulation from Figures 3.2, 3.3 and 3.4 achieved on anti-CD3 plus anti-CD28 (panel A), anti CD3 plus anti-CD28 (panel B) and PMA plus ionomycin (panel C) is shown.
3.3.4 Inter-sample variation.

In spite of a tendency of a dose response in the production of IL-2 in all the populations of T cells studied here, there was variability in this response (Figure 3.6). IL-2 production was not always detected in the analysed samples, even at the highest concentration of PMA (50ng/ml) or ionomycin (10μM) as Figure 3.6 shows. Stimulation with 50ng/ml PMA and 1μM ionomycin is shown in this figure because it was optimal for induction of detectable IL-2 in all cell types. All samples were viable (assessed by trypan blue exclusion before the experiment and proliferation to anti-CD3 plus anti-CD28 Ab, see Section 3.3.5). Whereas CD45RO+ cells always had the highest frequencies of IL-2 producing cells, the CD45RA+ T cells had frequencies comparable with those from the resting population of T lymphocytes.
Figure 3.5. Naïve (CD45RA+) cells are strictly dependent on ionomycin plus high doses of PMA to produce IL-2. Memory, resting and pure naïve T cells were obtained and stimulated as described in Figures 3.2, 3.3 and 3.5. All stimulations were performed in the presence of monensin. The cells were then stained extracellularly for CD3 and intracellularly for IL-2 and analysed by flow cytometry, as described in Figure 3.2. The results are represented as the frequency of IL-2 production by all the T cells populations with all the stimulations. PMA and ionomycin was the only stimulation that induced detectable IL-2 production in all populations.
3.3.5 CD45RA+ T cells derived from cord blood produce low levels of IL-2 per cell.

In spite of the ability of a large proportion of naïve cells to produce IL-2 with high concentration of PMA and ionomycin, the amount of IL-2 produced by these cells (expressed as the mean fluorescence intensity, MFI) was always lower that the amount of IL-2 produced by memory or resting T cells (Figure 3.7). This was seen in all the experiments and was independent of the frequency of IL-2+ cells. When the naïve T cells made this cytokine, the frequency of cells which made the cytokine could be equal to that of adult cells, whereas the level of cytokines produced was always lower (Figure 3.7). Thus, the naïveté of CD45RA+CD3+ cells was observed only in the reduced ability of each cell to produce the IL-2. These results show that, under appropriate stimulation, a high proportion of naïve T cells can produce IL-2. However, the amount of IL-2 produced by cell in the CD45RA+CD3+ population is lower compared with the amount of IL-2 produced by cells from other T cell populations.

3.3.6 Proliferative response of resting, memory and naïve T cells.

To determine whether the T cell populations were responding appropriately to the specific T cell stimulation used (anti-CD3 plus anti-CD28 Ab), proliferation assays for each experiment were performed (Figure 3.8).

Resting T cells within the PBMC microenvironment and the memory CD45RO+ T cells proliferated with increasing concentrations of immobilised anti-CD3 Ab (Figure 3.8). Immobilised anti-CD28 monoclonal Ab enhanced the anti-CD3 induced response, but this Ab did not cause a proliferative response alone. The naïve cells (CD3+CD45RA+) could not be induced to proliferate with the anti-CD3 monoclonal Ab alone, even at high concentrations (200μg/ml), whereas co-stimulation with anti-CD28 (30μg/ml) monoclonal Ab enabled the cells to proliferate, as reported in the literature (Koulova, Yang et al. 1990; Dubey, Croft et al. 1996; Yashiro, Tai et al. 1998).
Figure 3.6. Inter-sample variation of the frequency of IL-2 producing T cells. Variation in the frequencies of IL-2 producing populations between samples of T cells belonging to the same populations (naive, resting and memory) are shown. Cells were stimulated for 6 hrs with 50ng/ml PMA plus 1μM ionomycin and stained for IL-2 as described in Figure 3.2. The results are expressed as the frequency of IL-2 producing cells per population.
Figure 3.7. Amount of IL-2 produced per cell. Cells from memory (CD45RO+), resting (PBMC) and naive (CD45RA+) populations were stimulated for 6 hrs with PMA (0, 0.5, 5 and 50 ng/ml) and ionomycin (0, 0.1, 1 and 1μM), in the presence of monensin, stained for CD3 (extracellularly) and IL-2 (intracellularly) and acquired in a flow cytometer as explained in Figure 3.2. The results are expressed as the amount of IL-2 produced per cell (mean fluorescence intensity, MFI) for the different populations of T cells. This figure is representative of all the experiments performed.
Stimulation via CD3 and CD28 mediates IL-2 secretion and subsequent cell proliferation (June, Ledbetter et al. 1987; Turka, Ledbetter et al. 1990) although IL-2 independent T cell proliferation has also been reported (Tuosto and Acuto 1998). Thus, although by ICS the production of IL-2 was only detected in the memory T cells (Figure 3.2), the ability of all three cell types to proliferate to anti-CD3 and anti-CD28 Ab suggested that this stimulation induced IL-2 production.

3.3.7 Proliferation of fresh PBMC to anti-CD3 plus CD28 ± anti IL-2 Ab

To verify that the anti-CD3 plus anti-CD28 stimulation enabled IL-2 production, anti-IL-2 neutralising Ab was added to a T cell proliferation assay in which cells had been stimulated with these antibodies (Figure 3.9). Stimulation of PBMC with anti-CD3 and anti-CD28 gave a proliferative response of greater than 100,000 cpm. This proliferation was significantly (P<0.02) reduced with the addition of anti-IL-2 neutralising Ab, but not an isotype matched control. Thus resting T cells were making IL-2 with anti-CD3 plus anti-CD28 stimulation, but not at sufficient levels to be detected using ICS. Thus, stimulation via the CD3 and CD28 antigens induced proliferation mediated partially by IL-2 production in peripheral T cells. However, using ICS, this cytokine was not detectable with this stimulation.

3.3.8 Only activated T cells produce IL-2.

The low detection of IL-2 in all T cell populations with anti-CD3 plus anti-CD28 and anti-CD3 plus PMA, suggested a poor activation of the cells. To test this, the activation in CD3⁺ cells from PBMC (a mixed population of CD45RA⁺ and CD45RO⁺ T cells), was measured by the expression of CD69, an early marker of stimulation in T cells (López-Cabrera, Santis et al. 1993).

IL-2 was only produced by activated CD69⁺ cells in the CD3 gate. Thus, T cells without stimulus (Figure 3.10 panel A) or stimulated with CD3 plus CD28 (Figure 3.10 panel B), had a low CD69 and IL-2 expression (<0.5% T cells were IL-2⁺ in this experiment). Despite the CD69 expression induced by CD3 plus PMA, this stimulation was not strong enough to induce IL-2 production in the stimulated population (Figure 3.10 panel C). Finally, the addition of ionomycin to PMA induced detectable IL-2 production in the CD69⁺CD3⁺ cells (22.5%) (Figure 3.10 panel D). These results indicated that only activated CD69⁺CD3⁺ cells are able to produce IL-2 and that PMA and ionomycin is the only stimulation able to induce detectable IL-2 production in resting T cells from peripheral blood cells. Thus, CD3 plus CD28 stimulation induced activation in a low
frequency of T cells measured as CD69 expression (3.5%) but the stimulus was not strong enough to induce production of IL-2 at detectable levels by ICS.
Figure 3.8. Proliferative response of memory, resting and naïve CD3+ T cells. Three different cell populations, memory T cells (CD45RO+), resting T (PBMC) cells within a peripheral blood mononuclear cells environment and naïve T cells (CD45RA+) cells were plated in 96-flat bottom well plates, previously coated with varying dilutions of anti-CD3 Ab and anti-CD28 Ab. Cells were incubated for three days in a humidified atmosphere at 37°C and CO₂. 1 μCi/well [³H] thymidine/well was added 18 hours before harvesting the wells on glass-fibre filters and radioactivity was counted with a liquid scintillation counter as explained in Chapter 2 Section 2.4. Results are expressed as the mean of triplicate counts per minute (cpm) ± SD. This figure is representative of three experiments.
**Figure 3.9.** IL-2 is required for the proliferation of resting T cells. Resting T (CD3⁻) cells were plated in 96-flat bottom well plates, previously coated with 50μg/ml anti-CD3 Ab plus 10μg/ml soluble anti-CD28 Ab and with or without 0.01, 0.1 and 1 μg/ml of anti-IL-2 neutralising Ab or an isotype matched control Ab. Cells were incubated for three days in a humidified atmosphere at 37°C and CO₂. Radioactivity was added and the cells were harvested as described in Figure 3.8. Results are expressed as mean of triplicate counts per minute (cpm) ± SD. *P<0.02 by 2 tailed Student's t-test compared to no Ab. Here, the inhibition of the proliferation is shown only for the anti-IL-2 neutralising Ab and control Ab at 1 μg/ml, because lower concentrations of neutralising Ab (0.01 and 0.1 μg/ml) did not have an inhibitory effect in the proliferation.
Figure 3.10. Frequencies of activated (CD69 expressing) cells producing IL-2. PBMC from healthy donors were left unstimulated (A) or were stimulated with (B) plate immobilised anti-CD3 Ab (50μg/ml) plus anti-CD28 Ab (10μg/ml), (C) anti-CD3 Ab plus PMA (50ng/ml) or (D) PMA and ionomycin (1μM). The cells were then harvested, washed and stained extracellularly for CD3-PerCp and CD69 FITC, and intracellularly with anti-IL-2 or an isotype control Ab (not shown). The cells were then acquired in a flow cytometer and analysed as explained in Figure 3.2. The quadrants were set according to the negative controls of staining: non-stained cells and cells stained with the isotype control Ab for IL-2 staining (not shown) and with anti-CD69 Ab, in a CD3⁺ gate. The numbers in the boxes represent the frequencies of CD69 and IL-2 positive cells in the quadrants.
3.3.9 Analysis of the frequency of cytokine producing memory T cells within the resting PBMC environment

To determine whether the CD45RO+ T cells within the PBMC environment had the same response as the activated memory T cells within a T cell line, PBMC were stimulated with varying dilution's of different combinations of PMA, ionomycin, anti-CD3 Ab and anti-CD28 Ab. Cells were then stained extracellularly with anti-CD3 plus anti-CD45RO and intracellularly with anti-IL-2 or isotype matched control Ab. A gate on live CD3'CD45RO' cells was used to determine the frequency of IL-2 producing cells in the memory population. The pattern of response obtained with the resting CD45RO+ cells was very similar to that of the activated CD45RO+ cells from the other experiments. Figure 3.10 shows than less than 3.0% IL-2 producing cells were detected with anti-CD3 and anti-CD28 Ab stimulation, whereas with 5ng/ml and 50μg/ml anti-CD3 Ab stimulation (but not less) there was a increase in the frequency of IL-2 producing CD3'CD45RO' cells (6.1%). Similarly, increasing concentrations of ionomycin with 5ng/ml PMA induced higher frequencies of IL-2 producing memory T cells from resting populations. Thus, only PMA and ionomycin induced the detectable production of IL-2 by a high frequency of memory T cells within the periphery.
Figure 3.11. The frequency of IL-2 producing CD45RO⁺ T cells within a PBMC microenvironment. PBMC were stimulated for 6 hours with A) 50μg/ml immobilised anti-CD3 Ab ± soluble anti-CD28 Ab, B) 50μg/ml immobilised anti-CD3 Ab ± soluble PMA or C) 50 ng/ml PMA ± ionomycin. All stimulation’s were performed in the presence of monensin. Cells were then stained extracellularly for CD3 and CD45RO then intracellularly for IL-2 and analysed by flow cytometry, as described in Figure 3.2. Quadrants were set on both isotype matched controls and unstained cells. Results in the top right hand corner of each profile show the percentage of double positive (CD3⁺/IL-2⁺) cells in the CD45RO⁺ gate. Where no value is given the percentage is 0.0%.
3.4 Discussion and conclusions

The sources of IL-2 are Th1 and Tc1 cells (Mosmann, Schumacher et al. 1991; Salgame, Abrams et al. 1991) and those T cells not yet committed to a type 1 phenotype (Tp) (Sad and Mosmann 1994). In periphery, IL-2 is produced mainly by primed (CD45RO⁺) T cells (Jung, Schauer et al. 1993; Woodside, Long et al. 1999), whereas the IL-2 production by non primed T cells naïve (CD45RA⁺) from peripheral blood, is low compared to CD45RO⁺ cells (Akbar, Salmon et al. 1991; Ferrer, Plaza et al. 1992). CD45RA⁺ T cells are considered naïve because their cytokine production and proliferative response to Ag is low compared with primed cells (Merkenschlager, Terry et al. 1988; Salmon, Kitas et al. 1989).

Because lymphocytes from CB are 80-90.0% CD45RA⁺ (Rabian-Herzog, Lesage et al. 1992; D'Areana, Musto et al. 1998), the cells obtained from CB are considered naïve. CB lymphocytes secrete less IL-2 that adult peripheral cells upon stimulation with alloantigens (Kawano, Noma et al. 1987), mitogens (Watson, Oen et al. 1991) and superantigens (Kruse, Neustock et al. 1993; Takahashi, Imanishi et al. 1995). In addition, CB lymphocytes have a poor proliferative capacity on mitogenic stimulation with anti-CD3, anti-CD28 or anti-CD2 Ab alone or in combination (Gerli, Bertotto et al. 1989; Bertotto, Gerli et al. 1990; Hassan and Reen 1996). The low responsiveness of these cells may be due to the high proportion of CD45RA⁺ cells. To explain the lower response to stimulus observed in lymphocytes from CB, they have been described as "defective" (von Freeden, Zessack et al. 1991; Pirene-Ansart, Paillard et al. 1995; Chalmers, Janossy et al. 1998; Miscia, Di Baldassarre et al. 1999; Schultz, Rott et al. 1999).

However, some reports have shown that the immune responsiveness of CB cells is not defective. Indeed, the proliferation (Uchiyama, Kamagata et al. 1987; Gerli, Agea et al. 1993), IL-2 transcription (Lewis, Yu et al. 1991), IL-2 production (Miyawaki, Seki et al. 1985; Saito, Saito et al. 1988; Anderson, Anderson et al. 1990; Bertotto, Gerli et al. 1990; Lewis, Yu et al. 1991; Kruse, Rink et al. 1992; Pirene-Ansart, Paillard et al. 1995; Hassan and Reen 1997; Sautois, Fillet et al. 1997; Ito, Koide et al. 1998), IL-2 kinetics of production and bioactivity of CB derived IL-2 (Bessler, Sirota et al. 1993) can reach similar or higher levels that those reached by adult cells under certain stimulation. Furthermore, the frequency of CD4⁺CD45RA⁺ cells producing IL-2 and the amount of IL-2 produced by cells is not significantly different in CB and adult CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ cells (Anderson, Anderson et al. 1990; Chipeta, Komada et al. 1998).
In this Chapter the capacity of IL-2 production by naïve CD45RA⁺ T cells derived from umbilical CB was compared with the ability of control populations of T cells at other developmental stages (pure memory CD45RO⁺ T cells and resting T cells from peripheral blood). The production of IL-2 in all three populations was induced with a variety of stimulus.

A CD3⁺ gate was used in this study to determine the frequency of IL-2 producing T lymphocytes. There were four reasons for this. Firstly, although the CD4⁺ cells are an important source of IL-2, CD8⁺ cells also produce IL-2 detectable by intracellular staining (Picker, Singh et al. 1995). Therefore, by gating on the CD3⁺ lymphocyte population, the total IL-2 production was measured because it included the CD4⁺ and CD8⁺ contribution (Jason and Larned 1997). Secondly, CD3 is expressed on CB cells at the same levels as adult cells (Deacock, Schwarer et al. 1992) which enables a direct comparison between the CD3⁺ populations at different developmental stages. Thirdly, adequate detection of the CD3⁺ CD4⁺ population is not possible when the cells are cultured in PMA, since it reduces CD4 expression on T cells, as shown in Chapter 2 Section 2.7.3 (Anderson and Coleclough 1983). In contrast, the expression of the CD3 antigen is not reduced significantly with PMA (Anderson and Coleclough 1983). Finally CD3 staining can exclude NK cells from a lymphocyte gate with no further staining.

The data in this Chapter indicates that a high frequency of CD3⁺CD45RA⁺ from CB can produce IL-2 once the stimulation threshold had been reached. This was achieved using 50ng/ml PMA and 1μM ionomycin. Indeed, the frequencies of IL-2 producing cells in the CD3⁺CD45RA⁺ population could reach or exceed the frequency of IL-2⁺ resting T cells (Figure 3.6). This supports the notion that CD45RA⁺ T cells from CB are not defective in their capacity of IL-2 production compared with other subsets of T cells from other developmental stages. Nevertheless, the level of IL-2 produced by the naïve cells, expressed as MFI, was always lower compared with the level of IL-2 produced by memory and resting T cells (Figure 3.5). Thus, in spite of the capacity of a large proportion of cells from the naïve population to produce IL-2 under PMA and ionomycin stimulation, the amount of IL-2 produced per cell was low. Therefore, a culture of naïve cells from CB can secrete high amounts of IL-2 if a high proportion of these cells produce IL-2 (even low amounts per cell). Thus, under CD2 plus CD28 stimulation, CB lymphocytes as well as the pure CD4⁺CD45RA⁺ fraction of cells from CB secrete less IL-2 that their adult counterparts (Hassan and Reen 1996; Hassan and Reen 1997), but when CD4⁺CD45RA⁺ cells from CB and adult received 50ng/ml PMA as additional stimulation, both populations secreted similar levels of IL-2 (Hassan and Reen 1997). In comparison with the results from this Chapter, Hassan et al stimulate the cells via the CD2 and CD28 co-stimulatory molecules, and did not include stimulation
via the TCR/CD3 complex that is known to effectively induce IL-2 production, (Wacholtz and Lipsky 1993). The most effective stimulation to induce IL-2 production in resting and in CD45RA+ T cells from periphery is CD3 plus CD28 (Yashiro, Tai et al. 1998; Woodside, Long et al. 1999) because CD28 stimulation enhances PKC stimulation (Van Lier, Brouwer et al. 1991). Thus, the stimulation used by Hassan et al was suboptimal, but when optimal stimulation (50 ng/ml PMA) was included, the CD4+CD45RA+ cells from CB produced as much IL-2 as the CD4+CD45RA+ cells from adult blood. These results therefore suggest that with certain stimulation, CB derived naïve cells have the capacity to produce equal amount of IL-2 as their adult counterparts. However, the capacity of IL-2 secretion by CB mononuclear cells and PBMC or purified CD3+CD45RA+ cells from both compartments under a gradient of stimulation, to ascertain the stimulation threshold for IL-2 production secretion by both populations of cells, has not been determined in the literature.

The results from this Chapter contradict studies that reported that the frequency of CB mononuclear cells and CD4+CD45RA+ producing IL-2 under PMA plus ionomycin stimulation is lower compared with their adult counterparts, estimated by ICS (Chalmers, Janossy et al. 1998; Schultz, Rott et al. 1999). Whereas Schultz et al found reduced levels of IL-2 produced by CB CD3+ cells compared with adult CD3+ cells, Chalmers et al showed that, CD4+CD45RA+ lymphocytes from CB produced similar levels of IL-2 compared with adult CD4+CD45RA+ cells and only the CD8+ cellular fraction from CB produced IL-2 at lower frequencies than CD8+ cells from adult. The conclusion reached by Chalmers et al was that CB cells are defective in their cytokine production, implying that this may be the reason for a lower incidence and severity of GvHD seen using cells from CB (Chalmers, Janossy et al. 1998). In the study of Chalmers et al, only one concentration of PMA and ionomycin (5 ng/ml and 1 μM respectively) was used, whereas it has been shown here that a minimum of 50 ng/ml of PMA plus 1 μM ionomycin is needed to induced IL-2 production by a large proportion of CD45RA+ T cells from CB. The finding of a higher stimulation threshold in CD45RA+ cells derived from CB is been supported by other studies: Chipeta et al (Chipeta, Komeda et al. 1999) found similar frequencies of IL-2 producing CD4+CD45RA+ and CD8+CD45RA+ cells from CB and adult blood, using 30 ng/ml PMA and 1 μM ionomycin. Chipeta et al also found that CB and adult derived cells produce the same amount of IL-2 per cell. The concentration used by Chipeta et al may be in the critical range, as other study used 28 ng/ml PMA, contradictory founding that only the frequency of CD8+CD45RA+ IL-2 producing cells from CB and adult (and not in CD4+CD45RA+ cells) is lower and statistically significant in cells from CB compared with cells from adult blood (Krampera, Tavecchia et al. 2000).
It has been recently reported that allogeneic stimulation can optimally activate lymphocytes from CB (Trivedi, HayGlass et al. 1997; Matthews, Wadhwa et al. 2000). This stimulation induced higher levels of proliferation in CB lymphocytes compared with lymphocytes from adult blood (Matthews, Wadhwa et al. 2000). Furthermore, the proliferating lymphocytes from CB had higher frequencies of IL-2, TNFα and IFNγ, producing cells compared with proliferating cells from adult, upon 50ng/ml PMA and 0.5 μM ionomycin stimulation (Matthews, Wadhwa et al. 2000).

The recent results from Matthews et al strengthen the concept that naïve cells are not functionally defective but are able to secrete cytokines (such as IL-2) once their stimulation threshold has been reached. Other studies which have focused on T lymphocytes from neonates have provided similar results. Naïve cells from the periphery of neonatal mice stimulated through CD3 have a lower proliferative capacity and IL-2 production compared with cells from adult mice. However, addition of PMA and ionomycin induced neonatal and adult T cells to proliferate and produce IL-2 to comparable levels, stimulating similar functional responses in freshly isolated T cells from neonates and adults (Adkins and Hamilton 1992). A substantial increase in the IL-2 produced by naïve T cells from transgenic mice can be induced upon stronger signalling induced with APL with stronger affinity for the MHC (Manickasingham, Anderton et al. 1998), and optimal co-stimulation can lower the threshold of stimulation in CB T cells (Borrione, Peola et al. 1999).

The data of this Chapter also indicates that the only stimulation that could induce a high frequency of IL-2 producing cells in the naïve population of T cells PMA was plus ionomycin. In comparison, anti-CD3 plus PMA stimulation only allowed significant detection of IL-2 producing cells in memory CD45RO+ T cells from a T cell line (19.0%) and CD45RO+ T cells within a PBMC environment (2.9%) (Figure 3.2 and 3.11). Naïve T cells from CB had the lowest frequency in response to anti-CD3 plus PMA stimulation (Figure 3.4). Regardless of the ability of PMA to stimulate the cells and up-regulate CD69 expression (Borrego, Peña et al. 1993; D'Ambrosio, Trotta et al. 1993), (Figure 3.10 Panel C) IL-2 was not detected in resting T cells stimulated with anti-CD3 plus PMA (Figure 3.10 panel C) and only the addition of ionomycin induced these cells to produce enough IL-2 to be detected (Figure 3.10 panel D). It seems likely that the low frequency of IL-2 producing T cells in the resting PBMC population after stimulation with anti-CD3 Ab plus PMA was due to the high levels of CD45RA+ T cells in peripheral blood. Indeed, CD45RO+ from the resting population of T cells had a dose response similar to CD45RO+ T cells in their IL-2 production, as can be seen by comparing Figures 3.3 and 3.11.
T cell stimulation by cross linking the CD3 and CD28 antigens (Harding, McArthur et al. 1992) was included in this Chapter, to stimulate the cells in a more physiologic manner, as co-stimulation is required for optimal T cell responses (Harding, McArthur et al. 1992). Plate immobilised anti-CD3 and anti-CD28 Ab was sufficient to induce T cell proliferation in all states of T cell differentiation studied here. However, this stimulation did not enable the detection of IL-2 by ICS in naïve cells (Figure 3.5) and only induced a low frequency of IL-2+ resting and memory T cells (Figures 3.2 and 3.4). Nonetheless, stimulation with anti-CD3 and anti-CD28 Ab has been reported to induce cytokine production detectable by ICS in T cell clones and in CD45RO+ T cells from periphery (Groux, O’Garra et al. 1997; Woodside, Long et al. 1999). The frequency of IL-2 producing cells was not shown in the study of Groux et al and the efficiency of their stimulation is uncertain. Woodside et al (Woodside, Long et al. 1999) stimulated the T cells in a different way from this Chapter, as the authors used Ab immobilised to petridishes. In Woodside’s work, a gated on CD45RO+ T cells was used, where 32.0% of IL-2 producing cells was detected with 1mg/ml OKT3 and 4μg/ml of anti-CD28 (Leu 28, B & D) Ab stimulation. Although in this Chapter CD3 plus CD28 stimulation only induced high frequencies of IL-2 producing cells (11.2 %) in the memory T cells from lines (with 5μg/ml anti-CD3 plus 30 μg/ml anti-CD28 Ab), all populations proliferated with CD3 plus CD28 stimulation. As shown in Figure 3.9, proliferation in resting T cells was mediated by IL-2, because anti-IL-2 Ab partially reduced T cell proliferation. Anti-IL-2 plus anti-IL-2R neutralising antibodies can not totally inhibit proliferation because co-stimulation via CD28 also promotes IL-2 independent-proliferation (Tuosto and Acuto 1998; Boonen, van Dijk et al. 1999; Boulougouris, McLeod et al. 1999) by inducing the expression and activation of cell-cycle proteins and down-regulating cell-cycle protein inhibitors (Boonen, van Dijk et al. 1999). Therefore, there can not be an absolute inhibition of CD3 plus CD28 mediated proliferation with the addition of anti IL-2 antibodies or anti IL-2R Ab (Boonen, van Dijk et al. 1999), which explains why the use of an anti-IL-2 neutralising Ab did not inhibit all the T cell proliferation in Figure 3.9. As ICS was not able to detect this IL-2, it is concluded that this technique had limited sensitivity to detect IL-2 under CD3 plus CD28 stimulation.

The poor ICS detection of IL-2 production induced by CD3 and CD28 stimulation could have been partially due to a poor stimulation delivered by the Ab used in these experiments. However, the same anti-CD28 Ab has been used in other experiments, where higher levels of intracellular IL-2 were detected (Groux, O’Garra et al. 1997; Woodside, Long et al. 1999). The OKT3 Ab used here was purifed from ascites and may not be good enough to stimulate IL-2 production even at high concentrations (200 μg/ml). Nevertheless, the fact that PMA plus CD3 stimulation also induced a low
frequency of IL-2 producing cells in all populations of T cells enforced the idea that ICS is a low sensitivity technique that demands strong stimulation to the cells to detect cytokines.

The results from this Chapter suggests that differences between CB derived CD45RA+ T cells and mature T cells (resting or CD45RO+ memory T cells) must exist upstream of or at PKC, as 50ng/ml PMA plus ionomycin successfully stimulated a large proportion of CD45RA+ T cells to produce IL-2. Attempts to explain the differential responsiveness of naïve cells from CB and adults have focus in the expression of effector proteins. Whereas no differences have been found in the PKCβ isoforms expressed in pure CD45RA+ and CD45RA+ T cells from CB and adults (Hassan, Rainsford et al. 1997), Lck 56 is expressed at lower levels in T cells from CB compared with adult T cells (Miscia, Di Baldassarre et al. 1999). Upon TCR activation, p56 lck is engaged with the CD4+ or CD8+ co-receptors (Barber, Dasgupta et al. 1989) and couples TCR signalling to PLCγ (Weber, Bell et al. 1992). Although it has been reported that the expression of the PLC isoforms γ1 and β2 is lower in CB mononuclear cells compared with adult cells, PCLγ1 and ZAP-70 cellular localisation and activation was similar in CB and adult cells (Miscia, Di Baldassarre et al. 1999), implying a similar capacity of activation. NF-κB activation from CD28 stimulation and is involved in IL-2 expression (Ghosh, Tan et al. 1993). In spite of optimal CD2 and CD28 stimulation that induced cells proliferation to adult levels, it was found at lower levels in T cells from CB compared with adult cells (Hassan, O'Neill et al. 1995) although the levels of IL-2 mRNA (but not secretion) can be higher on CB derived lymphocytes compared with adult samples on stimulation with KLH (Hassan and Reen 1996). Data indicates that calcium ionophores induce higher levels of NF-κB activation in CB CD4+ T cells compared with adult CD4+ T lymphocytes (Kilpinen, Henttinen et al. 1996). These results suggests that the expression of cytokine genes may have a differential regulation in naïve cells from CB and adult cells and the differences between CB and adult blood naïve cells may lye in early signalling and in other stages of the signalling cascade.

In conclusion, CD45RA+ T cells from CB have a reduced response to stimulation compared to resting and memory T cells, but a high frequency of CD45RA+ T cells from CB do have the capacity to produce IL-2 once their threshold of stimulation has been reached. Nevertheless, the amount of IL-2 produced per cell is reduced, compared with T cells at other stages of maturation. As the IL-2 produced by CD3 plus CD28 stimulation by T cells was not detected by ICS, it is also concluded that this technique is not sensitive to detect cytokine production in CD3 and CD28 stimulated T cells.
3.5 Future experiments

It has been previously suggested that CD45RA+ T cells derived from umbilical CB are more naïve than their counterpart from peripheral blood (Hassan and Reen 1997). Although the results from this Chapter indicate that CD45RA+ T cells from CB are not impaired in their capacity to produce IL-2, a directly comparison of CD45RA+ T cells derived from CB and from periphery was not performed. This comparison can be done in future experiments by isolating pure CD45RA+ T cell populations from CB and peripheral blood, and stimulating the cells with a combination of anti-CD3, anti-CD28, PMA and ionomycin all at different concentrations, in the same manner used in this Chapter. This has not been done, as published work have compared frequencies of IL-2 producing CD45RA+ T cells from CB and adult blood at a single concentration of stimulus (see below). A extension of this work in this direction should include a bigger number of samples and may also include further lymphocyte phenotyping.

Furthermore, a comparison on the capacity of cytokine production by T cell subsets (CD4+ and CD8+ ) can be included in future work. The proportion of CD3+, CD4+ and CD8+ cells in CB and adult blood is controversial, since some studies found similar levels of expression whereas others found lower levels of expression in CB cells compared with adult cells, (Berry, Fine et al. 1992; Rabian-Herzog, Lesage et al. 1993; Han, Hodge et al. 1995; Mills, TG et al. 1996). Nevertheless, comparisons on the function of these subsets have been done (Chalmers, Janossy et al. 1998; Chipeta, Komada et al. 1998; Chipeta, Komeda et al. 1999; Krampera, Tavecchia et al. 2000). In adults, the regulation and capacity of cytokine production differs between CD4+ and CD8+ T cells (Aune, Penix et al. 1997) (Jason and Larned 1997) and it has been proposed that this capacity of cytokine production is also different in CB derived cells (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Krampera, Tavecchia et al. 2000). Some of these studies propose that CD4+ and CD8+ cells from CB have different frequencies of IL-2 producing cells, with CD4+ cells having a larger capacity of IL-2 production (Chalmers, Janossy et al. 1998; Krampera, Tavecchia et al. 2000). This has been controversial because other studies have not found this difference (Chipeta, Komeda et al. 1999). However, these studies have compared the capacity of cytokine production of CD4+ and CD8+ cells after PMA (5 and 30 ng/ml) plus ionomycin stimulation (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Krampera, Tavecchia et al. 2000) and the CD4 expression is down-regulated with as low as 5 ng/ml PMA (see Chapter 2 Section 2.7.3) and the proportion of CD4+ cells in generally underestimated with the commercial Ab used
for flow cytometry (Mark Lowdell, personal communication). Thus, the capacity of CD4\(^+\) cells to produce cytokines has been underestimated in these studies. As CD8 and CD3 expression is unaffected by PMA stimulation (Anderson and Coeclough 1983), only staining for these markers can be performed in studies including PMA stimulation and CD4 staining should not be included. To study the capacity of cytokine production by CD4\(^+\) and CD8\(^+\) subsets, however, they can also be isolated by negative sorting. The advantage of this approach is that other markers of activation (such as CD69 or CD25, Fas, CD40L or class II molecules) and other cytokines can be detected as CD3, CD4 and/or CD45RA staining is not needed.

A direct comparison between the capacity of CB and adult naïve cells to produce cytokine could also be done by multiple staining of unfractioned mononuclear cell populations from CB or adult blood. This could include CD3, CD8, CD45RA and IL-2 staining. CD45RA\(^-\) T cells can not be defined as CD45RO\(^-\) cells, although some studies have compared the frequencies of memory and naïve IL-2 producing cells from peripheral blood defining CD45RA\(^+\) cells as CD45RO\(^-\) (Jung, Schauer et al. 1993; Woodside, Long et al. 1999). In these studies, it was assumed that the CD45RO negative cells were CD45RA\(^+\). However, the definition of CD45RA\(^+\) cells as CD45RO\(^-\) cells may not reflect the true percentage of CD45RA\(^+\) cells in a populations. Since there is a proportion of double positive (CD45RA\(^+\)/RO\(^-\)) T cells from peripheral blood (6.0-7.8%, mean 6.8%) seen both this thesis and in the literature (Okumura, Fujii et al. 1993). Double positive cells were also seen in T cells from CB before purification (double positive : 0.1-4.7%, mean 3.2 %) but were never detected in T cell lines (Chapter 2 Section 2.7.2).

There is enough evidence that naïve cells derived from CB and adult blood differ in their stimulation requirements. To explain these differences, some studies have compared the expression and function of some of the proteins involved in early cells signalling in adult and CB T cells, such as Lck and PLC\(\gamma\)1, whose are expressed differently in both cells (Miscia, Di Baldassarre et al. 1999). Data in this Chapter indicates that differences between naïve T cells from CB and other T cell subsets reside upstream PKC, which is supported by Miscia et al results with CB cells (mainly composed of CD45RA\(^+\) cells (Rabian-Herzog, Lesage et al. 1993)). Although there was a low expression of Lck and ZAP-70 in stimulated CB T cells, the results from Miscia et al indicate that the PLC\(\gamma\)1 response to stimulation is not impaired. Nevertheless, the differences in the expression and activation levels of these proteins using purified CD45RA\(^+\) T cell populations derived from CB and adult blood have not been studied. To further understand the cellular response to stimulation of
different strength, this studies can be done with different concentrations of mitogenic stimulation of the cells (see below).

As the study of early signalling in CB and adult CD45RA+ T cells has not reached a conclusive explanation on their functional differences, other levels of signalling can be studied, such as the different capacity of signalling inhibition between both subsets. This feature has been described for naïve cells (CD45RBhigh) when compared with memory (CD45RBhigh) cells (Farber, Luqman et al. 1995). Because it has been found that the levels cytokine’s mRNA are higher in CB CD4+CD45RA+ cells compared with their adult counterpart (Hassan and Reen 1996), it may be that the different capacity of inhibition is reflected at cytokines mRNA levels. As CD28 co-receptor engagement upregulates and destabilises IL-2 mRNA (Lindstein, June et al. 1989; Ragheb, Deen et al. 1999), not only molecules involved in TCR signalling but those involved in CD28 co-receptor signalling (such as Lck and Fyn, PI3-K, ITK and GRB-2 (August, Gibson et al. 1994; Raab, Cai et al. 1995)) can deliver different signals in CB or adult naïve T cells that may result in cytokine mRNA of a longer life. Thus, the study of expression and activation of these molecules can also be addressed after stimulation by receptor and co-receptor crosslinking with Ab. Additionally the determination of different thresholds of stimulation at the level of early signalling can be done with different concentrations of crosslinking antibodies and antibodies with different stimulatory capacities, which depends on the cross-linking capabilities of the antibody used and the epitope recognised (Nunes, Klasen et al. 1993; Siefken, Klein-Hessling et al. 1998). (Yang and Parkhouse 1998).

Allogeneic stimulation has been shown to provide optimal stimulation to CB derived T cells for proliferation and cytokine production (Trivedi, HayGlass et al. 1997; Matthews, Wadhwa et al. 2000) and it has been shown that strong TCR signalling enables cytokine production by naïve T cells (Manickasingham, Anderton et al. 1998). As these models provide optimal stimulation to CB T cells (which respond with higher proliferation and cytokine production compared with adult T cells), a comparison in the association of early proteins with TCR and co-receptors after a physiological stimulus can be performed using purified populations of CD45RA+ T cells. The determination of the potential of CB cells responsiveness to stimulus is relevant to CB transplantation, because although CB transplants have a lower incidence of GvHD and permit a larger number of HLA mismatches compared with uBMT (Rocha, Wagner et al. 2000), CB transplants are not extent of GvHD. There may be only certain levels of mismatches below the stimulation threshold of CB T cells. Thus, further work can include the study of the frequency of cytokine producing
pure CD45RA⁺ T cells from CB under certain alloantigen, including mismatches in class I and/or class II antigens.

Matthews et al used cultured DC as allogeneic stimulus for CB derived T cells and mitogens to induce cytokine production. As mentioned before, the ICS technique requires the cells to be strongly stimulated (except in memory cells, which have lower stimulation thresholds (Curtsinger, Lins et al. 1998). Thus, it could be interesting to determine the frequency of IL-2 producing T cells from CB stimulated with alloantigen using a more sensitive technique, such as the ELISPOT assay.

The low responsiveness of CB seen in a variety of studies may also be due to the particular milieu that these cells exist in and not only due to intrinsic characteristics of the cells at this developmental stage, because a general low cellular responsiveness has been observed in T cells, NK cells and macrophages from CB (Glover, Brownstein et al. 1987; Weatherstone and Rich 1989; Racadot, Schaal et al. 1993; Sautois, Fillet et al. 1997). Thus, another approach to the study of the immunity of CB cells can include the effects of this milieu (i.e. CB serum) on cellular responses of adult cells such as proliferation and cytokine production. This is discussed further in Chapter 5 of this thesis.
4. Chapter 4. Naïve T cells from CB have high frequencies type 1 and type 2 cells and low frequencies of type 0 cells.

4.1 Introduction

The populations of CD4+ T cells that produce IL-2, IFNγ, IL-4 and IL-10 have been classified as Th0 (Maggi, Del Prete et al. 1988; Paliard, de Wall et al. 1988; Yssel, De Waal Malefyt et al. 1992) and represent partially differentiated primed cells consisting of multiple discrete subsets of cytokine secreting cells (Bucy, Panosklitsis-Mortari et al. 1994). There are also single type 0 T cells with the capacity to produce IFNγ and IL-4 simultaneously (Openshaw, Murphy et al. 1995; Picker, Singh et al. 1995). Depending on the environmental conditions of stimulation, such as the strength of TCR stimulation, co-stimulation and cytokines present during proliferation (Swain, Weinberg et al. 1990; Hsieh, Macatonia et al. 1993; Iezzi, Scotet et al. 1999; Rogers and Croft 1999) the non-committed Th0 cell can then progress to develop a Th1 or a Th2 phenotype (Nakamura, Kamogawa et al. 1997).

CD45RA+ T cells are non-primed cells (Wallace and Beverley 1990) and represent a 14.6-53.0% of peripheral T cells and a 22.1-87.6% of T cells from CB (see Figure 3.1 Chapter 3 Section 3.3.1 and also (Harris, Schumacher et al. 1992; Rabian-Herzog, Lesage et al. 1993; D’Arena, Musto et al. 1998). Cells from CB have traditionally been considered unable to secrete high levels of IFNγ or IL-4 (Kruse, Rink et al. 1992; Sautois, Fillet et al. 1997; Spinozzi, Agea et al. 1997; Trivedi, HayGlass et al. 1997). However, the capacity of IFNγ and IL-4 production by CB cells has been demonstrated by ICS (Anderson, Anderson et al. 1990) and occurs in T cells from CB and adult blood with the same kinetics (Anderson, Anderson et al. 1990; Chalmers, Janossy et al. 1998). Additionally, it has been observed that naïve cells from neonates have the potential to develop into high IL-4 producers (Adkins and Hamilton 1992) compared with a poor capacity of CD45RA T cells from the periphery to become high IL-4 producers (Sasama, Vyas et al. 1998).

The aim of this chapter was two fold: firstly, to study the potential of CD45RA+ T cells derived from CB to produce IFNγ, IL-4 and IL-10. To do this, pure CD45RA+ T cells were stimulated with various concentrations of different mitogens, because it was seen in the previous chapter that naïve cells from CB can produce cytokines at high frequency if their stimulation requirements are reached. This was performed by the detection of intracellular cytokines at the single cell level. Secondly, to determine the frequencies of
type 1 type 2 and type 0 cells in this naïve population. Type 1 cells were defined as IFN\(\gamma^+\), type 2 cells were defined as IL-4\(^+\) cells and type 0 as IFN\(\gamma^+\)IL-4\(^+\) or IL-10\(^+\), as IL-10 is considered a Type 0 cytokine in humans (Yssel, De Waal Malefyt et al. 1992; Del Prete, De Carli et al. 1993; Brookes, Cohen et al. 1996; Cohen, Webb et al. 1996; Gerosa, Paganin et al. 1996; Mingari, Maggi et al. 1996; Sornasse, Larenas et al. 1996). High frequencies of type 1 and type 2 cells but not type 0 cells were found in the CD3\(^+\)CD45RA\(^+\) lymphocytes purified from CB.
4.2 Materials and Methods

4.2.1 Cells

CB was obtained from normal and caesarean full-term deliveries by puncturing the umbilical vein (Domínguez, Madrigal et al. 1998). Mononuclear cell populations were isolated by separating the bloods over Ficoll (Lymphoprep; Nycomed, Oslo, Norway) by standard techniques. Naïve T cells (CD3'CD45RA') were derived by negative depletion from umbilical cord blood using magnetic separation (as explained in Chapter 2 Section 2.3.1). The CD14/CD16/CD19/CD56/CD45RO cells depleted cells were always >90% CD45RA' using this method (as shown in Chapter 3, Figure 3.1). Cells were washed and used immediately after depletion.

4.2.2 Antibodies

Anti-CD3 antibody (OKT3) and anti-CD28 Ab (PharMingen, Cambridge Biosciences, Cambridge UK) were used to stimulate T cells as described in Chapter 3 Section 3.2.2.

Antibodies for flow cytometry were anti-CD3-PercP (Becton and Dickinson Ltd, Oxford, UK). Antibodies used to stain for intracellular cytokines (and their isotype matched controls) were all obtained from PharMingen. The anti-cytokine antibodies were: anti-IFNγ FITC, anti-IL-4-Pe and anti-IL-10 Pe.

4.2.3 Stimulation of T cells for intracellular cytokine detection and cell staining.

5x10^5 cells /well were placed in 48 well plates for 6 hrs stimulation with either immobilised anti-CD3 Ab plus immobilised anti-CD28 Ab, immobilised anti-CD3 Ab plus PMA, or PMA plus ionomycin (as explained in Chapter 2 Section 2.5) all in the presence of 3μM monensin. After 6 hr incubation, cells were harvested from the plates, washed and stained for the extracellular antigen CD3 (as described in Chapter 2 Section 2.7.1). For detection of intracellular cytokines, cells were fixed, permeabilised and stained intracellularly with anti-IFNγ, anti-IL-4 or anti-IL-10 Ab or isotype matched control Ab (as detailed in Chapter 2 Section 2.7.5).

4.2.4 Flow cytometric analysis

Cells were analysed within 48 hours of staining by FACScan flow cytometer (Becton and Dickinson) using Cell Quest Software (Becton and Dickinson), after acquiring >20000 cells. Results are expressed as the frequencies of type 1, type 2 and type 0 cells. These
T cell types were defined by their exclusive production of IFNγ (type 1), IL-4 or IL-5 (type 2), both IFNγ and IL-4 or IL-10 (for type 0) within the CD3+ gate.

4.3 Results

4.3.1 Definition of type 1, type 2 and type 0 cells.

A quadrant plot representing events in the FL-1 (IFNγ) and FL-2 (IL-4) fluorescence channels or the FSC and FL-2 (IL-10) channels were gated in the CD3+ large lymphocyte population of purified CD45RA+ T cells. IFNγ- cells were defined as type 1 cells, IL-4- cells were defined as type 2 cells and IFNγ-/IL-4- or IL-10- cells were classified as type 0 cells (Figure 4.1 panel A and D). The negative values in the dot plots were defined by non-stained cells and with values given by the isotype matched control Ab (Figure 4.1 panels B and E). Staining with control Ab was included for each concentration of stimulus.

4.3.2 Frequencies of type 1 CD45RA+ T cells.

CD45RA+ T cells purified from CB were stimulated with CD3 ± CD28, CD3 ± PMA and PMA ± ionomycin for cytokine production, as explained in Chapter 2 section 4.2. After flow cytometry analysis, it was found that CD45RA+ T cells from CB were able to produce IFNγ. Cells from only one sample produced IFNγ with anti-CD3 Ab stimulation alone (5.5%) or CD3 plus PMA (5.6%) (Figure 4.2 Sample 1). In the rest of the samples, the frequency of IFNγ producing cells was always low (below 1%) under anti-CD3 ± anti-CD28 or PMA stimulation (Figure 4.2). However, these cells produced IFNγ when PMA and ionomycin stimulation was used and this stimulus induced the highest frequencies of IFNγ producing CD45RA+ cells. In one sample the frequency of IFNγ producing naïve cells was 21.0% (Figure 4.2 Sample 1), with 50 ng/ml PMA and 0.1μM ionomycin. The results represented in Figure 4.2 indicate that the combination of 5ng/ml or more PMA plus ionomycin (0.1-10 μM) induced IFNγ production in these CD45RA+ T cells, as these concentrations were required to induce frequencies of type 1 cells above 2.0% in samples 2 and 3. In the last sample analysed, only the highest concentrations of mitogens (50 ng/ml PMA plus 10μM or ionomycin) allowed detection of IFNγ.

Thus, mitogenic stimulation enabled the detection of type 1 (IFNγ+) CD45RA+ T cells from CB, PMA and ionomycin being the most effective stimulation to induce IFNγ production.
Figure 4.1 Definition of type 1, type 2 and type 0 CD3-CD45RA- cells. Naïve CB cells were purified by negative depletion as described in chapter 2 section 2.3.1. The pure naïve T cell population was then stimulated with various concentrations of stimulus for 6 hrs in the presence of monensin (as described in Chapter 2 Section 2.7.4). Cells were then stained extracellularly with anti-CD3 (PerCp) and intracellularly with anti-IFNγ Ab (FITC) and anti IL-4 Ab (Pe) or the appropriate control Ab or anti IL-10 (Pe) or the appropriate control Ab. Cells were acquired within 48 hrs staining and analysed using Cell Quest Software (B&D). The analysis was done gating in the blasting CD3⁺ lymphocytes. >95% cells in the lymphocyte gate were CD3⁺. A) Type 1, type 2 and type 0 T cells were defined by a quadrant where IFNγ⁺ (FITC) and IL-4⁺ (Pe) cells were defined type 1 and type 2 respectively, and double positive (IFNγ⁺/IL-4⁺) cells were defined as type 0 cells. B) IFNγ plus IL-4 control Ab staining C) IFNγ and IL-4 staining D) IL-10⁺ cells were also classified as type 0 T cells. E) IL-10 control Ab F) IL-10 staining.
Figure 4.2. Frequencies of type 1 CD45RA+ T cells. Naïve (CD45RA+) T cells were stimulated for 6 hours with varying concentrations of immobilised anti-CD3 Ab ± immobilised anti-CD28 Ab, immobilised anti-CD3 Ab ± soluble PMA or PMA ± ionomycin, all performed in the presence of monensin, as described in material and methods (chapter 2 section 2.17). Cells were then stained extracellularly for CD3 and intracellularly for anti-IFNγ and anti-IL-4 Ab and analysed by flow cytometry as described in Figure 4.1. The results represent the frequency of type 1 cells in the large CD3 lymphocyte gate, defined by the exclusive production of IFNγ (As described in Figure 4.1 panel A). The positive values were set according to negative staining of both unstained cells and a IFNγ isotype matched control Ab for each point of the stimulus dilution. The values for the isotype control antibody were always 0.0%.
4.3.3 Frequencies of type 2 CD45RA\(^+\) T cells.

Whereas 200 \(\mu g/ml\) CD3 plus 10 \(\mu g/ml\) CD28 stimulation induced IL-4 production in one of the samples studies here (4.8% in Sample 2, Figure 4.3), CD3 plus PMA and PMA plus ionomycin stimulation induced IL-4 production at high frequencies in two samples (Figure 4.3, Samples 1 and 2). Sample 3 did not produce IL-4 at high frequency under any stimulation. The cells represented in Figure 4.3 were exclusively IL-4\(^+\) and therefore, belonged to the type 2 group of cytokine producing cells. High concentrations of anti-CD3 or ionomycin induced the highest frequencies of IL-4 production. In some samples, ionomycin alone (1 or 10\(\mu M\)) induced IL-4 production by high frequencies of CD45RA\(^+\) T cells and the addition of PMA decreased the percentage of cells producing IL-4. The highest frequency of IL-4 producing cells in these CD3\(\cdot\)CD45RA\(^+\) samples was 13.0\% using 1\(\mu M\) ionomycin.

These results indicate that high frequencies of CD3\(\cdot\)CD45RA\(^+\) have the capacity to produce IL-4, mainly with anti-CD3 plus PMA and PMA plus ionomycin stimulation.
Figure 4.3. Frequencies of type 2 CD45RA+ T cells. Naive (CD45RA+) T cells were stimulated for 6 hours with varying concentrations of stimulus in the presence of monensin, stained and analysed by flow cytometry, as described in Figure 4.2. For analysis, a gate on blasting CD3+ lymphocytes was set. Results represents the frequency of type 2 cells, defined by the exclusive production of IL-4. The positive values were set according to negative staining set on both unstained cells and a IL-4 isotype matched control Ab for each point of the stimulus dilution. The values for the isotype control antibody were always 0.0%.
4.3.4 Frequencies of type 0 CD45RA<sup>+</sup> T cells.

The simultaneous production of IFNγ and IL-4 by CD3<sup>+</sup>CD45RA<sup>+</sup> CB cells (type 0 cells) was a rare event in these samples except when high concentrations of ionomycin ± PMA were used. Whereas anti-CD3 (50 μg/ml) plus anti-CD28 (30 μg/ml) induced the production of both cytokines (with a frequency of 3.4%) in only one sample (Sample 2, Figure 4.4), the frequency of type 0 cells was high when naïve cells were stimulated with 10μM ionomycin (up to 27.0% in Sample 1, with 50ng/ml PMA and 10μM ionomycin). Nevertheless, 10μM ionomycin induced cell death: cells from these samples decreased in size (defined by FSC), whereas the region of dead cells and debris increased. The frequency of double positive cells in the FL-1/FL-2 quadrant was also high when these cells were stained with the isotype matched control Ab (this value was subtracted in the samples stained with anti-cytokine Ab but the frequency of double positive cells remained high). The effect of culture in 10μM ionomycin on the frequency of the CD3<sup>+</sup> population of cells from CB or adult blood is shown in Figure 4.5, although this concentration of ionomycin did not eliminate all the large CD3<sup>+</sup> cells from adult samples, it did drastically decreased the population of large CD3<sup>+</sup> cells in CB samples. Although in all concentrations of mitogens used control Ab staining with isotype matched were included, the fact that this concentration of ionomycin is toxic for these cells leaves open the possibility that cells damage may cause non specific binding of the anti-cytokine Ab.

Next, and because human IL-10 is produced by type 0 T cell clones (Yssel, De Waal Malefyt et al. 1992; Del Prete, De Carli et al. 1993; Katsiskis, Cohen et al. 1995; Brookes, Cohen et al. 1996; Sornasse, Larenas et al. 1996) the cells were stained for IL-10 to verify the frequency of type 0 populations within the CD3<sup>+</sup>CD45RA<sup>+</sup> lymphocytes. High frequencies of IL-10<sup>+</sup> CD45RA<sup>+</sup> T cells from CB were no detected after CD3 plus CD28 or plus PMA stimulation. Only a low frequency of IL-10<sup>+</sup> cells was found in the samples stimulated with PMA and ionomycin (see Figure 4.6). It can be seen in the Figure 4.6 that only 10 μM ionomycin induced the detection of IL-10<sup>+</sup> cells (a maximum frequency of 2.5%). However, 10 μM ionomycin may be too strong for cells derived from CB derived cells, as discussed above. In conclusion, type 0 cells in CD3<sup>+</sup>CD45RA<sup>+</sup> cells derived from CB have a low frequency of type 0 cells, but high frequencies type 1 and type 2 cells can be obtained.
Figure 4.4. Frequencies of type 0 CD45RA+ T cells. Naïve (CD45RA+) T cells were stimulated for 6 hours with varying concentrations of stimulus in the presence of monensin, stained and analysed as described in Figure 4.2. The results represent the frequency of positive events in both FL-1 and FL-2 channels (double IFNγ+ /IL-4+ T cells). The positive values were set according to negative staining on both unstained cells and IFNγ and IL-4 isotype matched control Ab for each point of the stimulus dilution. The values for the isotype control antibodies were either 0.0% or were subtracted from the results.
Figure 4.5. Cells from CB die at high concentrations of ionomycin. CD45RA cells from CB where cultured in 50ng/ml PMA and increasing concentrations of ionomycin for 6 hrs as described in Figure 4.1. The cells were harvested and acquired in a flow cytometer. This figure represents the dot plots of FSC vs CD3 expression after stimulation in the lymphocytes. The square inside each dot plot in the right hand column indicates the CD3' lymphocytes in each sample. This figure is representative of 3 CB samples.
Figure 4.6. Frequency of IL-10 producing CD45RA+ T cells. Naïve (CD45RA+) T cells were stimulated for 6 hours with varying concentrations of CD3 ± CD28, CD3 ± PMA or PMA ± ionomycin in the presence of monensin for 6 hrs and stained with anti-IL-10 (Pe) or an appropriate isotype matched control Ab. The samples were then acquired and analysed as described in Figure 4.2. A gate was set on the large CD3+ lymphocytes for analysis. As CD3 ± CD28, CD3 ± PMA did not induce cytokine production, this figure represents the frequency of IL-10 producing cells after stimulation only for PMA ± ionomycin. The values for the isotype control antibody were always 0.0%.
4.4 Discussion and conclusions

T cells that have not encountered Ag have low responsiveness to stimuli and have higher requirements for activation (Croft, Bradley et al. 1994; Kuiper, Brouwer et al. 1994; Curtsinger, Lins et al. 1998; Cho, Wang et al. 1999). Once these cells are activated they divide, making IL-2, IFNγ and IL-4 genes accessible to transcription (Agarwal and Rao 1998; Bird, Brown et al. 1998). Whereas there is evidence that IFNγ and IL-4 can be produced by naïve T cells from periphery (Budd, Cerottini et al. 1987; Sanders, Makgoba et al. 1998; Lewis and Wilson 1990; Ehlers and Smith 1991), it was originally proposed that CD45RA⁺ T cells derived from CB are unable of IFNγ or IL-4 production at high levels. This was because several studies consistently reported lower levels of IFNγ and IL-4 production by cells derived from CB compared to adult cells (Miyawaki, Seki et al. 1985; Lewis, Larsen et al. 1986; Chin, Ank et al. 1988; Watson, Oen et al. 1991; Kruse, Rink et al. 1992; Takahashi, Imanishi et al. 1995; Trivedi, HayGlass et al. 1997). It was also reported that the expression of IFNγ and IL-4 mRNA is lower in T lymphocytes from CB compared to adult T cells after mitogenic stimulation (Lewis, Yu et al. 1991) which was proposed to be the reason for the low production of these cytokines by CB T cells (Lewis, Yu et al. 1991). But high concentration of mitogens (50ng/ml PMA and 0.5μM ionomycin) elevates the level and kinetics of production of the IFNγ and IL-4 mRNA in CB to adult levels (Lewis, Yu et al. 1991) and optimal stimulation induces similar levels of IFNγ secretion by CB and adult blood cells (Kesson and Bryson 1991). More recent papers have indicated that although CB derived CD45RA⁺ naïve T cells can produce IFNγ and IL-4, they have lower frequencies of cytokine producing cells compared with CD45RA⁺ T cells from adult blood (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Schultz, Rott et al. 1999; Krampera, Tavecchia et al. 2000), which then explains the low levels of cytokine production. However, the low frequencies of IFNγ and IL-4 producing cells can be due to the high threshold of stimulation of CB derived CD45RA⁺ T cells that, under appropriate stimulation, can produce IFNγ and IL-4 at high frequencies (Perez-Cruz, Fallen et al. 2000).

In this chapter, anti-CD3 plus anti-CD28 or PMA and PMA plus ionomycin were used to detect type 0, type 1 and type 2 cells in pure CD45RA⁺ T cells derived from CB. Anti-CD3 plus anti-CD28 (the most physiological stimulus used here) was not a significant stimulation to detect frequencies of type 1, type 2 or type 0 CD3⁺CD45RA⁺ cells: 5.5% of type 1 cells were detected in only one sample (Figure 4.2), 4.8% type 2 cells in only one sample (Figure 4.3) and 3.8% type 0 cells were detected in only one sample (Figure 4.4) using CD3 plus CD28 stimulation. However, high frequency of type
2 cells were detected in CD3\(^+\)CD45RA\(^+\) cells with CD3 plus PMA stimulation, which suggests that there is a high frequency type 2 cells within the naive T cell population (10.5% with 50ng/ml PMA plus 50μg/ml anti-CD3 represented in Figure 4.3), contrary to previous reports, where low frequency of IL-4 producing CD45RA\(^+\) T cells from CB has been described (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Schultz, Rott et al. 1999; Krampera, Tavecchia et al. 2000). Furthermore, the highest frequency of type 1, type 2 and type 0 cells were found with PMA and ionomycin (Figures 4.2, 4.4 and 4.5). The discrepancies between these results and those reported in the literature may be because the naiveté of the cells within cord blood may be overcome when CD3 signalling and simultaneous stimulation of PKC (with PMA) are provided, or when higher concentrations of PMA ± Ionomycin that those utilised in the literature (from 5 to 30 ng/ml PMA) (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Schultz, Rott et al. 1999; Krampera, Tavecchia et al. 2000) are used for stimulation.

The finding of the capacity of naive T cells from CB to produce type 2 cytokines agrees with suggestions that naive T cells could be predominately Th2, based on the observation that CD4\(^+\)CD45RA\(^+\) cells derived from CB can become efficient cytokine secreting cells (Yang, Byun et al. 1995). Yang et al have found that although both adult and CB naive cells can be cloned to become high IL-4 and IL-5 producers, under CD3 and CD28 stimulation plus IL-2, CB derived CD4\(^+\)CD45RA\(^+\) cells are more responsive than their adult counterparts to IL-4 stimulation and preferentially develop into Th2 cells (Yang, Byun et al. 1995). Although no detectable IL-4 was found in this Chapter with anti-CD3 plus CD28 stimulation, CD45RA\(^+\) T cells proliferate to this stimulus (Chapter 2 Section 3.3.5), which indicates that the cells are responsive to this stimulation. Moreover, the low level of detection may be due to the sensibility of the assay, as it was described for the intracellular detection of IL-2 in Chapter 2 section 3.3.6. In agreement with Yang results, Webb et al showed the capacity of CD4\(^+\)CD45RA\(^+\) T cells from CB to become high IL-4 producers under CD3 and CD28 stimulation in the presence of IL-4 (Webb and Feldmann 1995). In addition, it has also been published that both CD4\(^+\) and CD8\(^+\) CB derived cells have the capacity to become efficient IL-4 producers in vitro (Byun, Demeure et al. 1994; Demeure, Yang et al. 1995). Furthermore, under activation, naive T cells from transgenic mice can secrete enough IL-4 to support their differentiation into Th2 cells (Croft and Swain 1995) although the development of polarising phenotype in cells from these mice is accelerated in the presence of exogenous cytokines (Ben-Sasson, Makedonski et al. 2000). However, the capacity of cytokine production by CB CD45RA\(^+\) T cells is not restricted to IL-4, because CB derived CD4\(^+\)CD45RA\(^+\) cells also have the ability to become IL-2, IL-4 and IFN\(\gamma\) producing cells faster than adult CD4\(^+\)CD45RA\(^+\) cells (Early and Reen 1999) and CD8\(^+\) cells derived
from CB cultured IL-12 with can secrete high levels of IFNγ (Byun, Demeure et al. 1994). Here, the capacity of CD45RA⁺ T cells to produce IFNγ in high frequency was mainly seen with PMA and ionomycin (Figure 4.2).

Although PMA and ionomycin enabled the detection of type 1 and type 2 cytokines in fresh naïve T cells purified from CB (see Figures 4.2 and 4.3) the CD45RA⁺ cells used here were more fragile to the stimulation toxicity compared with other populations of T cells (such as T cells from adult peripheral blood): the highest concentration of ionomycin used (10µM) induced cell dead, according to FSC criteria, and in some samples this concentration reduced significantly the proportion of cells in the live gate (Figure 4.5). This was not observed in lymphocytes from adult blood, although at this concentration of ionomycin, the proportion of live cells in the lymphocytes gate was slightly diminished in some adult samples. Frequencies of type 0 cells in CD45RA⁺ T cells from CB were only obtained when the cells were incubated with 10µM ionomycin (± PMA) with one exception (see Figure 4.4), and the highest frequency was observed in sample 1 (see Figures 4.4 and 4.6), which was in particular susceptible to the toxicity of this concentration of ionomycin. At concentrations below 10µM ionomycin, no type 0 cells were detected in CD3⁺CD45RA⁺ cells from CB, measured by two criteria, IFNγIL-4⁺ and IL-10⁺ T cells (Figure 4.4 and 4.6). These results suggest that CD45RA⁺ T cells from CB have the capacity to produce IFNγ or IL-4 but not both cytokines simultaneously, and that whereas type 1 and type 2 cells can be found in healthy samples, type 0 cells are found at very low frequencies.

Comparing CB and adult derived cells, lower frequencies of CD4⁺ and CD8⁺ cells with the ability to produce of IFNγ or IL-4 have been reported (Chalmers, Janossy et al. 1998; Chipeta, Komada et al. 1998) and low frequencies of Th0 and Tc0 cells from CB compared with adult cells using ICS were also found by Chipeta et al (Chipeta, Komada et al. 1998). However, these studies used ionomycin (1µM) plus low concentrations of PMA (5-6.16 ng/ml) to induce cytokine secretion, leaving open the possibility that suboptimal concentrations of mitogens were used for cytokine production in these cells. In this Chapter this possibility was explored, and healthy IFNγ⁺ and IL-4⁺ CD45RA⁺ T cells were detected at high frequency. However, type 0 cells were only detected in populations with high cell dead. Thus, whereas CD3 CD45RA cells from CB have the capacity to produce IFNγ and IL-4, these cytokines are not expressed simultaneously in healthy cells.

Based on the detection of IFNγ and IL-5 mRNA expressed by Ag-stimulated cells from CB, Prescott et al have proposed that CB lymphocytes have high frequencies of type 1 and type 2 cells and suggest that these profiles develop into Th0 in older children.
Chapter 4

( Prescott, Macaubes et al. 1997). However, Prescott's study did not analyse the frequency of cytokine production at the single cell level and therefore these cytokines can be produced by different groups of cells. Indeed, results from this chapter indicate that the production of IFNγ and IL-4 is segregated in naïve cells from CB and that there is a very low frequency of type 0 cells in CD3+CD45RA+ cells from CB. Prescott's study additionally detected high IL-10 production by ELISA whereas in this chapter IL-10 producing cells were hardly detected. This may be because due to differences in the detection technique (ELISA and ICS) and because in this chapter, CB cells were stimulated for 6 hrs and Prescott et al stimulated for 24 hr with PHA or Ag. However, 6 hr incubation time has enabled the detection of IL-10 by adult T cells in this laboratory (Zamauskaite, Perez-Cruz et al. 1999).

The results from studies of the frequency of cytokine producing naïve cells from CB and adult blood indicate that there are differences not only in the stimulation requirements or the ability to produce cytokine between both groups, but also in the cytokine profile developed after stimulation (Frequencies of type 1, type 2 and type 0 T cells). In T cells from the periphery, 5 ng/ml PMA plus 1μM ionomycin can induce up to 7.9% of double IFNγ+IL-4+ (type 0) cells (this is shown and extensively discussed in Chapter 5 of this thesis), whereas this concentration of mitogens induce a 0.0, 0.1 or a maximum of 0.5% of IFNγ+IL-4+ type 0 naïve T cells from CB (Figure 4.4).

Importantly, the unveiled capacity of naïve cells from CB to produce cytokines also reveals their ability to develop into type 1 or type 2 T helper and cytotoxic phenotypes. This capacity is relevant to the use of CB as a source of stem cells for transplantation as the capacity of CB cells to develop the GvHD cytokine storm reaction (dominated by type 1 cytokines) has recently been questioned (Cohen, Wang et al. 2000). Although it has been reported that allogeneic stimulation can induce the development of IFNγ producing cells at higher frequencies in CB samples compared with adult samples (Matthews, Wadhwa et al. 2000), this study used broadly mismatched APC as stimulators to drive the allogeneic reaction, whereas it has been reported that a low frequency of cells from CB cells can induce GvHD like reactivity towards an stimulator mismatched in not more that six HLA mismatches (Wang, Sviland et al. 1998). Whereas Matthews et al study gives an indication of the capacity of CB cells to become high IFNγ producers, Wang et al 's work is a functional assay of the reactivity of CB and adult cells to induce GvHD-like reactions in vitro. These studies reached different but not excluding conclusions: Matthews et al proposed that a high frequency of CB cells have the capacity to be high IFNγ producers compared with adult cells and Wang et al concluded that CB cells have a high frequency of allogeneic-tolerant cells. As clinical results indicate that aggressive allogeneic reactions are less frequent with CB compared.
to bone marrow cells from adults, assays that use allogeneic antigens to induce cytokine production (such as Wang’s study) provide more information relevant to the clinical study of CB derived cells.

4.5 Future experiments

The results from this chapter indicate that type 1 and type 2 T cells, but not type 0 T cells, can be found in naïve CD45RA+ T derived from CB. Using PMA and ionomycin, Chipetta et al found a low frequency of type 0 cells from adult blood. Because a with a comparative study of the frequency of type 0 cells in CD45RA T cells from CB and adult using with purified populations stimulated with various concentration of mitogens has not been done, this can be considered for future work. This is because Th1 or Th2 polarised populations can be obtained using cells from CB (Sornasse, Larenas et al. 1996) that may be due to the capacity of these cells to produce type 1 or type 2 cytokines but not both simultaneously. The determination of the frequency of IL-10 producing cells can inform of further differences in the profile of cytokine expression in CB derived cells, as a subset of CD4+ IL-10 producing cells that produce only low amounts of IL-2 have been isolated from CB (Groux, O'Garra et al. 1997). The determination of frequencies of IL-10 producing cells by flow cytometry of can be done by staining at later times or alternatively, the cytokine production can be compared ELISA.

Additionally, a direct comparison of the capacity of CD45RA+ cells from CB or adult blood to become type 1 or type 2 cells under polarising conditions has not been done. Yang et al obtained naïve cells from CB and adult blood and cloned them with anti-CD3 and anti-CD28 stimulation plus IL-2 and found that all the clones did not produced IFNγ but produced IL-4 and IL-5 at high levels (Yang, Byun et al. 1995). Webb et al showed that CB derived naïve cells have the capacity to become type 2 cytokine producers after driving the cells for 7 days with anti-CD3 plus anti-CD3 Ab and IL-4, stimulating them with PMA (10μg/ml) plus Ionomycin (1μg/ml) to induce cytokine secretion (Webb and Feldmann 1995), but the authors did not compared the capacity of CD45RA+ T cells from CB and adult blood to become cytokine producers. Sasama et al compared the ability of CD4+CD45RA+ and CD4+CD45RO+ T cells from adults to develop a Th1 or Th2 profile after culture in PMA (10ng/ml) and ionomycin (400ng/ml) in the presence of IL-4 and found that at re-stimulation with PMA plus ionomycin, CD45RA+ were not developing a type 2 profile based in the low secretion of IL-4 (Sasama, Vyas et al. 1998). In spite of the documented importance of CD28 stimulation in naïve cells to induce the development of a type 2 phenotype (Webb and Feldmann 1995; Rulifson, Sperling et al. 1997), Sasama et al did not stimulate the naïve or
CD45RO⁺ cells with CD28 and the results from this study may only reflect the
CD45RO⁺ cells responsiveness to cytokines and mitogens. King et al have published a
study where the presence of CD28 re-stimulation in the cell cycle was found crucial for
IL-4 and IFNγ secretion by CD4⁺ T cells from CB and adults (King, Stupi et al. 1995).
However, King et al did not separate naïve and not naïve cells by any criteria and
therefore the adult CD4⁺ used may have been already primed cells. Furthermore, these
authors did not directly compare the capacity of IFNγ and IL-4 production or the
development of type 2 cells in CB and adult samples (King, Stupi et al. 1995). Although
there is growing evidence that naïve cells from CB can become efficient cytokine
producer cells that can support their own development into different T helper
phenotypes (Matthews, Wadhwa et al. 2000), the different ability between CD45RA⁺
from CB or adult blood to be polarised remains unsolved.

Different stimulation may deliver a signal of different strength in naïve cells from CB or
adult blood, and thus in the capacity of the development of different Th phenotypes. It
has been proposed that Th2 differentiation in adult cells is inhibited by TCR stimulation
(Schulze-Koops, Lipsky et al. 1998), which may be because strong stimulation favours
the development of IFNγ producing cells (Rogers and Croft 1999) but this has not been
explored in cells derived from CB. Therefore, the work described in this chapter can be
extended by directly comparing the development of CD45RA T cells from CB and adult
blood into different T cell phenotypes with different developmental conditions. This
can include T cell differentiation with CD3 plus CD28 stimulation (with Ab as done
some studies or with CD80 transfected cells (Webb and Feldmann 1995; Yang, Demeure
et al. 1995; Rulifson, Sperling et al. 1997). OX40 and CD40L stimulation can also be
studied, as it has been shown that OX40 enhances the maturation of naïve cells into high
IL-4 producers and CD40L stimulation that lowers the thresholds for the development
different cytokine phenotypes in T cells (Peng, Kasran et al. 1996; Ohshima, Yang et
al. 1998). Alternatively, Th differentiation can be induced with allogeneic APC, that can
provide a more complete stimulation compared with Ab and is more relevant to the
study of CB as a source of stem cells for transplantation. The selection of determined
HLA- mismatches can be included to correlate a stimulation threshold for CB cells to
induce frequencies of cytokine producing cells and to compared this threshold with adult
cells.

As mentioned, the results indicate that CB cells are more fragile to high concentrations of
ionomycin compared with adult cells from periphery (Figure 4.5). The viable dye
ethidium monoazide can be used to exclude dead cells from analysis, as stains and
binds covalently to DNA after exposure to light (Riedy, Muirhead et al. 1991). As the
cells positive for the dye are excluded from the analysis gate, this dye can be included in future work to assure analysis in the live population that produce cytokines.
5. Chapter 5. Analysis of the role of the CB microenviroment on T cell function.

5.1 Introduction

As extensively discussed in the previous Chapters, the major theory regarding the reduced immunological response of CB T cells are that these cells are naïve, not primed for activation and therefore defective or unable to respond to stimulus. However, in Chapters 3 and 4 it was shown that a high frequency CB derived CD45RA⁺ T cells can produce IL-2, IFNγ and IL-4 under certain stimulatory conditions and has been suggested that CB T cells have a high threshold for activation compared with T cells at other developmental stages (Bogunia-Kubik, Perez-Cruz et al. 2000). In addition to characteristic intrinsic to the cells, the microenvironment the CB mononuclear cells have been taken from is different from that of the adult PBMC and it is probable that it is, at least in part suppressing the immune response. Thus, the lower responsiveness of CB cells to stimulus proliferate capacity and cytokine production of T cells isolated from CB compared to adult cells may also be due to the environment the cells exist in.

CB serum is known to have different soluble factors compared to adult serum. Although only few studies have directly compared the cytokine profiles of cord and adult sera, the literature suggest that there are differences between CB and adult serum cytokine levels. TGFβ, IFNγ (Webb, Bochan et al. 1994), IL-10 (Hata, Kawamura et al. 1997), IL-6 and erythropoietin (EPO) (Westgren, Ek et al. 1995), are found at lower levels in CB compared with adult. IL-4 has been detected in 47.6% of CB serums (Hata, Kawamura et al. 1997) and higher levels of granulocyte macrophage colony stimulating factor (GM-CSF) have been found in CB sera compared with adult sera (Westgren, Ek et al. 1995).

In this Chapter, the role of CB serum factors on T cell function was studied and the role of CB sera in inhibiting T cell proliferation was analysed. In particular macrophage colony stimulating factor (M-CSF) in the CB sera inhibited T cell proliferative responses. These results could explain why, at least in part, CB lymphocytes have a reduced immunological response immediately after removal from the placental cord, compared to adult lymphocytes.
5.2 Material and methods

5.2.1 Antibodies

Anti-CD3 Ab used to stimulate T cells (OKT3) was made in house as described in Appendix Section 10.3.

Anti-CD3-PercP was obtained from Becton Dickinson UK Ltd (Oxford, UK), anti-CD3-FITC was obtained from Serotec (Oxford, UK) and anti-CD25-PE was obtained from Harlan Sera-Lab Ltd (Loughborough, UK). All antibodies used to stain for intracellular cytokines (and their isotype matched controls) were all obtained from Pharmingen (Cambridge Biosciences). The anti-cytokine antibodies were: anti-IL-4-PE and anti-IFNγ FITC.

Anti-MSCF Ab (for M-CSF neutralisation) and the isotype matched control Ab (both Mouse IgG2a) were obtained from R&D systems (Abingdon, UK).

5.2.2 Blood, sera and cells

CB was obtained from normal and caesarean full-term deliveries by puncturing the umbilical vein (Domínguez, Madrigal et al. 1998). All mothers had a normal pregnancy history. The reason for the caesareans was either the baby was too big, the mother had a previous caesarean or that it was convenient for the mother. Peripheral blood was collected from healthy adults of both sexes asuffy coats as described in Chapter 2 Section 2.2.1.

PBMC’s were separated from buffy coats by standard Ficoll separation as described in Chapter 2 Section2.2.1.

T cell lines. PBMC were plated at 10^6/ml of complete medium plus 20ng/ml IL-2 and a previously determined optimal concentration of anti-CD3 Ab (OKT3 culture supernatant) for 7 days. Cells were then supplemented with fresh medium and 20ng/ml IL-2 for another 7 days. At the end of this 14 day cycle the resulting T cells were placed with irradiated PBMC (as described in Chapter 2 Section 2.3.2) plus anti-CD3 Ab and IL-2 for 7 days and then supplemented with fresh medium and 20ng/ml IL-2 for another 7 days. Cells were maintained on this 14 day cycle. T cells were always used in assays at day 14 of the cycle, when the T cell population is always >90%.
Sera. Blood (CB or peripheral blood taken from healthy adults) was placed at 37°C for 1h. The blood clot obtained was then spun down at 1800 rpm for 20 minutes and the serum removed from the upper layer. Normal human AB' sera (to grow cell lines) was obtained from the North London Blood Transfusion Centre, London.

5.2.3 Proliferation assay

Adult PBMC: 1x10^6 viable PBMC/well were plated in serum free medium in 96 well plates with 5 x 10^4 irradiated PBMC's plus varying concentrations of CB or adult sera. In some cases recombinant M-CSF (a donation from Dr M. Wadhwa at the National Institute of Biological Standards and Control; NIBSC, Herts, UK), anti-MSCF Ab (10µg/ml) or an isotype matched control Ab (10µg/ml) was added to the cultures. The final volume was always 200µl. Cultures were maintained for 6 days with alloantigen, at 37°C in 5% CO₂ and pulsed with 0.5 µCi /well of methyl ³H-thymidine 18 hours before harvesting onto glass fibre filters, to measure proliferation as described in Chapter 2 Section 2.4. All cultures were performed in triplicate.

Human T cell line. 2x10^4 viable T cells/well were plated in RPMI medium plus 10 IU/ml penicillin and 10mg/ml streptomycin in 96 well plates. Some plates were previously immobilised with a predetermined optimal concentration of anti-CD3 Ab (See Appendix Section 10.4.). Alternatively T cells were stimulated with IL-2 (10ng/ml). All T cell stimulation's were done in the presence or absence of varying concentrations of CB or adult sera. The final volume was always 200µl. Cultures were maintained for 3 days at 37°C in 5% CO₂ and pulsed with ³H-thymidine 18 hours before harvesting as described in Chapter 2 Section 2.4. Scintillation counting was performed as described above. All cultures were performed in triplicate.

The proliferation of cells of the CTLL-2 T cell line was done plating 1x10^4 viable CTLL-2 cells/well in CTLL-2 medium with a predetermined optimal concentration of IL-2 ± varying concentrations serial dilutions of CB or adult sera. Cultures were maintained for 24 hours at 37°C in 5% CO₂ and pulsed with 0.5 µCi /well of ³H-thymidine 8 hours before harvesting onto glass fibre filters as described in Chapter 2 Section 2.4.1. Scintillation counting was performed immediately. All cultures were performed in triplicate.

5.2.4 Analysis of CD25 expression

1x10^5 viable PBMC/well were plated in serum free medium in 96 well plates with either 5 x 10^4 irradiated PBMC's (Chapter 2 Section 2.3.2) or an optimal concentration of PHA with CB or adult sera. The final volume was always 200µl.
Cultures were maintained at 37°C in 5% CO₂. Cells were then spun down at the end of incubation (time points are defined in the results Section) and assessed for expression of the IL-2 receptor α chain (CD25) on the T cell population.

Cell staining, acquisition and analysis was done as described in Chapter 2 Section 2.7.1. Cells were gated on live CD3⁺ cells. Data is expressed as the percentage of CD3⁺ cells which express CD25.

5.2.5 Analysis of the frequency of IL-2 producing T cells

1x10⁶ PBMC/ml were plated in RPMI plus antibiotics (as described above) in 1:10 serum and a predetermined optimal concentration of PHA (Appendix Section 10.2). Cultures were maintained in a humidified atmosphere at 37°C for 7 days. Cells were spun down and restimulated with PMA and ionomycin in the presence of monensin, as described in Chapter 2 Section 2.5, to induce cytokine production. After 6 hr incubation at 37°C in a humidified atmosphere, the cells were taken out of the wells and stained extracellularly for the CD3 marker and intracellularly for IL-2 cytokines as described in Chapter 2 Section 2.7. The cells were always stained immediately. The live CD3⁺ gated lymphocytes were analysed and the percentage of T cells producing IL-2 was assessed by the amount of anti-cytokine Ab staining which was above the staining of the negative control antibodies, as described in Chapter 2 Section 2.7.5.

5.2.6 Activation of T cells to analyse IL-2 protein production

1x10⁶ PBMC/ml were plated in serum free medium (as described above) in 1:10 dilution of serum and a predetermined optimal concentration of PHA (Appendix Section 10.2). Cultures were maintained in a humidified atmosphere at 37°C for 24 hours (the peak in IL-2 protein detection (Cohen, Parry et al. 1997)). Cells were spun down and the supernatant assayed for IL-2 by ELISA. The corresponding serum dilution's were also assayed for cytokine.

5.2.7 Detection of cytokines

The IL-2 ELISA was a commercial kit obtained from Diaclone Research, (Besançon, France) and used as described by the manufacturer, the detection limit of this assay was 10pg/ml.

IL-10 was detected using an ELISA, as previously described (Mullins, Cohen et al. 1995), the JES3-9D7 Ab (DNAX, Palo Alto, CA) was used for capture and the JES3-
12G8 biotinylated Ab (DNAX) for detection. The WHO reference reagent for IL-10 (93/722) was from NIBSC. The assay detection limit was 20pg/ml.

IL-13 was detected using the TFI cell line, as described (Wadhwa, Bird et al. 1995). The WHO reference reagent used as a control for IL-13 (94/622) was from NIBSC. The detection limit of the assay was 100pg/ml.

M-CSF was detected using the MNFS-60 bioassay (Wadhwa, Bird et al. 1995). The WHO international standard for M-CSF (89/512) was from NIBSC. The detection limit of the assay was 50pg/ml.

5.2.8 Statistical analysis

All samples were compared by Students-test using two tailed analysis, unless stated otherwise.
5.3 Results

5.3.1 The effect of adult and CB sera on the CTLL-2 cell line

The IL-2 dependant cell line CTLL-2 proliferated to IL-2 in the presence and absence of the different sera (Figure 5.1). However at low concentrations (dilution of 0.0004 and 0.004) the adult sera enhanced this IL-2 dependant proliferation. This increase was not seen with equivalent concentrations CB sera. With increasing concentrations of both sera there was a decrease in the IL-2 specific proliferate response, presumably due to inhibition of proliferation by excessive concentrations of protein. This response was always lower with the CB sera. At all dilution’s of sera there was a statistically significant difference in the proliferative response of the CTLL-2 cells when comparing assays performed in CB and adult sera (P<0.005 in all cases). Thus, the proliferative response of the CTLL-2 lines is inhibited by CB sera (Cohen, Morgan et al. 2000).

5.3.2 The effect of adult and CB sera on human T cell lines

To determine the effect of the different sera on pure T cells the ability of T cell lines to proliferate to immobilised anti-CD3 in the presence of the different sera was assessed. The proliferative response of a pure T cell line increased with increasing concentrations of adult sera, however CB sera had no effect (Figure 5.2). Indeed at each dilution greater than 1:1000 the difference between the proliferation in the presence of adult and CB sera were significant. In the experiment represented in Figure 5.2 there is wide error at 1:2 dilution as 1/8 CB serum did enhance the anti-CD3 specific proliferative response.

Thus, on memory T cells stimulated through the T cell receptor adult serum can act as a growth factor, whereas CB serum had no effect. In the absence of anti-CD3 stimulation there was no T cell proliferation, regardless of the presence of different sera.

The effect of the different sera on the IL-2 specific response of activated T cell lines was next analysed. When IL-2 was added to the T cell lines the cells proliferated as expected (Figure 5.3 Panel A). However this was not significantly increased with the addition adult sera. This was expected as the major growth factor in this assay was IL-2 and not the serum. Unexpectedly, when the cells were stimulated with IL-2 and different CB sera, the proliferative response increased, with a peak in response at a dilution of 1:100 CB sera. The differences in the proliferative responses in the
presence of cord and adult sera were significant at dilution's of 1:10 (P<0.005), 1:100 (P<0.005), and 1:1000 (P<0.05) (Figure 5.3 Panel B). This experiment was repeated 5 times and in each case a similar profile of response was seen. Thus CB sera enhanced the IL-2 specific proliferative response of human T cell lines.
Figure 5.1. Effect of different sera of the IL-2 specific response of CTLL-2 cells. 1x10^4 viable CTLL-2 cells/well in RPMI medium were plated with a predetermined optimal concentration of IL-2 ± varying concentrations serial dilutions of CB or adult sera as described in Chapter 2 Section 2.4.2. Results are expressed as the mean counts per minute of the 8 adult and 8 cord blood sera + SD.
Figure 5.2. Effect of different sera on the mitogen specific proliferation of pure T cells. 2x10⁴ viable T cells/well were plated in RPMI medium plus antibiotics in 96 well plates immobilised with a predetermined optimal concentration of anti-CD3 Ab ± varying concentrations of CB or adult sera. Cultures were maintained for 3 days then pulsed with methyl ³H-thymidine 18 hours before harvesting onto glass fibre filters, as described in Chapter 2 Section 2.4. All cultures were performed in triplicate. Results are expressed as the mean of 8 adult or 8 CB + SD. This figure is representative of 3 experiments. *P<0.05, **P<0.005 comparing adult and human sera by Student's t-test.
Figure 5.3. Effect of different sera on the IL-2 specific proliferation of a human T cell line. Human T cells were plated at $5 \times 10^4$ cells/well with varying concentrations of adult and CB sera plus 10ng/ml rIL-2. All cultures were incubated in a humidified chamber at 37°C, 5% CO$_2$ for 3 days. The cells were pulsed with 1μCi/well of methyl 3H thymidine 18 hours before harvesting on to glass fibre filters, and the proliferation measured by the incorporation of 3H thymidine. A. 3H thymidine incorporation of IL-2 stimulated T cells incubated with individual adult and CB sera. B. Mean 3H thymidine incorporation of all adult and CB sera + SD. This figure is representative of one of 5 experiments. *P< 0.05, **P< 0.005 comparing adult and human sera by Student’s t-test.
5.3.3 The effect of adult and CB sera on the mixed lymphocyte reaction

As expected there was no alloresponse until sera were added to provide essential growth factors. With the addition of increasing concentrations of adult sera, there was an increase in the proliferation of the responder cells (Figure 5.4 Panel A). At dilutions greater than 1:100 all adult sera enhanced the allospecific proliferative response. This increase in proliferation was also seen when cells were incubated with some CB sera, but the response was always lower than with adult sera. In 3/8 adult sera the response peaked at a dilution of 1:10 and then declined. With the addition of CB sera there was a peak in response at this dilution with only 1/8 serum. The differences in the proliferative responses in the presence of adult and CB sera were significant at dilutions of 1:10 and 1:4 (Figure 5.4 Panel B). Thus there was a reduced alloproliferative capacity of adult mononuclear cells in the presence of CB sera when compared to adult sera.
Figure 5.4. Allogeneic stimulation of PBMC in the presence of adult and CB serum. PBMC’s (responder cells) were incubated with irradiated PBMC’s (stimulator cells) at a ratio of 2:1 (1 x 10^5 : 5 x 10^4 cells/well), with varying concentrations of adult and CB sera. All cultures were incubated in a humidified chamber at 37°C, 5% CO₂, for 6 days. They were then pulsed with 1μCi/well of methyl 3H thymidine 18 hours before harvesting on to glass fibre filters, and the proliferation measured by the incorporation of 3H thymidine. All cultures were performed in triplicate. A) 3H thymidine incorporation, measured in counts per minute of PBMC incubated with individual adult sera and CB sera. B) Mean 3H thymidine incorporation of all the adult sera and CB sera. This figure is representative of 5 experiments each containing between 8 and 10 samples. **P< 0.005, ***P< 0.0005 comparing adult and human sera by Student’s t-test.
5.3.4 The expression of activation markers on adult PBMC following allostimulation in the presence of adult and CB sera

To determine whether the sera were effecting CD25 expression on T cells, adult PBMC were activated with alloantigen in the presence or absence of different dilution’s of various sera and analysed the CD25 expression on CD3+ gated cells after 6 days of incubation (Figure 5.5). For both serum dilution’s analysed (1:100 and 1:1000) there was statistically significant difference for CD25 expression on T cells between cells incubated in the presence of adult and CB sera. Thus incubation in CB sera enhanced the CD25 expression on T cells in comparison to cells stimulated in the presence of adult sera.

Because alloactivation requires 6 days of culture for an optimal response and gives a milder proliferative response than mitogen stimulation it is possible that the effect of the different sera using the assay described above could have not been detected. Therefore, the experiments described above were repeated using the mitogen PHA as a stimulus. In addition, it was determined whether the sera were effecting CD25 expression on T cells after activating in the presence of the different sera over a range of time points. Figure 5.6 show the change in CD25 expression after PHA activation on CD3+ gated cells after incubation in a 1:100 dilution of adult or CB sera. As with allostimulation there was an increased expression of CD25 on the T cells which had been incubated in the CB sera compared to those cells which had been incubated in the adult sera. This was seen at 2, 6 and 168 hours and was statistically significant.
Figure 5.5. Expression of IL-2 receptor α chain (CD25) on adult PBMC's following allostimulation in the presence of adult and CB sera. Adult PBMC's (responder cells) were incubated with or without irradiated allogeneic PBMC's (stimulator cells) at a ratio of 2:1 in the presence of different concentrations of adult and CB sera. The cells were incubated in a humidified chamber at 37°C in 5% CO₂ for 6 days. Cells were then double-stained for CD3 and CD25. The cells were analysed by flow cytometry using a FACScan. Results are expressed as the percentage of CD25 expressed on T (CD3⁺) cells. This figure is representative of 3 experiments. P values were obtained by comparing cells incubated in adult and human sera by Student's t-test.
Figure 5.1. The effect of different sera on CD25 expression on T (CD3) cells stimulated with PHA. 10⁵ adult peripheral blood mononuclear cells were plated per well in a 96-well plate. Cells were stimulated with a pre-determined optimal concentration of PHA (1/1000) and incubated in 1:100 dilution of cord or adult serum at 37°C in 5% CO₂. At different time points, a plate was removed and the cells stained extracellularly for CD3 and CD25 as described in materials and methods. The cells were analysed by flow cytometry. Results are expressed as the percentage of CD25 expressed on T (CD3⁺) cells. P-values were: 2 hours=0.0039, 6 hours= 0.0052 and 168 hours=0.0007. These values were obtained by comparing cells incubated in adult and cord sera by the Students t-Test.
5.3.5 The effect of adult and CB sera on the cytokine production from stimulated adult PBMC

Because the different sera may effected T cell cytokine production which would explain the differences in the T cell functions, the IL-2 production of T cells resulting from 24 hour incubation with PHA in the presence and absence of different sera was analysed. To verify that the production of cytokine by stimulated T cells and not derived from the different sera was assayed, the IL-2 within the dilution's of sera used for the experiment was also assayed. An ELISA was used to measure the cytokine levels, as the proliferative response of the CTLL-2 cells used for the IL-2 bioassay is inhibited by CB sera (Figure 5.1) Not significant differences were detected in the IL-2 production between PBMC stimulated with the CB or adult sera. The mean ± SD IL-2 production by the stimulated PBMC incubated in adult sera (n=10) was 1612.7 ± 576.6 pg/ml compared to 1519.0 ± 888.0 pg/ml when the cells were incubated in CB sera (n=10). No IL-2 was detected in any serum. To verify these results the frequency of IL-2 producing T cells after stimulation in adult and CB sera was analysed (Figure 5.7). Again, the CB sera did not inhibit IL-2. Indeed the frequency of IL-2 producing cells was higher in populations which had been stimulated in the presence of CB sera compared to adult sera and this difference was statistically significant.
Figure 5.7. Analysis of the frequency of IL-2 producing T cells. PBMC/ml were plated in RPMI plus antibiotics in 1:10 serum and a predetermined optimal concentration of PHA. Cultures were maintained for 7 days. Cells were then restimulated with phorbol-12-myristate-13-acetate plus soluble Ionomycin and monensin (3 µM; Calbiochem). After 6 hr incubation, the cells were taken out of the wells and stained extracellularly for CD3 markers and intracellularly for IL-2 cytokines (as described in Chapter 3 Figure 3.2). Live CD3+ gated lymphocytes were analysed and the percentage of T cells producing IL-2, assessed by the amount of anti-cytokine Abstaining which was above the staining of the negative control antibodies. Statistically analysis compared the samples incubated in adult sera compared to CB sera by Paired t-test.
5.3.6 The effect of adult and CB sera on the frequency of cytokine producing adult PBMC

To determine whether the CB sera effected the frequency of cytokine producing T cells, PBMC were stimulated with alloantigen, in the presence or absence of various dilutions of different sera. Then at day 6, the T cells were further stimulated with PMA plus ionomycin for 6 hours and subsequently assessed for intracellular cytokines and analysed on gated T (CD3+) cells. The changes in the frequency of IFNγ and IL-4 producing T cells were analysed. When cells had been incubated in CB sera the frequency of T cells which produced IFNγ or IL-4 was reduced compared to when the cells had been incubated in the presence of adult sera (Figure 5.8). This difference was statistically significant by 1 tailed student’s t-test at 0.001 dilution for IL-4 and 0.01 dilution for IFNγ (P<0.05 in both cases).
Figure 5.8. Intracellular cytokine production by PBMC after activation in the presence of adult and CB sera. PBMC’s (responder cells) were incubated with irradiated PBMC’s (stimulator cells) at a ratio of 2:1 with varying dilutions of adult and CB sera. Cultures were incubated in a humidified chamber at 37°C, 5% CO₂ for 6 days. Cells were then washed and stimulated for 6 hours with mitogen and stained extracellularly for CD3 and intracellularly for IFNγ or IL-4. The results are expressed as the percentage of T cells which produce IFNγ or IL-4. *P<0.05 by 1 tailed Student’s t-test.
5.3.7 Serum cytokines

There has been much analysis of the cytokines within CB sera (for review see (Perez-Cruz, Bogunia-Kubik et al. 2000)). However the present literature does not explain the differences in the function of the sera noted here. To determine whether there was an anti-inflammatory cytokine within the CB sera which could be causing, at least some of the results that we have described, serum analysis of IL-10 (which inhibits T cell function, both indirectly (de Waal Malefyt, Haanen et al. 1991) and directly (de Waal Malefyt, Yssel et al. 1993). IL-13 (which has been shown to inhibit some T cell function, predominantly by inhibition of macrophage presentation (Cash, Minty et al. 1994) and M-CSF (which plays a major role in placental development and function (Jokhi, Chumbley et al. 1993) and can inhibit alloreactivity in the mouse (Sakurai, Yamada et al. 1996) were done. Figure 5.9 shows that IL-13 could not be detected in any of the sera analysed, whereas IL-10 could be detected in adult (mean ± SD was 104.0 ± 55.5 ng/ml) but not in CB sera and this difference was statistically significant (P<0.002). There was also a significant difference in the levels of M-CSF detected in adult and CB sera (P<0.01). M-CSF was detected in 6/7 CB sera (mean ± SD was 3.8 ± 2.3 ng/ml) and 0/5 adult sera. IL-2 is well recognised as an important T cell growth factor. The level of IL-2 in the sera was also assayed to determine whether there was less IL-2 in CB compared to adult sera. As with IL-13, IL-2 was not detect in any of the sera analysed (Figure 5.9).
Figure 5.9. Concentration of cytokines in different serum. The presence of cytokines in different CB and adult sera were determined by bioassay and ELISA (as described in the materials and methods Section). * P<0.01 and **P<0.002 comparing adult and CB sera by Student’s t-test.
5.3.8 Role of M-CSF in CB sera

M-CSF has been shown to inhibit the mouse allospecific response (Sakurai, Yamada et al. 1996). To determine whether M-CSF could inhibit the human allosresponse, increasing concentrations of recombinant M-CSF were added to a mixed lymphocyte reaction. Figure 5.10 shows that with increasing concentrations of M-CSF a mixed lymphocyte reaction could be inhibited. However this result was not always consistent and we only achieved inhibition with M-CSF (>1ng/ml) in 4 out of 6 assays.

To determine whether M-CSF in the CB sera played a role inhibiting the allogeneic response, anti-M-CSF neutralising Ab (or an isotype matched control) were added to MLR incubated in CB sera (Figure 5.11). There was a statistically significant increase (P<0.05 by paired t-test) in the proliferative response of PBMC’s which had been incubated in CB sera when anti-M-CSF Ab was added, compared to cells incubated in the presence of an isotype matched control. Anti-MCSF antibodies in the presence of adult sera were also tested but the results were inconsistent. In some experiments there was an increase in the allosresponse with anti-MCSF antibody, but not with control Ab (as seen with the CB sera), but in other experiments this increase was not seen. Thus, the increase in the allosresponse seen with anti-MCSF Ab and some adult sera may be due to the anti-MCSF Ab neutralising endogenously produced M-CSF (i.e. M-CSF which is produced as a result of the alloreaction).
Figure 5.10. M-CSF can inhibit the human alloresponse. $5 \times 10^4$ responder cells were incubated with M-CSF for 2 hours at 37°C in 5% CO$_2$. Irradiated stimulator cells were then added to the plates which were subsequently incubated at 37°C in 5% CO$_2$ for 6 days. These were pulsed with 20°C of methyl $^3$H Thymidine, before harvesting on to glass fibre filters and were incubated for a further 6-8 hours and then frozen overnight. $^3$H Thymidine incorporation was measured using a Scintillation counter. The results represent the mean of the triplicates at each M-CSF concentration + SD.
Figure 5.11. The role of M-CSF in CB sera. To determine whether M-CSF in the CB sera played a role inhibiting the allogeneic response anti-M-CSF neutralising Ab (or an isotype matched control (Ctr) Ab) was added to an MLR incubated in 1:10 CB sera (as described for Figure 5.4). The results represent the mean of the thymidine incorporation (as a parameter of proliferation) in triplicate cultures of cells incubated in different sera (in the presence of the control Ab or the M-CSF neutralising antibody). Statistical analysis was performed using a paired t-test.
5.4 Discussion

The results from this Chapter indicate that there are differences in the soluble factors present in CB and adult serum and that these differences play a role in T cell function. The alloantigen specific proliferative response of adult T cells was increased with increasing concentrations of both adult sera and CB sera, but to a lesser extent with CB sera. When the effect of the sera on pure T cell lines was analysed, the proliferation induced by stimulation through the T cell receptor alone (via CD3 stimulation) was enhanced with adult but not CB sera. However, CB sera enhanced the IL-2 specific proliferative response of pure T cells whereas adult sera did not, possibly due to up-regulation of CD25.

In support of this is the observation that CB lymphocytes have been shown to be more sensitive to the effect of IL-2 stimulation. For example, CB exhibits higher levels of non-specific lysis of tumour cell targets than PBMC in response to high concentrations of IL-2. At lower IL-2 doses CB is more responsive than adult PBMC to non-specific lysis (Harris 1995). In addition NK and LAK cells in CB express more IL-2 receptor compared to the equivalent cells in adult blood (Harris 1995). Thus, the data of this Chapter suggests that this may be due to the presence of, as yet unidentified soluble serum factors.

It was assumed that the CB serum was acting by inhibiting IL-2 production (thereby inhibiting T cell proliferation in response to T cell receptor stimulation (mitogen or alloantigen) and internalisation of the IL-2 receptor (CD25), thus increasing CD25 expression on the cell surface, enabling pure T cells to respond to exogenous IL-2 but there was not any evidence of this. Using intracellular cytokine staining, an increasing the frequency of IL-2 producing T cells after stimulating cells in the presence of CB sera compared to stimulating them in adult sera was obtained. Thus, the mechanism of action can not be fully explained.

There are other soluble factor differences reported between adult and CB. Within CB sera there is more soluble IL-2 receptor (Lencki, Maciulla et al. 1994), 1,25-dihydroxyvitamin D, r-T3, thyroid stimulating hormone (Sulovic, Siljak et al. 1984), soluble HLA class I and less TGF-β (Webb, Bochan et al. 1994) than in adult sera. However, none of the differences seen in the literature explain the findings of this Chapter. Nevertheless, there are cytokines which have similar, but not identical function. For example IL-10 will suppress T cell activation by a variety of mechanisms. It inhibits antigen presenting cell function to the T cell (Fiorentino, Zlotnik et al. 1991; Ding and Shevach 1992) and also inhibits IL-2 production by pure
T cells (de Waal Malefyt, Yssel et al. 1993). However, in activated T cells, IL-10 will synergise with IL-2 to enhance T cell proliferation by up-regulation of CD25 (Cohen, Katsikis et al. 1994). Although this is very similar to what has been observed in these experiments, the factor present in CB sera can not be IL-10 as the CB sera did not inhibit the alloresponse or mitogen specific T cell proliferation, it just did not enhance the response (which adult sera did). Furthermore the CB serum did not inhibit T cell IL-2 production. Lastly, but most conclusively, we did not detect any IL-10 in the majority of CB sera tested.

Since there are cytokines which have similar, but not identical function to CB sera (such as IL-1 (Fiorentino, Zlotnik et al. 1991; Ding and Shevach 1992; de Waal Malefyt, Yssel et al. 1993; Cohen, Katsikis et al. 1994) other cytokines were studied to determine if they could be responsible of the observed effect of CB sera.

M-CSF has been shown to inhibit the mouse allospecific response (Sakurai, Yamada et al. 1996). This cytokine is a disulphide-bonded glycoprotein, which is encoded by a single 20kb gene on the short arm of chromosome 1 (Saltman, Dolganov et al. 1992). The protein is a dimer, with each sub-unit consisting of a bundle of four α-helices with an inter-chain bond. It is one of the few cytokine/growth factors that has been found in measurable concentrations in various tissues including plasma, urine, cerebrospinal fluid and placenta (Ralph and Sampson-Johannes 1990). However, M-CSF was difficult to detect in human sera whereas 6/7 of the CB sera had between 1-10ng/ml M-CSF. We showed that M-CSF could inhibit the human alloresponse and that neutralising the M-CSF in CB sera restored the allogeneic response of the PBMC (Figures 5.9 and 5.10). However, the presence of M-CSF in the CB sera does not explain the observation that the OKT3 specific proliferation of pure T cells was not enhanced with CB sera, whereas it was with adult sera (Figure 5.2). T cells do not express the receptor for M-CSF (Stanley, Berg et al. 1997) and therefore M-CSF in the sera could not have been acting on the pure T cell population. Thus, there is not only one soluble factor responsible for all the effects seeing or the CB sera. Other factors seen in abundance in CB sera (such as soluble CD25) may play a role.

Because CB sera had no effect on the IL-2 specific growth of the mouse IL-2 dependant T cell line CTLL-2 cells (Figure 5.1) (Cohen, Morgan et al. 2000), the effect of CB sera on the IL-2 specific response of activated T cells with a T cell line were studied. The results could be due to CB sera enhancing CD25 expression on the human T cells, but having no effect on the established CTLL-2 cell line. Although this has been attributed to the cells immaturity, it could equally be seen as a specialised response to the needs of the neonate. It has been shown here that by incubating adult
cells in CB sera (mimicking the foetal microenvironment) imitating this CB cell ‘phenotype’. These results suggest that the microenvironment that the cells are derived from could influence the cells proliferative response, keeping the neonatal T cells in a non-responsive state. These results suggest that CB sera may lack an activation factor which is present in adult sera and that there are suppressor factors (such as M-CSF) in CB sera which are absent in adult sera. Nevertheless, the role of other soluble factors within the sera has not been excluded, for example soluble CD25 within CB sera may play a role in the observed effects. Whether these results are sufficient to explain the lack of an alloresponse by the foetus to the mother or the reduced GvHD seen with CB transplantation (Madrigal, Cohen et al. 1997) is yet to be determined.

5.5 Future experiments.

The results from this Chapter and those published in the literature (discussed though this Chapter) suggest that the microenvironment from which the lymphocytes are derived may influence their activity, such as alloproliferation or their capacity to make cytokines. However, only few studies have characterised the differences between adult and CB serum.

To determine if the cells that have been in a CB environment are tolerised to alloantigens, more experiments must be done. After culturing cells in CB serum the cells must be removed and then returned to an “adult” environment (adult serum). With this experiment, it could be determined if the CB serum induced a long lasting effect in the alloreactivity of the cells. The CB and adult serum must be stored at 4°C to avoid degradation of soluble factors.

MCSF was identified as a cytokine that modulate the alloreactivity. In future work, its effects of Ag specific cells can be tested to confirm the induction of tolerance mediated by this cytokine. This work can also be extended by the study of the mechanisms responsible of this modulation of cell activity and the dissection of the effects of MCFS on different cell types. Additionally, this work can be extended by the analysis of other cytokines, such as IL-7, present in CB and adult sera. There is the possibility that novel factors in serum are found. Thus, a long term goal is to identify and ultimately isolate and characterise these serum factors for future use in stem cell transplantation. This work may enable us to identify the molecular mechanisms which are associated with a lower GvHD in CB compared to BM transplanted individuals.
6. Chapter 6. The frequency of type 1, type 2 and type 0 memory T cells depends on the stimulation used.

6.1 Introduction.

Naïve T cells encounter Ag in vivo in the context of MHC and get primed (Ebnet, Kaldjian et al. 1996). T cells then differentiate into cytokine producing cells of the type 1 or type 2 phenotypes depending on polarising conditions (Le Gros, Ben-Sasson et al. 1990; Hsieh, Heimberger et al. 1992; Hsieh, Macatonia et al. 1993; Manetti, Parronchi et al. 1993; Seder, Gazzinelli et al. 1993; Szabo, Jacobson et al. 1995; O’Garra 1998). These primed T cells can migrate to the site of inflammation (Mackay, Marston et al. 1992) and release cytokines and/or perform cytotoxic activities. Some of these cells become memory cells acquiring the ability to respond rapidly to a second challenge of the antigen (Bruno, Kirberg et al. 1995).

The Th1 and Th2 cell phenotypes described by Mosmann et al (Mosmann, Cherwinski et al. 1986) were well established memory murine CD4+ T cell clones, as discussed in Chapter 1 Section 1.6 and represent highly committed cells. Type 0 cells, that are not fully committed, have been found when T cells from peripheral blood are stimulated with mitogens (Maggi, Del Prete et al. 1988; Paliard, de Wall et al. 1988; Wierenga, Snoek et al. 1990; Parronchi, Macchia et al. 1991; Piccinni, Macchia et al. 1991) and can be maintained in vitro without losing their cytokine heterogeneity (Kelso and Gough 1988). At the single cell level, murine memory Th0 cells have also been described in studies where mitogenic stimulation is used to induce cytokine production (Openshaw, Murphy et al. 1995; Miner and Croft 1998). In contrast with these results, T cells derived from L. major infected mice did not display a Th0 phenotype when they were re-stimulated with Ag or mitogens and analysed at the single cell level (Sommer, Meixner et al. 1998). Thus, the functionality of Th0 cells as a differentiated subset of T lymphocytes is still controversial (Bucy, Karr et al. 1995).

The analysis of cytokines at the single cell level (Sanders, Andersson et al. 1991), simplifies the analysis of frequency of specific cells producing cytokine, by the detection of extracellular and intracellular antigens. Phorbol esters and calcium ionophores have been used in the ICS technique to enhance the cytokine detection (Schauer, Jung et al. 1996; Carter and Swain 1997). It is assumed that this stimulation does not change the cytokine profile of the T cell, but enables the detection of the cytokines that are
otherwise difficult to identify (Jung, Schauer et al. 1993). Thus, ICS reveals the potential of cytokine production of a cell. However, the strength of stimulation may determine the profile of cytokine production (Chaturvedi, Yu et al. 1996; Cohen, Webb et al. 1996; Schountz, Kasselman et al. 1996; Ausubel, Krieger et al. 1997) in spite of the specific regulation for the expression of cytokine genes in differentiated T cells (Penix, Weaver et al. 1993; Szabo, Gold et al. 1993; Todd, Grusby et al. 1993; Lederer, Liou et al. 1994; Young, Ghosh et al. 1994).

The aim of this chapter was to determine if the cytokine profile of memory T cells determined by ICS, can differ depending on the stimulation used. CD3⁺CD45RO⁺ memory T cell populations were used in this chapter because the cytokine profile of memory cells is more stable compared with other developmental stages (Perez, Lederer et al. 1995; Szabo, Jacobson et al. 1995; Murphy, K et al. 1996).

6.2 Material and Methods

6.2.1 Cells

T cell lines

Non specific T cell lines were generated by PHA stimulation using PBMC from healthy individuals in complete medium with a predetermined optimal concentration of PHA (as described in Chapter 2, Section 2.3.2). The cell lines were maintained in IL-2 for 7 days and then re-stimulated with IL-2, PHA and irradiated PBMC as feeders. At least two cycles were completed before T cell lines were used in experiments, when >99.0% of the cells were CD3⁺ and >98.0% were CD45RO⁺ (as explained in Chapter 2 Section 2.3.2). One of the lines was predominantly CD8⁺ (Line 1) whereas a second line was predominantly CD4⁺ (Line 2).

T cell clones.

Th1 clones (PK15, PIL 35) were derived by non specific stimulation with PHA or anti-CD3 Ab (Cohen, Katsikis et al. 1995). The cytokine production of two of these T cell clones had been previously characterised by anti-CD3 stimulation for 24 hrs and ELISA of the supernatants (see Table 6.1). The Th2 clone (122.1) used was a gift from Dr. C. Navarette (North London Blood Transfusion Centre) (see Table 6.2) and its cytokine profile was characterised by the percentage of IFNγ and IL-4 positive cells assayed by ICS (see Table 6.2). All T cell clones aliquots were stored at -120°C in N₂ and only
thawed once to be used. The cells were then expanded for one week with 20ng/ml IL-2 and then re-stimulated to determine their cytokine production.

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<th>Clone</th>
<th>IFNγ</th>
<th>IL-4</th>
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<tr>
<td>PK15</td>
<td>&gt;1000 pg/ml</td>
<td>&lt;40 pg/ml</td>
<td>Th1</td>
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<tr>
<td>PIL 35</td>
<td>5041 pg/ml</td>
<td>&lt;40 pg/ml</td>
<td>Th1</td>
</tr>
</tbody>
</table>

Table 6.1. Cytokine phenotype of Th1 cell clones. The cytokine profile of the PK15 and PIL35 CD4+ T cell clones was determined by Cohen et al (Cohen, Katsikis et al. 1995), using the following method: The cells were stimulated with immobilised anti-CD3 stimulation for 24 hrs and the supernatant was collected. The production of IFNγ and IL-4 was determined by ELISA and the clones were classified as Th1 according to the ratio of cytokines produced (pg/ml). These clones were frozen by standard methods (see Chapter 2 Section 2.6) upon reception until their use here.

<table>
<thead>
<tr>
<th>Clone</th>
<th>IFNγ</th>
<th>IL-4</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>122.12</td>
<td>2.4 %</td>
<td>62.0%</td>
<td>Th2</td>
</tr>
</tbody>
</table>

Table 6.2. Cytokine phenotype of Th2 cell clones. The cytokine profile of the 122.12 CD4+ T cell clone was determined by Dr C. Navarrete using the following method: After stimulation with an optimal dilution of anti-CD3 (OKT3) Ab and 20 U/ml IL-2, the clone was stained with anti-IFNγ and anti-IL-4 Ab and the frequency of cytokine producing cells was analysed by ICS. After this, the clone was classified as a Th2 according to the ratio of frequencies (expressed as the percentage of positive cells) of cytokines produced. The clone was frozen upon reception by standard methods (see Chapter 2 Section 2.6) until it use here.

6.2.2 Stimulation of T cells for intracellular cytokine detection.

5x10^5 cells/well from the T cell lines were placed in 48 well plates for stimulation with either immobilised anti-CD3 Ab (OKT3) plus immobilised anti-CD28 Ab (Pharmingen), immobilised anti-CD3 Ab plus PMA, or PMA plus ionomycin (as explained in chapter 2 Section 2.5) in the presence of 3μM monensin for 6 hr. The cells were then harvested and stained extracellularly with anti-CD3 and intracellularly with anti-IFNγ and anti-IL-4 or isotype matched control Ab (as described in chapter 2 Section 2.7.5).

5x10^5 cells/well from the T cell clones were stimulated with 5ng/ml PMA and 1μM ionomycin for 6 hrs in the presence of 3 μM monensin. The cells were then harvested and
stained extracellularly with anti-CD3 and/or anti-CD69 Ab (Chapter 2, Section 2.7.1f) and intracellularly with anti-IFNγ and anti-IL-4 or isotype matched control Ab.

6.2.3 Flow cytometric analysis

40000 events were acquired within 48 hours of staining in a FACScan flow cytometer (Becton Dickinson) and analysed using the Cell Quest Software (Becton and Dickinson). Antibodies for flow cytometry were anti-CD3-PercP (Becton and Dickinson) and all antibodies used to stain for intracellular cytokines (and their isotype matched controls) were all obtained from Pharmingen. Results are expressed as the frequencies of IFNγ, IL-4 or both IFNγ and IL-4 producing cells within the CD3+ gate in the T cell lines and the activated lymphocyte-gate in the T cell clones. All clones were CD3+.

To analyse the frequency of cytokine positive cells, a quadrant plot was set, where the negative values were defined by non-stained cells and by the staining of isotype matched control Ab (detailed in Chapter 2 Section 2.8.1). Staining with isotype control Ab was included for all the stimulations used.

6.2.4 Definition of type 1, type 2 and type 0 cells.

The frequencies of IFNγ (FL-1), IL-4 (FL-2) or IFNγ and IL-4 positive cells in the CD3+ activated lymphocyte-gate were determined for each sample of the CD45RO+ populations. The IFNγ positive cells were defined as type 1, the IL-4 positive cells were defined as type 2 and cells positive for both cytokines were defined as type 0 CD3+ CD45RO+ cells, as described in chapter 4 Section 4.3.1.
6.3 Results

6.3.1 Phenotype of non-specific T cell lines and clones

The two T cell lines used in this work were characterised by their expression of extracellular Ag by flow cytometry (see Table 6.3). To do this, a gate was set in the CD3⁺ lymphocytes to analyse the frequencies of CD4⁺, CD8⁺ CD45RA⁺ and CD45RO⁺ positive cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CD3(%)</th>
<th>CD4(%)</th>
<th>CD8(%)</th>
<th>CD45RA(%)</th>
<th>CD45RO(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 1</td>
<td>&gt;98.3</td>
<td>5.1</td>
<td>93.5</td>
<td>0.0</td>
<td>86.0</td>
</tr>
<tr>
<td>Line 2</td>
<td>&gt;99.1</td>
<td>71.2</td>
<td>10.0</td>
<td>0.0</td>
<td>99.0</td>
</tr>
</tbody>
</table>

Table 6.3. Phenotype of T cell lines. Non Ag specific T cell lines were generated from PBMC by PHA stimulation as detailed in Chapter 2, Section 2.3.2. After at least three rounds of stimulation, the cells were harvested and stained with anti-CD3 (PerCp) plus anti-CD4 (FITC) and anti-CD8 (Pe) Ab, or anti-CD3 plus anti-CD45RA (FITC) and anti-CD45RO (Pe) Ab, to determine the phenotype of each line. The staining was performed as detailed in Chapter 2 Section 2.7.1, after acquiring 10,000 cells for analysis. The results represent the percentage of CD3⁺ cells in the large lymphocyte gate and the percentages of CD4⁺, CD8⁺, CD45RA⁺ and CD45RO⁺ cells in the gate of large CD3⁺ lymphocyte.

6.3.2 Frequencies of type 1 cells in T the cell lines.

The cells from the T cells lines were stimulated with different concentrations of anti-CD3 ± CD28 Ab, anti-CD3 Ab ± PMA or PMA ± ionomycin as described for stimulation of other T cells populations as in Chapters 3 and 4 of this thesis.

After stimulation, type 1 cells were detected by their exclusive IFNγ production, in both CD3⁺ CD45RO⁺ T cell lines. Anti-CD3 plus anti-CD28 Ab stimulation induced 31.0% of the T cells to produce IFNγ when 5 μg/ml of anti-CD3 plus 1 μg /ml of anti-CD28 were used (Figure 6.1, Line 1), although low frequencies of type 1 cells (<5.0%) were observed in the Line 2 with this stimulation (Line 2 in Figure 6.1).

Compared with anti-CD3 plus anti-CD28 stimulation, the frequencies of type 1 cells in both samples increased when anti-CD3 and PMA were used to induce cytokine production. However, in sample 2 the frequency was always below 5.0%. When PMA
and ionomycin were used to induce cytokine production, the frequency of type 1 cells increased dramatically in both samples and reached a maximum (57.0%) with 50 ng/ml PMA and 1 μM ionomycin in Line 1 and 31.8 % with 0.5 ng/ml PMA and 10 μM ionomycin for Line 2 (Figure 6.1).

The amount of cytokine produced by the cells was determined by the MFI on the FL-1 channel for both samples. This parameter indicated that cells for Line 1 produced high levels and similar amounts of cytokine per cell with all the stimuli (Figure 6.2). T cells from Line 2 produced little IFNγ with anti-CD3 plus anti-CD28 Ab or anti-CD3 plus PMA stimulation, but produced higher amounts of IFNγ per cell with PMA and ionomycin.
Figure 6.1. Frequencies of type 1 CD3+CD45RO+ cells. Memory (CD45RO+) T cells from non-specific T cell lines were stimulated for 6 hours with varying concentrations of immobilised anti-CD3 Ab ± immobilised anti-CD28 Ab, immobilised anti-CD3 Ab ± soluble PMA or PMA ± ionomycin, all performed in the presence of 3 μM monensin, as described in chapter 2 section 2.17. Cells were then stained extracellularly with CD3-Per-Cp and intracellularly with anti-IFNγ(FITC conjugated) and anti-IL-4 (Pe conjugated) Ab or isotype matched control Ab (not shown) and analysed by flow cytometry, using a double gate in large CD3+ lymphocytes as described in chapter 4 figure 4.1. Dot plots of FL-1 (IFNγ) vs FL-2 (IL-4) staining were set to detect frequencies of cytokine producing cells (as described in chapter 4, figure 4.1). The positive values were set according to negative staining of both unstained cells and a IFNγ isotype matched control Ab for each point of the stimulus dilution. The results here represent the frequency of type 1 cells, defined by the exclusive production of IFNγ.
Figure 6.2. **Level of IFNy produced per type 1 T cell.** Memory (CD45RO+) T cells from two non-specific T cell lines were stimulated for 6 hr with various stimuli and acquired in a flow cytometer as described in Figure 6.1. The cells were analysed for the FL-1 (IFNγ) mean fluorescence intensity (MFI) values, obtained from the quadrant of IFNγ positive cells in each sample, excluding the double positive (IFNγ/IL-4) cells and IL-4 positive cells.
6.3.3 Frequency of type 2 cells in the T cell lines

The exclusive production of IL-4 was used to define type 2 T cells and was determined for both cell lines. For Line 1, the frequency of type 2 cells was lower than the frequency of type 1 cells with all the stimulation used (Compare Figure 6.1 and Figure 6.3). Nevertheless, type 2 cells were detected with all stimulations: anti-CD3 (5μg/ml) plus anti-CD28 (30μg/ml) Ab stimulation induced a 17.0% type 2 cells in this T cell line, whereas 5 ng/ml PMA with 50 μg/ml anti-CD3 induced the detection of 16.8% type 2 cells. However, the highest frequency of type 2 cells (22.3%) was detected with 1 μM ionomycin in the absence of PMA.

CD45RO+ T cells from Line 2 had spontaneous IL-4 production (7.2%) and the highest frequency of type 2 cells on stimulation (Figure 6.3). The stimulation with anti-CD3 plus anti-CD28 did not increase the frequency of type 2 cells in this sample. However, the addition of PMA to the anti-CD3 stimulation increased the proportion of cells producing IL-4 (reaching a maximum of 20.4% with 5ng/ml PMA and 200 μg/ml anti-CD3). The frequency of type 2 cells in this T cell line was the highest with PMA and ionomycin stimulation (44.1% with 50ng/ml PMA and 1 μM ionomycin).

The amount of IL-4 produced by the cells was estimated by the MFI and was higher in Line 1 than in Line 2 (Figure 6.4). Anti-CD3 plus anti-CD28 or anti-CD3 plus PMA stimulation increased the MFI mostly in Line 1, although this value did not augment dramatically with increasing concentrations of these stimulants in either line. In comparison, the addition of PMA and ionomycin increased the MFI by several fold in both samples (up to 9 fold in Line 1 and 2 fold in Line 2).
Figure 6.3. Frequencies of type 2 CD3+CD45RO+ cells after stimulation. Memory (CD45RO+) T cells from non-specific T cell lines were stimulated for 6 hours with various stimuli as described in Figure 6.1. Cells were then stained extracellularly for CD3 and intracellularly with anti-IFNγ and anti-IL-4 Ab or isotype matched control Ab and analysed by flow cytometry. The results represent the frequency of type 2 cells, defined by the exclusive production of IL-4, in the gate of large CD3+ lymphocytes.
Figure 6.4. Level of IL-4 produced per type 2 T cell. Memory (CD45RO+) T cells from two non-specific T cell lines were stimulated for 6 hr with various stimuli as described in Figure 6.1. The cells were analysed for the FL-2 MFI (IL-4) in the quadrant of IL-4 positive cells, excluding the double positive (IFNγ/IL-4) cells and IFNγ positive cells. The figure shows the MFI for IL-4 staining for the memory T cells for all the stimulations performed.
6.3.4 Ratio of type 2/type 1 CD45RO⁺ T cells under different stimulation.

The results described above contrast with the frequency of type 1 cells obtained for both cell lines. Thus, whereas Line 1 had higher frequencies of type 1 cells that type 2 cells, Line 2 displayed the opposite profile with higher frequencies of type 2 cells in all stimulation and spontaneous production of IL-4. The ratio of the frequencies of type 2 cells/type 1 cells was < 1 when PMA and ionomycin were used as stimulus (the stimulation that allowed high levels of detection of IFNγ and IL-4 in both cell lines) for Line 1 (Table 6.4), whereas this ratio was > 1 in Line 2 (Table 6.5). The difference in ratios may be due to the higher percentage of CD8⁺ cells in Line 1 and CD4⁺ cells in Line 2, as CD8⁺ memory T cells have a higher capability to produce IFNγ compared with CD4⁺ memory cells, whereas CD4 cells have higher capacity to produce IL-4 under the same stimulation (Sander, Cardell et al. 1991; Aune, Penix et al. 1997). The change in the ratio of type 2/type 1 cells (which defines the phenotype of the lines) depended on the concentration of stimulus used and the cell line and can be seen in Tables 6.4 and 6.5.

<table>
<thead>
<tr>
<th>Ionomycin (μM)</th>
<th>0.0</th>
<th>0.5</th>
<th>5.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-----</td>
<td>0.0</td>
<td>-----</td>
<td>0.0</td>
</tr>
<tr>
<td>0.1</td>
<td>1.4</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>1.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 6.4. Ratio of type 2: type 1 T cells in the T cell Line 1. The ratio of the frequencies of type 2/type 1 cells obtained with PMA and ionomycin is presented here. In this cell line, more cells belong to the type 1 phenotype as the ratio was always greater than 1. When no value is given, the ratio was a non-real number.
Table 6.5. Ratio of type 2: type 1 T cells in the T cell Line 2. The ratio of the frequencies of type 2/type 1 cells obtained with PMA and ionomycin is presented here. In this cell line, more cells belonged to the type 2 phenotype and the ratio was always greater than 1.

6.3.5 The frequency of type 0 T cells increases with PMA and ionomycin stimulation

The frequency of type 0 cells was determined in both CD45RO+ T cell populations by the assessment of simultaneous fluorescence in the FL-1 (IFNγ) and FL-2 (IL-4) channels.

For Line 1 in the absence of anti-CD3 plus anti-CD28 stimulation, there was a frequency of type 0 lower that 8.0% (Figure 6.5). When 5 μg/ml anti-CD3 and 30 μg/ml anti-CD28 were used, 14.4% of cells produced simultaneously IFNγ and IL-4. This stimulation induced the detection of lower frequencies of type 0 cells compared with the frequencies of type 1 (30.0% maximum) and type 2 (17.0% maximum) cells in this sample (Figures 6.1 and 6.3). The addition of PMA to CD3 stimulation increased the frequency of type 0 cells to 22.8% in this line (with 0.5 ng/ml PMA and 5 mg/ml anti-CD3). However, the frequency of type 0 cells increased dramatically with PMA and ionomycin, when there was a maximum of 46.9% cells which produced both cytokines with 50 ng/ml PMA and 0.1 μM ionomycin.

In Line 2, the frequencies of type 0 cells were low (below 3.0%) with anti-CD3 plus anti-CD28 Ab, when compared with the higher frequencies of type 1 and type 2 cells (6.4% and 9.5% respectively) obtained with those stimulations in the same sample. In the same sample, the frequency of type 0 cells was higher with PMA to CD3 stimulation (see Figure 6.5), reaching a maximum of 20.4% with 200 μg/ml anti-CD3 plus 5 ng/ml PMA. PMA and ionomycin increased the frequency of type 0 cells in this line noticeably. The
maximum frequency of type 0 cells in this line (12.0%) was reached with 5 ng/ml PMA plus 1 μM ionomycin.

Thus, whereas stimulation with anti-CD3 and anti-CD28 or anti-CD3 plus PMA induced detection of type 1 and type 2 cells in CD3'CD45RO' populations, PMA and ionomycin was the most powerful stimulus that increased the detection of type 1 and type 2 cells (Figures 6.1 and 6.3). In contrast, the frequency of type 0 CD3'CD45RO' cells obtained with CD3 plus CD28 or plus PMA plus CD3 was low, whereas PMA and ionomycin stimulation induced a high frequency of type 0 cells in Line 2 and to a lesser extent in Line 1. The CD3 plus CD28 stimulation induces a higher frequency of type 2 compared with type 1 (8.1/1.0=8.0 in a type 2/type 1 ratio), and a low frequency of type 0 cells (1.0%) (Figure 6.6 panel A). However, according to the results of CD3 plus PMA stimulation (Figure 6.6 panel B), the frequencies of these cell types are different (19.3/7.5=2.6), with more cells belonging to the type 0 group (2.6%). With PMA and ionomycin stimulation, (Figure 6.6 panel C), the ratios differ dramatically from the first stimulation (Figure 6.6 A) (37.5/17.9=2.1) and there was a big increase in type 0 cells (11.9%).

Thus, the frequency of cytokine producing cells in CD3'CD45RO' populations depended on the mode of stimulation used to induce cytokine expression.
Figure 6.5. Frequencies of type 0 CD3+CD45RO+ cells. Memory (CD45RO+) T cells from non-specific T cell lines were stimulated with CD3 plus CD28, CD3 plus PMA or PMA plus ionomycin, stained with anti-IFNγ and anti-IL-4 Ab or isotype matched control Ab and analysed by flow cytometry as described in Figure 6.1. The results here represent the frequency of type 0 cells in the gate of large CD3+ lymphocytes, defined by the simultaneous production of IFNγ and IL-4.
Figure 6.6. PMA and ionomycin stimulation increases the frequencies of type 0 CD3+CD45RO+ cells compared with stimulation via the CD3 ± CD28 or PMA. Memory cells from non-specific T cell lines were stimulated with 50 μg/ml anti-CD3 plus 10 μg/ml anti-CD28 Ab (A), 50 μg/ml anti-CD3 Ab plus 5 ng/ml PMA (B) or 5 ng/ml PMA plus 1 μM ionomycin (C), for 6 hrs and stained intracellularly with anti-IFNγ (FITC) plus IL-4 (PE) or isotype matched control antibodies. The cells were acquired as described in Figure 6.1. The results are expressed as the percentage of type 1 (IFNγ+), type 2 (IL-4+) and type 0 (IFNγ+/IL-4+), cells in the CD3+ large lymphocytes gate in the quadrants, that were defined according to non stained cells or cells stained with the isotype control antibodies.
6.3.6 Frequencies of cytokine producing memory cells in peripheral T lymphocytes

The previous results from the CD3'CD45RO' populations of T cell lines indicated that the cytokine profile of a population is modified by the mitogen used to stimulate cytokine production. The highest percentages of type 0 cells were always obtained with PMA and ionomycin, and this stimulation also enhanced the frequency of type 1 and 2 cells in the samples. As peripheral T cells from adult blood are a mixture CD45RA' and CD45RO' cells (Beck and Lam-Po-Tang 1994; Mills, TG et al. 1996; D'Arena, Musto et al. 1998) (and Figure 3.1, Chapter 3), and CD45RA' cells are not committed to a cytokine profile yet, it was hypothesised that the frequency of type 0 cells would be high in peripheral blood. Thus, the frequency of type 0 cells in T cells from periphery was determined, using combinations of the mitogens CD3, CD28, PMA and ionomycin used above, to induce cytokine production.

The results of stimulating three different samples of T cells within a PBMC environment (Figure 6.7), show a low frequency of type 0 cells when anti-CD3 plus anti-CD28 stimulation and CD3 plus PMA stimulation were used. These stimuli induced frequencies of type 1 and type 2 cells up to 11.2 and 12.7% respectively in some of these samples (See Table 6.6). Compared with other stimuli, PMA and ionomycin induced high and the highest frequency of type 0 cells in these samples (see Figure 6.7). This stimulation also induce the highest frequencies of type 1 and type 2 cells in these samples (see Table 6.6).

<table>
<thead>
<tr>
<th></th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBMC 1</td>
<td>PBMC 2</td>
</tr>
<tr>
<td>CD3+CD28</td>
<td>1.0</td>
<td>3.4</td>
</tr>
<tr>
<td>CD3+PMA</td>
<td>5.3</td>
<td>11.2</td>
</tr>
<tr>
<td>PMA+ionomycin</td>
<td>11.3</td>
<td>66.0</td>
</tr>
</tbody>
</table>

Table 6.6. Frequency of type 1 and type 2 T cells within a PBMC environment. Three different PBMC samples were stimulated for cytokine production as described in Figure 6.6. After CD3, IFNγ and IL-4 staining, the frequencies of type 1 and type 2 cells were obtained. The Table shows the highest frequency, represented as the
percentage, of type 1 or type 2 cells in a CD3⁺ activated lymphocyte gate with the optimal concentration for all the stimulations performed. ND means not determined.

To evaluate the effect of different stimulations on the frequency of type 1, 2 and type 0 cells in CD45RO⁺ T cells from the periphery, one sample of PBMC was studied by four colour staining. Stimulation with CD3 plus CD28 induced low frequencies of type 1 and type 2 and very low frequencies of type 0 cells (maximum 0.28%, see Figure 6.7 and see below). CD3 plus PMA induced higher frequencies of both type 1 and type 2 cells, but low frequencies of type 0 cells (maximum 1.3%, Figure 5.7) and PMA and ionomycin induced the highest frequencies of type 1 cells (45.7%) and type 0 (5.2% Figure 6.7), but not type 2 cells (see below).

CD3 plus CD28 or PMA stimulation induced cytokine production in the CD3⁺CD45RO⁺ cells from the periphery: the maximum frequency of type 1 cells with CD3 (50μg/ml) plus CD28 (50μg/ml) was 1.4%, whilst CD3 (50μg/ml) plus PMA (50ng/ml) induced a 5.2% and PMA (5ng/ml) plus ionomycin (10μM) induced a 45.7%. The maximum frequencies of type 2 cells were 2.2% with CD3 (50μg/ml) plus CD28 (5μg/ml), 5.3% (CD3 (50μg/ml) plus PMA (5ng/ml) and 3.5% with PMA (5ng/ml) plus ionomycin (0.1μM).

These results indicate that in CD45RO⁺ T cells from periphery, which are not chronically stimulated the frequency of type 1, type 2 and type 0 cells also depends on the stimulation used. Thus, CD3 plus CD28 or plus PMA were able to induce single cytokine production in both peripheral CD45RO⁺ T cells and in CD45RO⁺ cells from T cell lines, whereas PMA and ionomycin induced also frequencies of cells producing both cytokines, modifying the frequency of type 1, type 2 and type 0 cells.
Figure 6.7: Frequency of type 0 T cells in PBMC. Three PBMC were obtained from healthy donors and purified by Ficoll Hypaque as described in Chapter 2 Section 2.2.2. For induction of cytokine production, the cells were stimulated with plate immobilised anti-CD3 ± CD28, CD3 ± PMA or PMA ± ionomycin, for 6 hrs in the presence of 3μM monensin and then stained with extracellular anti-CD3 (PerCp) and intracellularly with anti IFNγ and IL-4 Ab or isotype matched controls (not shown), as described in Figure 6.1. The cells were harvested, and 40,000 cells were acquired for flow cytometric analysis in the CD3+ activated lymphocyte gate. The results represent the frequency of double positive (IFNγ and IL-4) cells within the gate.
Figure 6.8. Frequency of type 0 cells in CD3+CD45RO+ peripheral cells. PBMC were obtained from a healthy donor as described in chapter 2 Section 2.2.1. For induction of cytokine production, the cells were stimulated with plate immobilised anti-CD3 (50µg/ml) ± CD28 (0, 5 and 50µg/ml), CD3 (50µg/ml) ± PMA (0, 5 or 50 ng/ml) or PMA (5ng/ml) ± ionomycin (0, 0.1, 1 or 10µM), for 6 hrs as described in Figure 6.1. The cells were recovered and stained extracellularly with anti-CD3 (PerCp) and anti-CD45RO (APC) and intracellularly with anti-IFNγ (FITC) and IL-4 Ab (PE) or isotype matched controls (not shown). 20,000 cells were acquired for flow cytometric analysis in the double positive CD3+/CD45RO+ gate of activated lymphocytes. The results here represent the frequency of double positive (IFNγ and IL-4) cells within the gate.
6.3.7 PMA and ionomycin induces the expression of IL-4 in Th1 clones and the expression of IFNγ in Th2 clones in a high frequency of cells.

The above results (Figures 6.1, 6.3, 6.6 and 6.8) suggested that the cytokine profile of a population of memory CD45RO cells varies depending on the stimulation provided. Additionally, they suggested that PMA and ionomycin, contrary to other stimulations (such as CD3 plus CD28 or PMA) has the capacity to simultaneously induce the expression of IFNγ and IL-4 in a high frequency of memory cells, changing the cytokine phenotype of the population. In order to analyse the effect of these stimuli in the frequency of Th1, Th2 and Th0 cells in highly differentiated CD4+ memory T cells, Th1 and Th2 T CD4+ cell clones were stimulated, stained intracellularly for cytokines and analysed.

The T cell clones PK15, PIL35 (Th1) and 122.1 (Th2) were characterised by their cytokine secretion by ELISA or cytokine profile by ICS, as described in Material and Methods (see Tables 6.1 and 6.2).

**Frequencies of Th1, Th2 and Th0 cells in Th1 and Th2 T cell clones.**

The T cell clones were stimulated with the most common concentration of PMA (5 ng/ml) and ionomycin (1μM) used in the literature to detect type 1 and type 2 cells by ICS (see Table 10.1, Appendix Section 10.6). After 6 hrs stimulation, the cells were stained and acquired for analysis by flow cytometry. Figure 6.8 shows the FSC and SSC (panel A) and the CD3 expression (panel B) characteristic of these clones. After stimulation with PMA and ionomycin, a portion of the population was composed of bigger cells (Figure 6.8 A), where CD69 was expressed at high frequency: 89.2-99.8% (Figure 6.9 B). As these were the activated cells, the gate for analysis was set on this population (R1 in Figure 6.8 panel A and Figure 6.9 panel A).

Stimulation with PMA and ionomycin induced high frequencies of Th1, Th2 and Th0 cells in all three T cell clones (Figure 6.9 panel C). This stimulation of clone PK15 induced 21.0% of the cells to express IL-4 (classifying them as Th2) and a 1.6% to express IFNγ and IL-4 (Th0). The highest frequency of cells were Th1 cells (35.0%). The second Th1 cell clone studied (PIL 35) had a higher frequency of Th2 cells under stimulation with PMA and ionomycin (25.0%), only a 17.0% frequency of Th1 cells and a 6.4% of cells of the Th0 phenotype. The Th2 cell clone had similar frequencies of Th2
(22.7%), Th0 (23.2%) and Th1 (18.3%) cells with PMA and ionomycin stimulation. In all samples, a proportion of the cells did not express the cytokines analysed here.

Thus, the expression of IL-4 in established Th1 cells was stimulated with the use of 5ng/ml PMA and 1μM ionomycin and in some cells the expression of IFNγ was inhibited, while IL-4 expression was induced. The same phenomena was seen with the Th2 cell clone.
Figure 6.9. Phenotype of T cell clones. αβ CD4+ T cell clones were derived from PBMC from healthy donors by anti-CD3 cloning or PHA stimulation (Cohen, Katsikis et al. 1995). The figure shows a representative FSC and SSC profile of one of the clones (A) and in (B), a histogram of the frequency of CD3 positive cells from the R1 gate (shown in A). This figure is representative of three T cell clones.
Figure 6.10. Frequencies of IFNγ and IL-4 producing T cell clones. The PK15, PIL35 and 122.1 clones were thawed and cultured for one week with 20ng/ml hrIL-2. The cells were then washed and stimulated for cytokine production with 5ng/ml PMA and 1μM ionomycin in the presence of monensin as explained in Figure 6.1 for 6 hrs. The cells were then stained with anti-IFNγ and anti-IL-4 or with isotype matched control Ab, and analysed by flow cytometry as explained in Figure 6.1. The lymphocyte gate (R1) was set on the activated population (A). After stimulation 89.2-99.8% of the cells in the R1 gate were expressing CD69. (B) The frequencies of cytokine producing cells gated in R1 are shown in quadrant plots for IFNγ (FL-1), IL-4 (FL-2) or IFNγ and IL-4 producing cells. The results are expressed as the percentage of positive cells in the quadrants, that were defined according to non stained cells or cells stained with isotype control IFNγ and IL-4 Ab.
6.4 Discussion and conclusions

In this chapter, a comparison of the most common mitogenic stimulations used for the intracellular detection of cytokines, PMA and ionomycin, with PMA plus anti-CD3 stimulation and a more physiological and T-cell restricted stimuli, anti-CD3 plus anti-CD28 stimulation, has been performed to determine the cytokine profile of memory T cells by ICS. Pure CD3'CD45RO' cells were obtained from T cell lines and T cell clones. T cells from the periphery were also included due to their high content on CD45RO' cells.

It was shown that the frequency of cytokine producing cells (that defines the cytokine profile of a population) in memory T cell varies with the stimulation used. Thus, whereas CD3 plus CD28 induced the lowest frequencies of type 1 and type 2 cytokine producing cells, the use of CD3 plus PMA or PMA plus ionomycin increased both type 1 and type 2 frequencies in the T cell lines. Whereas CD3 plus CD28 stimulation induced the detection of type 1 cells, the frequency increased with CD3 plus PMA and the maximum frequency was obtained with PMA and ionomycin in both cell lines (Figure 6.1). Similarly, the maximum frequency of type 1 cells in Line 2 obtained with CD3 plus CD28 was 1.8%, 6.4% with CD3 plus PMA and 31.2% with PMA and ionomycin. Although Line 1 had similar frequency of type 2 cells when CD3 plus CD28 or CD3 plus PMA stimulation were used, the frequency of type 2 cells was always higher with PMA and ionomycin stimulation (Figure 6.3). In Line 2, the lowest frequency of type 2 cells was obtained with CD3 plus CD28 stimulation, then CD3 plus PMA and finally, the highest frequency was obtained with PMA and ionomycin stimulation (Figure 6.3).

The ratio of type 2/type 1 cells in memory T cell lines, induced with ionomycin, decreased with increasing concentrations of PMA (Tables 6.4 and 6.5): the highest ratios were obtained with ionomycin in the absence of PMA. However, this change in the ratio was because the combination of both mitogens induce the simultaneous expression of IFNγ and IL-4 in a high proportion of cells in all populations, revealed with ICS. This finding contradicts a study were it was found that PMA decreased IFNγ mRNA and increased IL-4 mRNA production in spleen cells (Monteyne, Renauld et al. 1992). However, Monteyne et al determined only mRNA, did not determine protein production and mRNA degradation or stabilisation was not addressed. Monteyne' study used resting cells from spleen whereas in this Chapter memory T cells were used, and both IFNγ and IL-4 production was increased by PMA in CD3 stimulated memory T cells (Figures 6.1 and 6.3). However, it has also been reported that the use of mitogens
modify the ratio of type 1 and type 2 cytokines secreted by memory T cell populations. In KLH immunised mice, whereas only 0.1U IL-4 and 296 U IFNγ were produced by anti-CD3 stimulation, 15 U IL-4 and only 0.9 U IFNγ/10⁶ cells were secreted with ConA stimulation (Kelso, Groves et al. 1995). In Kelso et al results, anti-CD3 stimulation induced more production of IFNγ whereas ConA stimulation induced a predominant IL-4 production in the same cells. The results in this Chapter shows that increasing concentrations of PMA does not decrease the frequency of IL-4 producing cells obtained with ionomycin stimulation (Figure 6.3 middle panels for both cell lines), but induces the production of IFNγ and IL-4 in the cells, decreasing the type 2/type 1 ratio. Thus, PMA and ionomycin increases the frequency of Th0 and Tc0 cells (Figure 6.5). In contrast, only low frequencies of Th0 cells were obtained under CD3 plus CD28 or CD3 plus PMA stimulation (Figure 6.5), although these stimulations induced type 1 or 2 cells in both cell lines (Figure 6.1 and 6.3).

The differences seen in the production of cytokines by memory T cells stimulated with different mitogens may reside in the strength of the mitogenic stimulation and in the capacity of the cells to regulate certain mitogenic stimulus. PMA and ionomycin stimulate PKC and elevate calcium levels in a sustained and non-regulated manner (Foreman, Mongar et al. 1973; Bennet, Cockcroft et al. 1979; Niedel, Khun et al. 1983; Truneh, Albert et al. 1985; Antonelli, Ruggiero et al. 1988), inducing stabilisation of IFNγ and IL-4 mRNA and favouring protein production (Antonelli, Ruggiero et al. 1988; Kaldy and Schmitt-Verhulst 1991; Dokter, Esselink et al. 1993; Todd, Grusby et al. 1993). In contrast with PMA and ionomycin stimulation, CD3 crosslinking in peripheral T cells induces a biphasic increase in intracellular Ca²⁺, PLCγ-1 phosphorylation and PKC activation (Friedrich, Cantrell et al. 1989; Park, Rho et al. 1991; Katayama, Miyazaki et al. 1993; Wacholtz and Lipsky 1993). The signalling induced by CD3 crosslinking is, furthermore autoregulated, as it induces cAMP production, inhibits IP production and regulates cytokine expression (Betz and Fox 1991; Bihoreau, Heurtier et al. 1991; Guse, da Silva et al. 1999). The cellular activation induced with phorbol esters and calcium ionophores is not only stronger but faster, compared with CD3 stimulation (Szamel, Kracht et al. 1990). This information gives insights into why IFNγ and IL-4 were expressed at higher frequencies in CD45RO⁺ T cells stimulated with PMA and ionomycin compared with CD3 plus CD28 Ab or PMA stimulation (Figures 6.1 and 6.3), as PMA and ionomycin gives a more sustained stimulation. In both the CD4⁺ and CD8⁺ T cell lines, PMA plus ionomycin stimulated more cytokine produced per cell (Figure 6.4), which can be due to the stabilising attributes of PMA on the IFNγ and IL-4 mRNA (Kaldy and Schmitt-Verhulst 1991; Dokter, Esselink et al. 1993). A higher increase in the amount of IFNγ produced per cell under PMA and ionomycin stimulation compared
with other stimulations was also seen in the CD4⁺ T cell line (Line 2 in Figure 6.2). In contrast, the amount of IFNγ produced per cell in the CD8⁺ T cell line under the stimulations used was not dramatically different (Figure 6.2). This may be because the intrinsic capacity of CD8⁺ T cells to express the IFNγ gene, whose transcriptional activity is enhanced in CD8⁺ memory T cells compared with CD4⁺ T memory lymphocytes (Aune, Penix et al. 1997).

The results from Figures 6.5 and 6.6 indicate that only PMA and ionomycin unveil the high frequency of type 0 cells in populations of memory T cells. In Figure 6.6, optimal stimulation by mitogens and the frequency of type 0 cells induced is compared: 1.0% type 0 cells are found with CD3 and CD28 stimulation, 2.6% with CD3 plus PMA stimulation and 11.9% with PMA and ionomycin stimulation. The same pattern was found in T cells from periphery, with a higher frequency of type 0 cells induced with PMA and ionomycin (Figure 6.7). In addition, as the high frequency of type 0 cells can be due to the use of a strong stimulus and may not reflect the true frequency of the type of cytokine producing T cells in the memory populations, Th1 and Th2 T cell clones were used to verify that stimulation with PMA and ionomycin does not alter the pattern of cytokine expression. It was found that 5ng/ml plus 1µM ionomycin (a common concentration of the stimulus in ICS, as it can be seen in Appendix Section 10.6) induced high frequencies of type 1, type 2 and type 0 cells in both Th1 and Th2 cells clones (Figure 6.10). Thus, the frequency of type 1, type 2 and type 0 cells in CD3⁺CD45RO⁺ cells depended on the stimulation used.

The results from in Figure 6.10 are supported by literature’s reports. Yssel et al have described that PMA and ionomycin enables IFNγ gene expression in Th2 clones within hours of stimulation (Yssel, Johnson et al. 1992), which may be because ionomycin can induce a sustained Ca²⁺ flux in cells such as Th2 that have lost the ability to engage a sustained Ca²⁺ influx in response to Ag stimulation (Boutin, Leitenberg et al. 1997; Sloan-Lancaster, Steinberg et al. 1997). Thus, strong stimulation (such as PMA and ionomycin) induce the aberrant expression of IFNγ and IL-4 genes in polarised T cells so that, as a result of the polarisation, cells express only one of those genes. It is now known that weaker or stronger stimulation of polarised T cells can induce the expression of genes otherwise silent in determined phenotypes. Thus, signalling of different strength induced by TCR engagement with antagonists (such as altered peptide ligands, APL), can induce changes in the patterns of cytokine production and in the frequency of cytokine producing cells in stabilised T cell lines and clones. In one study, the stimulation of memory Th0 cell lines with APL, specifically abrogated IFNγ production inducing a Th2 profile (Ausubel, Krieger et al. 1997). Similarly, stimulation with APL
can enhance proliferation and IFNγ production without altering IL-4 or IL-5 production in T antigen specific T cells in other study (Janssen, van Oosterhout et al. 2000). Thus, the strong stimulation used for the T cell clones in this Chapter induced a change in the clone’s pattern of cytokine production. Furthermore, with 5ng/ml PMA and 1μM ionomycin, some cells in the Th1 and Th2 clones “reverted” their cytokine profile and expressed only the cytokine of the opposite phenotype (21.0% and 25.0% of the Th1 clones expressed only IL-4 and 18.3% of the Th2 cells expressed only IFNγ, Figure 6.10). Nevertheless, there is the possibility that the T cell clones used here were classified as Th1 or Th2 inadequately, because only CD3 Ab was used to induce proliferation and cytokine production and there is the possibility that the clones were not completely polarised (as described in Section 6.2.1 Tables 6.1 and 6.3), which would explain heterogeneity in the cytokine production by these populations. In this case, the expression of the genes of the “opposite” phenotype would not has been completely silenced in both the Th1 and Th2 clones, allowing both IFNγ and IL-4 expression when the cells receive a stronger stimulation than CD3 crosslinking.

In humans, high frequencies of Th0 lymphocytes (assessed by ICS after PMA and ionomycin stimulation), have been found in T cells from peripheral blood (Anderson, Anderson et al. 1990; Picker, Singh et al. 1995; Jason and Larned 1997), in agreement with previous findings of a high frequency of Th0 cells at the population levels in mouse (Bucy, Panoskaltsis-Mortari et al. 1994; Kariv, Hardy et al. 1994; Bucy, Karr et al. 1995; Kelso, Groves et al. 1995). Here, a high frequency of type 0 T cells from periphery was also found (Figure 6.6). High frequency of memory Th0 cells in peripheral blood has been reported (Brown, Woods et al. 1993; Brown, Davis et al. 1994). Memory T cell clones and lines can also contain high frequencies Th0 cells (Kelso and Gough 1988; Paliard, de Wall et al. 1988; Kelso, Groves et al. 1995). Although Th0 lines can retain their cytokine profile when maintained in vitro under certain conditions (Miner and Croft 1998), the chronic in vitro antigenic stimulation of T cell lines without exogenous cytokines induce Th0 differentiation: in one study, the differentiation was towards Th2 subset, which furthermore reflected the immunity developed in vivo (Doncarli, Stasiuk et al. 1997). Recently and due to the use of highly sensitive detection techniques, the study of cytokine production at the single cell level has indicated that the co-expression of Th1 and Th2 cytokines is a relatively rare event in antigen stimulated T cell populations of memory cells. Thus, using Ag specific stimulation instead of mitogens, Th1 or Th2 cells are found at high frequencies whereas Th0 cells are rarely found in peripheral blood T cells from parasite infected patients or chronically immunised mice (de Boer, Fillie et al. 1998; Yip, Karulin et al. 1999; Karulin, Hesse et al. 2000). Karulin et al have put forward the notion that memory T cells from chronically infected mice express only one
type of cytokine per cell and not IFNγ and IL-2 or IL-4 and IL-5 simultaneously and practically never IFNγ and IL-4 simultaneously (Karulin, Hesse et al. 2000). Furthermore, it has been suggested that memory IFNγ and IL-5 producing cells derive from clonally expanded Th1 and Th2 and not Th0 cells, in a model of Th cell differentiation that used HEL, incomplete Freund's adjuvant and Pertussis toxin stimulation obtaining high frequencies of Th1 and Th2 cells (Shive, Hofstetter et al. 2000).

This and the results from this chapter suggest that the observed frequencies of type 0 cells in memory T cells from diverse organisms (mice and humans) can be due to the stimulation used for cytokine expression rather than reflecting the real frequency of type 0 cells in a population. Thus, the strength of the mitogen reveals the potential of a population to produce cytokine, but does not reflect the real cytokine profile generated by Ag stimulation and the mode of stimulation determines the cytokine profile of memory T cells.

6.5 Future experiments

This Chapter shows that the frequency of cytokine producing cells varies with the stimulation used. Although the results of this Chapter are consistent, the sample size of the memory populations used here should be increased. More T cell lines could be assayed for stimulation and determination of frequencies of cytokine production (Sections 6.3.3, 6.3.4, and 6.3.5). In particular, the experiment with CD3'CD45RO' cells from the periphery (Figure 6.7), should be done with more samples using randomly selected healthy adult donors. It will be equally important to include more Th2 cell clones, as only one was used in this chapter (Section 6.4).

The T cell lines used here were polyclonal and were developed with anti-CD3 stimulation and expanded with IL-2. No differentiating conditions were used (such as IL-4 plus anti-IL-12 Ab). Similarly, CD3'CD45RO' cells from periphery were not selected on the basis of their cytokine phenotype. Thus, these T cells most probably represent a heterogeneous population (defined by their cytokine production). In contrast, the T cell clones used here had been previously characterised as Th1 or Th2 cells. As their cytokine profile is in theory homogenous, the fact that some mitogenic stimulation induces expression of the cytokines of the opposite phenotype clearly shows that mitogenic stimulation does not indicate the true frequency of cytokine producing cells. Nevertheless, it may be that these T cells were not yet fully committed to either cytokine phenotype. This may be because only CD3 stimulation was used to defined the
clones as Th1 or Th2, but signalling though co-stimulatory molecules may be necessary to ascertain the cytokine profile of a memory T cell (as discussed in Section 6.4). As this possibility has not been ruled out, it could explain that under strong stimulation, these cells can still express cytokines of the opposite phenotype. Thus, it is important to include long term T cell clones stimulated via the CD3 and co-stimulatory molecules in this comparative study to ascertain if there is a point in development when mitogens do not induce the expression of negatively regulated cytokines.

Additionally, the T cell clones used in this Chapter were not Ag specific, and therefore, the magnitude of the change on the frequency of cytokine producing cells with Ag or with mitogens was not studied here. This can be done using Ag specific T cells stimulated with Ag and mitogens to induce cytokine production. Because cell division enables IFNγ and IL-4 gene expression (Bird, Brown et al. 1998) these genes may have been more available to PMA and ionomycin induced transcription because the clones used here were incubated in IL-2 before re-stimulation for ICS. The influence of cell division in a broad expression of cytokine genes facilitated by PMA and ionomycin stimulation can be studied by staining the cells with 5-carboxyfluorescein diacetate succinimidy l ester (CFSE). This is a fluorescent dye which dilutes out according to the number of division in a population of cells (Lyons and Parish 1994). The relationship between the production of cytokines and cells in division can then be done staining for intracellular cytokines in samples stained with CFSE.

Several aspects involved in T cell activation, such as affinity for Ag and Ag-dose dependency (Hosken, Shibuya et al. 1995; Kersh, Shaw et al. 1998; Rogers, Huston et al. 1998) are reflected in the capacity of T cells to produce cytokine. Thus, of special interest for this thesis was the determination of the frequency of cytokine producing alloreactive lymphocytes, without the use of mitogens to induce cytokine production, using Ag instead of mitogens. This could be done using allospecific T cell lines, because there is a big frequency of alloreactive T cells in periphery, compared with other antigens. The difference in frequencies obtained with Ag or mitogenic stimulation could also be determined. However, Ag-specific re-stimulation protocols require a highly sensitive assay for the detection of frequencies. One of these assays is the ELISPOT, in which the use of Ag to re-stimulate activated cells provides a more physiologic scenario of induction of cytokine production. This is discussed further in Chapter 7.
Chapter 7


7.1 Introduction

In spite of strategies to reduce graft rejection, such as recipient conditioning (Slavin, Nagler et al. 1998) and more extensive HLA typing (Spencer, Szydlo et al. 1995; Tay, Witt et al. 1995; Petersdorf, Longton et al. 1996), BMT still faces the risk of rejection caused by HLA incompatibilities (Gaschet, Lim et al. 1996). Both Class I (Davies, Shu et al. 1995; Spencer, Szydlo et al. 1995; Petersdorf, Longton et al. 1997) and class II (Petersdorf, Longton et al. 1995) mismatches in BMT increase the possibility to develop GvHD. Although improved HLA typing techniques and better donor and patient matching result in better transplant outcome in BMT (Hansen, Petersdorf et al. 1997; Madrigal, Scott et al. 1997), a perfect HLA match may not be achieved using siblings (Schipper, D’Amaro et al. 1996) and a large proportion of patients undergo BMT with an unrelated donor (Beatty, Dahlberg et al. 1988; Hansen, Petersdorf et al. 1997; Madrigal, Scott et al. 1997), which increases the probability of developing GvHD. As profilaxis to prevent GvHD, T cell-depleted grafts results in lower GvHD (Slavin, Or et al. 1985; Hale, Cobbold et al. 1988; Poynton 1988), although an increased risk of graft failure or leukemia relapse has been observed in T cell-depleted grafts for BMT (Mitsuyasu, Champlin et al. 1986; Slavin 1987; Vallera and Blazar 1989). Furthermore, there may be loss of the Graft versus Leukemia effect (a T-cell mediated phenomena) in T cell-depleted grafts (Horowitz, Gale et al. 1990; Jiang, Datta et al. 1991; Yang, Sergio et al. 1997). T cell depletion is therefore not always considered a profilaxis for a BMT. As T cells have been considered to play a central role in the development of the cytokine cascade responsible for GvHD (Antin and Ferrara 1992), a better understanding of alloreactive T cells can provide information of how to control those cells without eliminating them.

To provide predictive information of allogeneic reactions that may lead to GvHD in unrelated pairs of individuals, several strategies have been designed. One of them is the determination of cytokines produced in a MLC because IL-2, IFNγ and TNFα have been found in the supernatants of two ways MLC in mismatched pairs of PBMC but not in autologous MLR (Danzer, Kirchner et al. 1994; Tanaka, Imamura et al. 1995). Thus, it has been proposed that the enhanced expression of IFNγ mRNA or IFNγ released to the medium can indicate transplant complications (Tanaka, Imamura et al. 1994) whereas
that the determination of low levels of IL-2, IFNγ and TNFα in the MLC supernatant indicates identical HLA-DRB1 and HLA-DQB1 alleles (Danzer, A Campo et al. 1994). Nevertheless, the correlation between these parameters and post-transplant complications is low and these approaches have not been adopted in the clinic. In contrast, limiting dilution assays (LDA) have correlate high frequencies of precursors (alloreactive cells) with a poor transplant outcome. The frequencies of alloreactive cytotoxic (CD8+) or helper T cell (CD4+) precursors can be estimated using the CTLp and the HTLp assay respectively (Kaminski, Hows et al. 1989; Theobald, Nierle et al. 1992). The frequency of CTLp is dependant on the degree of mismatch (Kaminski, Sharrock et al. 1988) and high CTLp frequencies have been correlated with development of acute GvHD (Kaminski, Hows et al. 1989; Spencer, Brookes et al. 1995), whereas high HTLp frequencies have been correlated with development of chronic GvHD (Schwarzer, Jiang et al. 1993; Bunjes, Theobald et al. 1995). Both CTLp and HTLp have been used in combination to provide more information about alloreactivity (Schwarzer, Jiang et al. 1994; Wang, Proctor et al. 1996). An alternative way to predict GvHD has been the skin explant assay, where IFNγ and TNFα producing T cells induce histologic damage to a skin biopsy model, and the level of this damage was correlated with the degree of acute GvHD developed post-BMT (Dickinson, Sviland et al. 1991; Dickinson, Sviland et al. 1994).

However, the assays described above does not provide information about the balance of type 1 or type 2 cytokines produced in allogeneic reactions, that may determine the immunological outcome of a transplant. It has been proposed that the development of GvHD is due to the development of type 1 cytokines following a BMT (Antin and Ferrara 1992; Goldman 1994; Krenger and Ferrara 1996) and the production of IFNγ has been associated with alloreactivity (Hall 1991; Dallman 1995; Carayol, Bourhis et al. 1997). IFNγ is secreted by alloreactive CD8+ and CD4+ T cells from MLC cultures (Fong and Mosmann 1990; Pawelec, Rehein et al. 1996). Accordingly, there is a high precursor frequency of IFNγ producing alloreactive T cells in HLA-mismatched pairs (Matesic, Lehmann et al. 1998; Heeger, Greenspan et al. 1999). However, alloreactive T cells also secrete type 2 cytokines (such as IL-4, and IL-5) (Tanaka, Imamura et al. 1994; Li, Sad et al. 1997; Imami, Brookes et al. 1998; Matesic, Valujskikh et al. 1998). Although the role of type 2 cytokines in the allogenic reactions is not well understood yet, it has been proposed that type 2 cells induce tolerance in BMT (Krenger, Snyder et al. 1995; Imami, Brookes et al. 1998). In BMT, the association of class I or class II mismatches and the development of different T helper phenotypes in vitro has not been studied yet. Thus, it is unknown if the frequency of type 1 or type 2 cells would vary depending on certain mismatches.
The cytokines secreted by alloreactive T cells in most of the studies mentioned above have been detected by ELISA or bioassays. These techniques cannot determine frequencies of type 1, type 2 or type 0 cytokine producing T cells in a MLC. Although the cells can be cloned to determine cytokine profiles, clones are cells that have been selected by *in vitro* culture, which may reflect artefacts (Marini and Cohen 2000). In this chapter, the frequencies of type 1 and type 2 alloreactive short time T cell lines produced *in vitro* were determined by detection of their production of IFNγ and IL-4, using ICS. It was found that the mitogenic stimulation required to induce detectable cytokine production using this technique obscured the differences in frequency of specifically alloreactive T cells.

The ELISPOT assay is a highly sensitive technique and has been used to detect low frequencies of cytokine producing Ag specific T cells, by *in vitro* re-stimulation using Ag presented in APC (McCutcheon, Wehner et al. 1997; Yip, Karulin et al. 1999; Karulin, Hesse et al. 2000). It has also been applied to the detection of low frequencies of type 1 cytokine producing CD4⁺ T cells stimulated by the indirect (Valujskikh, Matesic et al. 1998) and by the direct (Lehmann, Graser et al. 1997) pathway of allore cognition. Here, using the IFNγ ELISPOT assay (that did not required mitogenic stimulation), it was possible to determine frequency of IFNγ producing alloreactive cells in single HLA-allele mismatched combinations.
7.2 Material and Methods

7.2.1 Cells

PBMC were obtained from buffy coats, members of the staff at the Anthony Nolan Research Institute or from healthy donors from the panel of bone marrow donors of the Anthony Nolan Bone Marrow Trust. All PBMC from donors and buffy coats were purified upon receipt by Ficoll Hypaque, as described in Chapter 2 Section 2.2.1 to 2.2.3. The blood from members of the staff and buffy coats were chosen randomly to obtain PBMC, the donors from the Anthony Nolan Bone Marrow Trust panel were selected on the basis of their HLA type, as explained below (Section 7.2.3). These donors received a letter inviting them to participate in this study by the donation of blood (see Appendix Section 10.5). Two donations of approximately 50ml of blood of these donors were received at the laboratory after 1-10 days of posting (average 2 days). After purification, all PBMC were stored in liquid Nitrogen in 10% DMSO-FCS, according to standard techniques (see Chapter 2, Section 2.6).

The PBMC were always thawed on the day of the experiment, obtaining always >90% live cells (calculated by trypan blue exclusion).

7.2.2 Typing of samples

Cells were typed at low resolution by serology for HLA class I antigens or by Sequence Specific Oligonucleotide probing (SSOP) at medium resolution for HLA class I alleles. High resolution typing of HLA class I and II alleles was performed by Sequence Specific Priming (SSP) or by Reference Strand Conformation Analysis (RSCA). All the HLA-typing was performed at the Anthony Nolan Round Table Laboratories or at the Anthony Nolan Research Institute (Chapter 2 Section 2.10). Where allele subtypes could not be identified unambiguously, linkage disequilibrium characteristics and ethnicity were taken into consideration (Imanishi, Akaza et al. 1991).

7.2.3 The selection of pairs of PBMC used to perform the MLC

Pairs of PBMC were chosen to be cultured in a MLC in order to determine frequencies of cytokine producing alloreactive lymphocytes. Randomly selected members of staff and buffy coats were paired, expecting a broad HLA-mismatch. These pairs were used for the ICS assay. Low resolution typing was done to confirm disparities in class I and class II alleles. For the ELISPOT assay, volunteers from panels of unrelated bone marrow donors were selected on basis of a common caucasian type
(HLA-A2 and HLA-B7) (Marsh, Parham et al. 2000). The donors selected were paired to obtain single mismatches in HLA-DR or a pair matched in all alleles according to the typing techniques used. All the samples from these donors were retyped upon arrival.

7.2.4 Allogeneic cultures (MLC).

For each experiment, one PBMC population was chosen as "stimulator" and the other was chosen as "responder". The stimulator PBMC were irradiated in a Cesium γ-irradiator (10,000 Rads) as described in Chapter 2, Section 2.3.2 and then washed twice in serum free medium. Irradiated stimulators and live responder PBMC were cultured together in a one way MLC, at a 1:1 ratio, in complete medium, in 12 (for ICS) or 48 (for ELISPOT) well plates. The final concentration of cells was $1 \times 10^6$ responder cells plus $1 \times 10^6$ stimulator cells/ml. After 6 days of culture the responder cells were harvested (as stimulators have disintegrated by that time as described in Chapter 2, Section 2.3.2).

7.2.5 Proliferation assay

200 μl of a concentration of $1 \times 10^6$ responder cells plus $1 \times 10^6$ stimulator cells/ml (1:1 responder:stimulator ratio), were plated in triplicate in 96 round bottom well plates and incubated for 5 days at 37°C in a 5% CO₂ humidified atmosphere. The last 18 hours of incubation, 1μ Ci/well [$^3H$] thymidine/well was added. The cultures were then harvested and the incorporated radioactivity counted as explained in Chapter 2 Section 2.4. Results are expressed as mean of triplicate cpm ± SD.

7.2.6 Determination of the frequencies of cytokine producing lymphocytes.

The frequencies of cytokine producing cells harvested from the MLC was determined by ICS or the ELISPOT assay. When ICS was used, the CD3⁺ or CD8⁺ populations were determined by extracellular staining of these antigens, setting a gate in blasting lymphocytes or in blasting CD3⁺ lymphocytes. Extracellular staining was performed as described in Chapter 2 Section 2.7.1, with anti-CD3 (PerCp), anti-CD4 (FITC) and anti-CD8 (Pe or PerCp).

7.2.6.1 ICS

The ICS technique was used for the detection of frequencies of IFNγ and IL-4 producing CD3⁺ or CD8⁺ lymphocytes. After one week of the MLC described in Section 7.2.4, cells were harvested from the cultures. $5 \times 10^5$ harvested cells were
placed in 48 flat-well plates to induce cytokine production with different stimulus. These included alloantigen, PMA plus ionomycin (50 ng/ml and 1μM respectively) or an optimal dilution of plate immobilised anti-CD3 Ab (OrthoDiagnostics) (determined by its ability to induce cytokine production by T cell lines, see Chapter 6, Section 6.3 and 6.4) plus 5ng/ml PMA. Al re-stimulations were performed in the presence of 3μM monensin. After 6 hrs of incubation, the cells were transferred to 96 round bottom plates, washed twice in staining buffer and stained extracellularly with anti-CD3 or anti-CD8 Ab and intracellularly with anti-IFNγ and anti-IL-4 or anti-IL-2 plus anti-IL-5 Ab, or isotype matched control Ab, as described in chapter 2 sections 2.7.1 and 2.7.5. Antibodies used to stain for intracellular cytokines (and their isotype matched controls) were all obtained from Pharmingen. Cells were then acquired (a minimum of 20,000) and analysed by flow cytometry, within 48 hours of staining. Results are expressed as the frequency of IFNγ and or IL-4 producing cells within the CD3⁺ or CD8⁺ populations, in the gate of activated lymphocytes (as described in Chapter 2 Section 2.7.5).

7.2.6.2 ELISPOT

The IFNγ-ELISPOT assay is described in Chapter 2, Section 2.9 and was used in this Chapter for the detection of frequency of IFNγ producing alloreactive lymphocytes. MLCs were set up to generate short term allogeneic T cell lines for each pair of responder and stimulator PBMC. After one week of culture in 24 well plates at a 1:1 responder:stimulator ratio, the responder cells were harvested and re-challenged with the same alloantigen at a 1:1 responder:stimulator ratio, or with a predetermined concentration of PHA (see Appendix Section10.2) (as positive control of the assay) or without stimulus (as negative control of the experiment) (McCUTCHEON, WEHNER et al. 1997) in the re-stimulation plates (96 well plates). After 20 hrs of re-stimulation, the cells were harvested and the frequency of IFNγ producing cells in the ELISPOT was determined as described in Chapter 2 Section 2.9. All the spots/well were counted using the NIH image 1.57 Analysis Software for Macintosh. As the number of cells was limiting, no duplicates were done per plate but the ELISPOT was done twice. The plates were counted two consecutive times and the average of spots were recorded. Results for the ELISPOT are expressed as the frequency of IFNγ spots/plated cells.
7.3 Results

7.3.1 Tissue type of the samples.

In randomly selected individuals, there is a low possibility of a perfect HLA match because of the HLA polymorphism. Table 7.1 shows the low resolution HLA type of three randomly selected buffy coats (BC). It can be seen that they do not match. The typing of these samples was not performed at high resolution and therefore it is not possible to determine the allele expressed in these randomly selected samples. The class I typing for BC number 2 (BC2) was done by serology. DNA typing indicates that HLA-C mismatches are common in HLA-A and -B serologically matched pairs (Scott, O'Shea et al. 1998) and HLA-C is considered less immunologically relevant for alloreactivity (Lawlor, Zemmour et al. 1990). Since HLA-A, B and/or DR were broadly mismatched in these pairs, HLA-C was not typed.
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Table 7.1. Tissue typing of buffy coats (BC) 1, 2 and 3. Class I and class II typing for BC1 and BC3 was done by medium resolution molecular typing by SSO. For the BC2, class I typing was done by low resolution (serology) and class II typing by medium resolution (SSO). Serology typing can not resolve amino acid differences but assigns a determinant group. Similarly, the resolution of the SSO performed for these samples do not resolve the HLA type, but offer a group of possible alleles. For BC1, the HLA typing 0101-06 means any allele from 0101 to 0106. Nevertheless, the low and medium resolution typing done for these samples permits to determine that non of these samples are matched.

XX means any allele (not resolved by the typing).
<table>
<thead>
<tr>
<th>Locus</th>
<th>A (Responder)</th>
<th>J (stimulator)</th>
<th>Gu (Stimulator)</th>
<th>Gr (Stimulator)</th>
<th>BC3* (stimulator)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>0201/09</td>
<td>0201/09</td>
<td>0201/09</td>
<td>0201/09</td>
<td>02XX, 0301/04</td>
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<td>0702</td>
<td>0702</td>
<td>0702/04/09, 5701</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>or 0709, 5704</td>
</tr>
<tr>
<td>Cw*</td>
<td>0702</td>
<td>0702</td>
<td>0702</td>
<td>0702</td>
<td>------</td>
</tr>
<tr>
<td>DRB1*</td>
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<td>0401, 1501</td>
<td>0403/06/07, 1501</td>
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<td>1501/04-07, 0701/03/04</td>
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<tr>
<td>DRB4*</td>
<td>0103101</td>
<td>0103101</td>
<td>0103101</td>
<td>0103101</td>
<td>0101/02/03/04/05/0201N</td>
</tr>
<tr>
<td>DRB5*</td>
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<td>0602, 0302</td>
<td>0602, 0302</td>
<td>0602/11, 03032</td>
</tr>
</tbody>
</table>

Table 7.2. HLA typing of responder A and stimulators. Volunteers from the panel of unrelated bone marrow donors were selected on basis of their HLA-A2 and HLA-B7 tissue type (determined by low serology typing). DNA was extracted from the samples received from these donors to perform medium and high resolution typing. Class I typing was done by RSCA and class II typing was done by SSO or SSP for DRB4 subtypes, as described in Section 7.2.2 of this chapter. Although typing by RSCA was not able to differentiate between the 0201* or 0209* HLA-A alleles, all the samples had the same mobility and therefore are matched for this allele regardless of the type.

*Sample BC3 is also shown in Table 7.1, typed by medium resolution typing (SSO).
<table>
<thead>
<tr>
<th>Locus</th>
<th>W (Responder)</th>
<th>Wd (stimulator)</th>
<th>Witt (Stimulator)</th>
<th>BC3 (stimulator)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>0201/09</td>
<td>0201/09</td>
<td>0201/09</td>
<td>02XX, 0301/04</td>
</tr>
<tr>
<td>B*</td>
<td>0702</td>
<td>0702</td>
<td>0702</td>
<td>0702/04/09, 5701 or 0709, 5704</td>
</tr>
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<td>0702</td>
<td>0702</td>
<td>------</td>
</tr>
<tr>
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<td>1501/04/05/06/07, 0701/03/04</td>
</tr>
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<td>0103101</td>
<td>01</td>
<td>0101/02/03/04/05/0201N</td>
</tr>
<tr>
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<td>0101</td>
<td>0101</td>
<td>0101</td>
<td>0101/05/09</td>
</tr>
<tr>
<td>DQB1*</td>
<td>0602, 0302</td>
<td>0602, 0302</td>
<td>0602, 0302</td>
<td>0602/11, 03032</td>
</tr>
</tbody>
</table>

Table 7.3. HLA typing of responder W and stimulators. Volunteers from the panel of unrelated bone marrow donor were selected on basis of a HLA-A2 and HLA-B7 HLA type and medium and high resolution typing was performed as explained in Table 7.2. Sample BC3 is also shown in Table 7.1.
7.3.2 Frequencies of cytokine producing irradiated T cells after mitogenic stimulation determined by ICS

As irradiation or mytomycin C treatment of PBMC does not block cytokine production by PBMC determined by ELISA (Leenaerts, Ceuppens et al. 1992; Toungouz, Denys et al. 1993), IFNγ/IL-4 production by the irradiated stimulator PBMC was analysed by ICS. The capacity of irradiated cells to produce these cytokines was analysed before irradiation (day 0) and at days 1, 4 and 6 after irradiation. These irradiated PBMC were harvested at the mentioned time points and stimulated with an optimal dilution of anti-CD3 Ab plus 5ng/ml PMA (as described in Section 7.2.6.1). The cells were stained and their cytokine production was analysed by flow cytometry. Figure 7.1 shows that irradiated cells leave the region of live blasting cells (R1), determined by FSC and SSC parameters (shown in Figure 7.1 panel A), as soon as 1 day after irradiation (Figure 7.1 panel B). From all the cells, the percentage of cells in the R1 region was 5.9% at day 0, 1.8% at day 1 and then the frequency decreased to 0.2% at day 4 and 6 in one experiment. The percentage of cells in the region of non-blasting small cells (R2) increased with the time from 92.8% at day 0, to 98.0% at day 1, 99.1% at day 4 and 99.4% at day 6 in one experiment. An 85-100% of the cells in these cultures were dead at day 3 as determined by trypan blue exclusion (two experiments). Before irradiation, the cells in R1 can produce detectable cytokines with anti-CD3 plus PMA stimulation (Figure 7.1 A). Aliquots of irradiated cells were taken at the indicated time points, re-stimulated, stained and analysed by flow cytometry for intracellular cytokines. After 1 day of irradiation, the cells that remained in the gate of live cells (R1 in Figure 7.1 panel B) did not produce detectable IFNγ or IL-4 on stimulation (Figure 7.1 panel C), and neither did the cells from the gate R2 (Figure 7.1 panel D). Thus, irradiated cells are unable to produce detectable cytokines under anti-CD3 plus PMA stimulation 24 hrs after γ-irradiation.
Figure 7.1. Irradiated cells do not appear in the blasting-lymphocyte gate and do not produce cytokines with mitogenic stimulation 6 days after irradiation. PBMC obtained from healthy donor’s buffy coats, were irradiated with 10,000 Rad and cultured for 6 days in 12 well plates in complete medium. Aliquots of 1x10⁶ cells were and on days 1, 4 and 6 after irradiation. The cells from aliquots taken before irradiation were placed in a 48 well plate with plate-immobilised anti-CD3 Ab plus 5ng/ml PMA to induce cytokine production. The cells were then harvested and stained extracellularly (anti-CD3 Ab PerCp) and intracellularly (anti-IFNγ plus anti-IL-4 or with isotype matched control antibodies). 20,000 events were acquired and analysed. A) FSC and SSC dot plot of non irradiated PBMC. A gate was set on the large blasting lymphocytes (R1) and a second gate (R2) was set in small condensed lymphocytes. B) FSC and SSC dot plots of irradiated PBMC at days 1, 4, and 6 after irradiation. The R1 gate was almost empty from day 1 through day 6, more cells appear in the R2 gate. C) and D) Frequencies of IFNγ -FL-1- and/or IL-4 -FL-2- producing CD3⁺ lymphocytes from gates R1 (C) or R2 (D). The bars in the FL-1 and FL-2 dot plots were set on the negative staining of both unstained cells and isotype matched control Ab (now shown). In the figure, the transversal bars of each plot show the percentage of positive (IL-4⁺ and/or IFNγ⁺) cells in the CD3⁺ R1 or R2 gates. The isotype matched control values were either 0.0% or subtracted from the values shown in this figure. Where no value is given the percentage is 0.0%. This figure is representative of two independent experiments.
7.3.3 Responder cells from an MLC require mitogenic stimulation to produce cytokines detectable by ICS

Cells from 6 day MLCs were recovered to determine if re-stimulation with alloantigen induced cytokine production detectable by ICS. Lymphocytes harvested from 6 days MLC were washed and split into groups. Responder cells from the MLC (at a final concentration of $1 \times 10^6$ responder cells/ml), were re-stimulated for 6 hrs with irradiated stimulators in the presence of 3 $\mu$M monensin at a 1:4 (panel A), a 1:1 (panel B) or a 2:1 (panel C) responder:stimulator ratio. The cells were then washed and stained with anti-CD3 and anti-IFN$\gamma$ plus anti-IL-4 or anti-IL-2 plus anti-IL-5 Ab (or isotype control Ab). No IFN$\gamma$ or IL-4 were detected in any sample. However, a frequency of 24.9% of Th1 (IFN$\gamma^+$) cells, 5.7% Th2 (IL-4$^+$) cells and 3.3% of Th0 cells (IFN$\gamma/IL-4^+$) was found in responder cells incubated for 6 hr with PMA plus ionomycin (panel C). Responder cells from the MLC without re-stimulation and irradiated stimulator cells has 0.0% frequencies of IFN$\gamma$ or IL-4 positive cells, thus did not produce detectable intracellular cytokines after 6 hr in culture. Similar results were seen at 24 hrs re-stimulation with alloantigen, where monensin was added the last 6 hr, because IFN$\gamma$ or IL-4 positive cells were not detected (frequencies where 0.0%).

A comparison between re-stimulation with alloantigen and CD3 plus PMA was performed. Cells from one week MLC were harvested and re-stimulated with alloantigen or plate immobilised anti-CD3 Ab plus PMA. The cells were then washed and stained with anti-CD3 and anti-IFN$\gamma$ plus anti-IL-4 or anti-IL-2 plus anti-IL-5 Ab (or isotype control Ab, with a frequency of positive cells below 0.1 always). As it is shown in Table 7.4, only mitogenic stimulation induced the production of detectable cytokines in T cells.
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IFNγ</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>Th0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alloantigen</td>
<td>0.1</td>
<td>0.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Anti-CD3+PMA</td>
<td>3.7</td>
<td>2.8</td>
<td>0.1</td>
<td>2.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 7.4. Frequencies of cytokine producing T cells under allogenic or mitogenic re-stimulation. Cells from two unrelated donors were cultured in a MLC as described in Figure 7.2.Responder cells were harvested at day 6 and were re-stimulated with irradiated stimulators (in a 1:1 ratio), or re-stimulated with plate immobilised anti-CD3 Ab plus 5ng/ml PMA for 6 hrs, in the presence of 3μM monensin. The cells were then stained for the intracellular detection of IFNγ and IL-4, or IL-2 and IL-5, as described in Figure 7.2. The results are expressed as the frequency of cytokine positive cells in a CD3+ lymphocyte gate. Type 0 cells were defined as IFNγ^IL-4^ T cells.

Therefore, IFNγ or IL-4 production by short time alloreactive T cells can not be detected by ICS when alloantigen is used to induce cytokine production, whereas mitogenic stimulation enables the detection of both cytokines.
Figure 7.1. Cells from a MLC culture do not produce cytokines detectable by ICS when they are re-stimulated with alloantigen. Cells from two unrelated donors were obtained as described in Section 7.2.1 and cultured in a MLC at different responder:stimulator ratios (Section 7.2.4). Responder cells were harvested at day 6 and were re-stimulated with irradiated stimulators for 6 (A, B and C), or re-stimulated with 50 ng/ml PMA plus 1 μM ionomycin (D). The cells were then harvested, and stained extracellularly with anti-CD3 Ab (PerCp) and intracellularly with anti-IFNγ plus anti-IL-4 or with isotype matched control antibodies as described in Figure 7.1. 40,000 events were acquired and analysed by flow cytometry. The figure shows the FSC and SSC (right dot plots) of cells for each condition. A double gate of CD3⁺ lymphocytes in the R1 region was set to analyse the cytokine producing T cells. The results in the right contour plot of each panel represent the frequency of IFNγ and/or IL-4 positive cells in R3. The negative and positive regions of the contour plots were set as described in Figure 7.1, and where no value is given the percentage is 0.0%. This figure is representative of two experiments.
7.3.4 Stimulation with PMA and PMA plus ionomycin down-regulates the expression of CD4 in short term allogeneic T cell lines.

Cells harvested one week MLC were stimulated with various mitogens, including anti-CD3 Ab, anti-CD3 plus PMA and PMA plus ionomycin stimulation and stained for CD3, CD4 and CD8. As described in Chapter 2 Section 2.7.3, whereas the expression of CD3 and CD8 in the lymphocytes in these alloreactive T cell lines remained basically constant in all experiments (Figure 7.3 panel A), the expression of CD4 was drastically reduced with PMA stimulation (anti-CD3 Ab plus PMA and PMA plus ionomycin) (Figure 7.3 panel B).

7.3.5 Proliferative response of mismatched PBMC.

PBMC fromuffy coats (BC3 as responder, BC1 and BC2 as stimulators) were typed to confirm total HLA disparity (see Table 7.1). A standard one way MLR was performed with PBMC from BC3 as responders and PBMC from BC1 and BC2 as stimulators. This assay was done in parallel to the MLC, that was performed to determine frequencies of cytokine producing cells and which is described in the following sections. As a broad class II mismatch was used in these pairs of PBMC, a high proliferative response was expected. Figure 7.4 shows the proliferative response of the responder cells (BC3) towards the allostimulus (BC1 and BC2) in a 5 days MLR. BC3 have a low autologous proliferative response (BC3 vs BC3), that is comparable to that of cells from buffy coat 3 “alone” (cells that were cultured without irradiated stimulators for 5 days). Thus, the differences in class II in the pairs of PBMC chosen induced alloreactivity as measured by proliferation.
Figure 7.3. Down-regulation of CD4 expression in alloreactive T cell lines stimulated with PMA. Cells from two unrelated donors were obtained and cultured in a MLC as described in Figure 7.2. Responder cells were harvested at day 6 and were re-stimulated for 6 hr with plate immobilised anti-CD3 Ab, anti-CD3 Ab plus PMA (5ng/ml) or PMA (5ng/ml) plus ionomycin (1μM) or left unstimulated. The cells were then harvested, washed and stained with anti-CD3 (PerCp), anti-CD4 (FITC) and anti-CD8 (Pe) Ab, and the frequency of positive cells was analysed in a flow cytometer. The figure shows the frequency of CD3+ cells in the gate of live lymphocytes (panel A) and the frequencies of CD4+ and CD8+ T cells (panel B) after the 6 hr culture. This experiment was repeated twice with similar results and a representative experiment is shown.
Figure 7.4. Proliferative response to allogeneic stimulus. Cells from buffy coat 3 were chosen as responders and were cultured in a MLR with irradiated PBMC from buffy coat 1 and 2 to assess the proliferative response to alloantigen. Keeping a 1:1 responder-stimulator ratio, a total of 1x10⁶ responder PBMC/ml were cultured by triplicates in a 96 round bottom well plate, in a final volume of 200µl, for 5 days. 18 hrs before the end of the culture, 1µCi/well [³H] thymidine/well was added. The cultures were then harvested and the incorporated radioactivity was counted in a γ-counter. Results are expressed as mean of triplicate cpm ± SD. A) and B) represent two independent MLR.
7.3.6 Frequencies of type 1, type 2 and type 0 cells and Tc1, Tc2 and Tc0 alloreactive T cells assayed by ICS.

The frequency of cytokine producing alloreactive T cells was estimated using short term T cell lines generated in a 6 days MLC. Because the frequency of type 1, type 2 and type 0 cells was to be evaluated, a broad mismatch between the samples was used. This was because whereas HLA-DR mismatches induce an increase in the frequency of IFNγ producing T cells clones (Pawelec, Rehbein et al. 1996), only fully mismatched allogeneic stimulus induces the secretion of both type 1 and type 2 cytokines (IFNγ, IL-4, IL-5) (Tanaka, Imamura et al. 1994).

The MLC cultures were set up as described in material and methods (Section 7.2.4), with an autologous culture as control (BC3 vs irradiated BC3). At day 6 after irradiation, cells are disintegrated and do not produce cytokines using ICS (Figure 7.2). At this time point, only responder cells are recovered from an MLC: an average of 50.0% (range 35.7-59.5) of the total events acquired at this time point were CD3⁺ and 80.3% (range 71.8-88.0) of cells in the lymphocyte gate were CD3⁺. Responder cells recovered from 6 day-MLC were stimulated with 5ng/ml PMA plus an optimal dilution of plate immobilised anti-CD3 Ab (as described in Section 7.2.6.1) for 6 hrs to induce cytokine production. This stimulation was chosen because it was able to induce production of type 1 and type 2 cytokines in T cell lines (as discussed in Chapter 6 Section 6.3.4). The cells were then stained for CD3 or CD8 extracellularly, and IFNγ plus IL-4 intracellularly (or isotype matched control Ab). The frequency of type 1 (IFNγ positive) type 2 (IL-4 positive) or type 0 cells (IFNγ and IL-4 positive) cells was determined by flow cytometry, as described in Chapter 2 Section 2.7.5.

As expected, the highest frequency of type 1 responder T cells corresponded to the allogeneic cultures (3 vs 1 and 3 vs 2, with a 24.2% and 18.8% of type 1 cells, respectively). However, in the autologous culture, 13.1% of T cells produced IFNγ on re-stimulation and furthermore, a 7.4% of “cells alone” (cells cultured without any allogeneic or autologous irradiated PBMC, see Figure 7.5 “3 alone”) were type 1 on re-stimulation. These cells survived the culture in the absence of any stimulation. The frequency of type 2 cells was low compared to the frequency of type 1 cells obtained and was <6.6% always, as well as the frequency of type 0 cells (< 0.1% always) in all cultures. Cells without re-stimulation did not produce detectable cytokines. This experiment was repeated and similar results were obtained.

In Figure 7.6 the frequency of Tc1, Tc2 and Tc0 responder cells from the MLC is shown. The frequency of IFNγ producing cells was higher in this population
compared with the frequency of type 1 cells (CD3+) in Figure 7.5. However, the
frequencies of type 2 and type 0 cells is similar in the CD3+ and the CD8+ population
of lymphocytes (For one experiment, in the 3 vs 1 pair: type 2 CD3+ cells were 2.34%.
and Tc2 were 1.05%. In the 3 vs 2 pair, type 2 CD3+ cells were 6.6% and Tc2 were
6.7%).

The above results indicate a high frequency of type 1 cells and lower frequencies of
type 2 and type 0 cells, in populations of activated CD3 and CD8 lymphocytes after
one week of culture, independently of allogeneic stimulation, autologous stimulation
or stimulation at all, but dependent on the re-stimulation with mitogens.

Therefore, the ICS technique was unable to determine different frequencies of type 1,
2 or 0 alloreactive T cells. It was not possible to correlate the development of type 1,
2 or 0 cells with allogeneic stimulation or control stimulation (which included
autologous or nil stimulation). As ICS could not be used for determination of
frequencies of cytokine producing alloreactive T cells, a modified IFNγ ELISPOT
(McCutcheon, Wehner et al. 1997) was used to determine the frequencies of IFNγ
producing alloreactive lymphocytes. This was because this technique uses re-
stimulation with Ag and not mitogens to induce cytokine production.
Figure 7.5. Frequencies of the type 1, type 2 and type 0 T cells after allogeneic culture. PBMC were obtained from buffy coats (BC) 1, 2 and 3 and purified as described in chapter 2, Section 2.2.1 and BC3 was the responder. They were cultured in a MLC with irradiated cells from BC 1 and 2 (indicated as 3 vs 1 and 3 vs 2, respectively), as described in Chapter 2, Section 2.3.2. An autologous MLC (3 vs 3) and one culture of responder cells without allogeneic stimulation was included (alone). A 1:1 responder:stimulator ratio was used in the MLC, with a final concentration of responders of 1x10^6 cells/ml in 12 well plates. After one week of culture, the cells were harvested, washed twice in serum-free medium and re-stimulated for cytokine production with a predetermined optimal dilution of plate-immobilised anti-CD3 Ab plus 5ng/ml PMA and 3 \( \mu \text{m} \) monesin, in complete medium. The cells were then recovered, washed twice in staining buffer and split for CD3 (PerCp) or CD8 (shown in Figure 7.6) extracellular staining. The cells were subsequently stained with anti-IFN\( \gamma \) plus anti-IL-4 intracellular Ab or isotype matched control Ab (not shown). The cells were acquired and analysed by flow cytometry. 40,000 events were acquired/sample. The results represent frequencies of type 1, type 2 and type 0 T cells in a CD3+ in a gate of CD3+ large lymphocytes for two independent experiments.
Figure 7.6. Frequencies of Tc1, Tc2 and Tc0 cells after allogeneic culture. PBMC from buffy coat 3 were cultured in a MLC as described in Figure 7.1. After one week of culture, the cells were harvested, stimulated for cytokine production, stained for CD8 (PerCp) and intracellular Ab and analysed by flow cytometry as described in Figure 7.1. A gate in the large CD8+ lymphocytes was used to determine the frequencies of Tc1, Tc2 and Tc0 cytokine producing cells, for two independent experiments. The results represent frequencies of type 1, type 2 and type 0 T cells in a CD3+ in a gate of CD8+ large lymphocytes for two independent experiments.
7.3.7 Determination of frequencies of IFN\(_\gamma\) secreting cells in a single mismatch MLC using the ELISPOT assay.

The frequency of IFN\(_\gamma\) producing alloreactive lymphocytes that develop in response to certain mismatches is unknown. Thus, IFN\(_\gamma\) ELISPOT was introduced in this study to determine such frequency.

Pairs of PBMC with a single mismatch in HLA antigens were chosen. In order to obtain matched pairs of PBMC or pairs with a single mismatch in class II antigens, the panel of unrelated bone marrow donors from the Anthony Nolan Bone Marrow Trust was screened, searching for donors with a common caucasian HLA type (HLA-A2). A group of donors (A2, B7), was retrieved from the panel. Within this group, some donors were matched for HLA-A, B, C, DR and DP whereas others had histoincompatibilities in class II alleles. Then, they were considered to be included in this phase of the study. As some of these donors were originally typed by low resolution techniques, the blood samples were retyped upon arrival by high resolution techniques at the Anthony Nolan Research Institute and the Anthony Nolan Round Table laboratories (as described in Chapter 2 Section 2.10 and in Tables 7.2 and 7.3).

Matched or one HLA-DR allele mismatched PBMC from donors were paired. For each pair, one PBMC acted as responders and the other PBMC acted as stimulator. Two responders (A and W) were chosen. For one of them (responder A), the stimulators were J, Gr, Gu and the buffy coat 3 (BC3, used in previous experiments and included to have a broad mismatch in the ELISPOT assay). For the second responder (W) the stimulators were Wd, Witt and BC3. These groups of pairs and the mismatches for each pair are described in Table 7.5.
Table 7.5 Mismatches in responder and stimulator pairs used in the ELISPOT assay.

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator</th>
<th>Mismatch</th>
<th>Responder</th>
<th>Stimulator</th>
<th>Mismatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td>Nil</td>
<td>W</td>
<td>Wd</td>
<td>Nil &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>Gr</td>
<td>Nil</td>
<td>W</td>
<td>Witt</td>
<td>DRB1, B4</td>
</tr>
<tr>
<td>A</td>
<td>Gu</td>
<td>DRB1</td>
<td>W</td>
<td>BC3</td>
<td>Broad &lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>Broad &lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Donors from the Anthony Nolan panel of donors were paired in basis of their HLA type. Whereas some pairs were matched (A vs J, A vs Gr), others had a single mismatch (A vs Gu, W vs Wd) or one mismatches (W vs Witt) in class II antigens.

<sup>a</sup> In this pair, the responder’s (W) DRB1 type is *0404, *1501, but the DRB1 typing of the stimulator (Wd) was not completely resolved (*0404/08/19, *1501). However, the low proliferative response of the MLR of this pair -similar to that of autologous proliferative response - (Figure 7.7 B) suggests a match in HLA-DRB1 in this pair (see text).

<sup>b</sup> A broad mismatch (in class I and II antigens) was obtained pairing the responders (A or W) with a buffy coat (BC3).

**Proliferative response**

The proliferative response of the pairs of PBMC selected for this phase of the study was measured in a standard one way MLR. Figures 7.7 and 7.8 shows two standard MLR for the responders A (Figure 7.7) and W (Figure 7.8) towards their stimulators and towards controls (these are autologous MLR, “A vs A” and “W vs W”, or cells cultured alone “A alone” and “W alone”). Whereas low proliferation was detected in autologous and HLA-matched pairs in both responders, high proliferative responses were detected in pairs with a class II mismatch and in the broad mismatched pairs (A vs BC3 and W vs BC3) (see Table 7.2 and 7.3 for the HLA type of the samples). Although the HLA-DR typing for Wd was ambiguous (DRB1* 0404/08/09, *1501), the most common type for this combination is the DRB1* 0404 allele (Imanishi, Akaza et al. 1991). Furthermore, the proliferation of W vs Wd in the MLR is comparable to the autologous one (Figure 7.7 B), in contrast with the proliferation towards Witt (DRB1* 0401, * 1501 and therefore mismatched).

The proliferative response of A was always higher that the proliferative response to W, towards the same mismatch (Figures 7.7 and 7.8). A vs Gui and W vs Witt were
both pairs with a mismatch in HLA-DR, but the proliferation of W was always lower compared with proliferation of A.

The MLC for the responders A and W were set up concurrently. The MLRs were done simultaneously to the MLC used for the ELISPOT assay, and were repeated on separate occasions with similar results. The proliferative response seen in the MLR was a parameter related to the alloreactivity studied in the ELISPOT assay.
Figure 7.7. MLR for responder A. A standard one way MLR was performed with PBMC from A as responders and irradiated PBMC from J, Gu, Gr and buffy coat 3 (BC3) as stimulators in two independent occasions (experiment 1 and experiment 2). The HLA-typing of these samples is shown in Table 7.2. For the MLR, responders and stimulators were cultured in a total volume of 200 µl using a 1:1 ratio at a final responders concentration of 1x10⁶ cells/ml, in triplicate wells in 96 round bottom well plates. After 5 days of culture, the cells were harvested. 18 hrs before harvesting, 1µCi/well [³H] thymidine/well was added. The incorporated radioactivity was counted as described in Figure 7.4. Results are expressed as mean of triplicate cpm ± SD.
Figure 7.8. MLR for responder W. A standard one way MLR was performed with PBMC from W as responders and irradiated PBMC from Wd, Witt and BC3 as stimulators in two independent occasions (experiment 1 and experiment 2) as described in Figure 7.7. The HLA-typing of these samples is shown in Table 7.3. Results are expressed as mean of triplicate cpm ± SD.
**IFNγ ELISPOT.**

The IFNγ ELISPOT assay was performed twice for both responders (A and W), with 2x10⁵ or 5x10⁵ responder cells/well in the re-stimulation plates.

The frequency of IFNγ producing cells was measured for responder A in two independent experiments (Table 7.6 and Figure 7.9). This responder was paired with matched stimulators (J and Gr), with a stimulator with one HLA-DRB1 mismatch (Gu) or stimulator with a broad mismatch (BC3). A negative control was the responder A cultured with autologous irradiated cells. A second negative control were irradiated stimulators alone to determine their contribution to the IFNγ spots, and a third negative control were cells without re-challenge (5x10⁵ responder cells/well), to determine if re-stimulation is necessary for the production of the cytokine. The positive control for each MLC were cells from the MLC restimulated with PHA, which always had a high frequencies of IFNγ spots (more than 50 spots in 1x10⁵ cells/well), indicating that the cells were viable and responsive to stimulation and that a low number of spots in the well truly indicates a low response.

There was a low frequency of spots in the negative controls: less than 15 for Experiment 1 and less than 20 for Experiment 2 (Table 7.6). In contrast, in responder cells cultured and re-stimulated with cells mismatched in one allele of HLA-DRB1 there was the highest frequencies of IFNγ spots (Figure 7.8, A vs Gu and Table 7.6, A vs Gu). The frequency of spots in the broad mismatched pair (A vs BC3, 52 spots/5x10⁵ cells) was lower than the frequency found in A vs Gu. Similarly, the proliferative response seen in two independent MLR (Figure 7.8) was lower compared with the proliferation of A vs Gu. The typing of BC3 did not to define the extent of the mismatch in the A vs BC3 pair. However in this pair there is at least one mismatched allele (DRB1*0401 and DRB1*0701/03/04) and DRB4 and DRB5 alleles can also be mismatched (see Table 7.2).
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th>Experiment 2</th>
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Table 7.6. Frequency of IFNγ spots in matched and HLA-DRB1 mismatched pairs for responder A. The ELISPOT assay was performed for responder A as described in Figure 7.8 in two separate experiments. The Table shows the frequency of IFNγ spots/well in different conditions of re-stimulation: for 5×10⁵ no re-challenged responder cells (500-), 5×10⁵ rechallenged responder cells (500), 2×10⁶ rechallenged responder cells (200) and 5×10⁶ irradiated stimulators alone (irrad). The numbers are the average of two consecutive counting on the same plate.

The IFNγ ELISPOT assay was performed twice for responder W (Figure 7.10). As described for responder A, the negative controls had low frequency of spots: below 11 in Experiment 1 and below 8 in Experiment 2, with the exception of the negative control from the W vs BC3 pair (cells without re-stimulation), where the frequency of spots was 25 and 38 spots per 5×10⁵/cells (see Table 7.7, W vs BC3 in non restimulated cells). This may indicate that these cells were stimulated strongly during the course of the MLC and that even in the absence of re-stimulation there is IFNγ production.

The stimulator Wd’s HLA-DR type was not completely resolved and the matching between W and Wd is unknown (as was mentioned in Table 7.4). The proliferation of this pair in the MLR was low compared with the mismatched stimulator (Witt) (Figure 7.8); this low proliferative response suggests a HLA-DRB1 match between W and Wd. In support of this, the frequency of spots for this pair is low (11 spots/5×10⁵ cells), similar to the frequency of spots in the negative controls (below 10 spots/5×10⁵ cells as mentioned above) (Figure 7.10). In contrast, the IFNγ spot
frequency in the single allele-HLA-DRB1 mismatch pair (W vs Witt), was 69 spots/5x10^3 cells.

A broad HLA-mismatch in the W vs BC3 pair, induced a high proliferative response compared with the rest of the pairs (as shown in Figure 7.8) in two independent experiments. The frequency of IFNγ spots for this pair was the higher in Figure 7.10, although the frequency of spots was similar in a independent experiment.

<table>
<thead>
<tr>
<th></th>
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<th>Experiment 2</th>
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<td>W vs BC3</td>
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Table 7.7. Frequency of IFNγ spots in matched and HLA-DRB1 mismatched pairs for responder W. The ELISPOT assay was performed for responder W as described in Figure 7.9 in two separate experiments. The Table shows the frequency of IFNγ spots/well in different conditions of re-stimulation as explained in Table 7.6. The numbers are the average of two consecutive counting on the same plate.

The number of spots in the negative control wells and in the matched pairs were similar for each assay. In contrast, the IFNγ spots were found at higher frequencies in wells of pairs mismatched in one HLA-DRB1 allele. High frequencies of IFNγ spots were also found in broad mismatched combinations. Thus, the production of IFNγ in response to a single HLA-DRB1 allele mismatch can be detected by the ELISPOT assay. The results suggest that HLA-DR mismatches induces high frequencies of IFNγ producing cells.
Figure 7.9. Frequency of IFNγ producing alloreactive lymphocytes in a HLA-DRB1 mismatch. PBMC from responder A and different stimulators (J, Gu, Gr and B7) were paired in basis of their tissue type -as explained in the text and in Table 7.2 and 7.5- and cultured in a 6 days MLC. Additionally, an autologous MLC was set up. The final concentration of cells in the MLC was 1x10^6 responder cells/ml in a 1:1 responder-stimulator ratio. At the end of MLC, the cells were harvested and washed twice in serum-free medium and 5x10^5 cells were rechallenged with the same alloantigen and keeping the same responders:stimulators ratio for 24 hours in 200ml/well in 96 U bottom well plates. The cells were then harvested and transferred to the ELISPOT capture plate. The capture plates were placed at 37°C in a CO2 humidified atmosphere for 20 hours. The cells were then discarded from the plates, the plate was washed and a previously determined optimal dilution of rabbit anti-human IFNγ polyclonal Ab was added, followed by the addition of the alkaline phosphatase-conjugated mouse anti-rabbit polyclonal Ab and finally by the addition of alkaline phosphatase substrate solution, as described in this chapter Section 7.2.6.2. The reaction was stopped by washing twice with water and the plate was air dried. All the spots/well were counted using an Image Analysis Software for Macintosh. As the number of cells was limiting for these experiments, no duplicates were done per plate but each plate was counted two consecutive times and the ELISPOT was performed twice in separate occasions, with similar results. Control wells included responder PBMC from each MLC re-stimulated for 24 hrs with an predetermined optimal concentration of PHA instead of alloantigen, and the number of spots was always greater than 50 spots per 1x10^5 cells. The plate was counted twice and an average number/well was obtained. The results represent the of the frequency of spots for 5x10^5 cells without rechallenge -black bars-, 5x10^5 re-stimulated cells -light grey bars-, 2x10^5 re-stimulated cells -dark grey bars-, or irradiated stimulators 5x10^5 -white bars- per well.
Figure 7.10. Frequency of IFNγ producing alloreactive lymphocytes in a HLA-DRB1 mismatch. PBMC from responder W and different stimulators (Wd, Witt and BC3) were selected in basis of their tissue type to be cultured in a 6 days MLC as described in Figure 7.8 -and Tables 7.3 and 7.5-. Then, cells were harvested and rechallenged with allogeneic stimulus, PHA or where not re-challenged, to perform an IFNγ ELISPOT as described in Figure 7.8. The spots for positive control cells stimulated with PHA, were always greater than 50 spots per 1x10^5 cells. The plate was counted twice and an average number/well was obtained. The results represent the of the frequency of spots for 5x10^5 cells without rechallenge -black bars-, 5x10^5 re-stimulated cells -light grey bars-, 2x10^5 re-stimulated cells -dark grey bars-, or irradiated stimulators 5x10^5 -white bars- per well.
7.4 Discussion and conclusions

Whereas the MLR assay has been dismissed because the low accuracy in predicting BMT outcome (Hows, Yin et al. 1986), frequency analysis, using LDA of IL-2 producing (Schwarzer, Jiang et al. 1993; Schwarzer, Jiang et al. 1994; Wang, Proctor et al. 1996) or cytotoxic (Kaminski, Hows et al. 1989; Roosnek, Hogendijk et al. 1993) anti-recipient T lymphocyte precursors in the peripheral blood of the bone marrow donor has been used as a predictive assay for the development of moderate-to-severe acute GvHD in HLA identical siblings and unrelated donor-patients pairs. In this chapter, the determination of the frequency of cytokine producing alloreactive lymphocytes was addressed, by proposing an in vitro assay to reveal the frequency of type 1 and type 2 T cells induced by allostimulation. Two approaches were taken. Firstly, the frequencies of type 1, type 2 and type 0 alloreactive CD3+ and CD8+ T cells were assessed by flow cytometry by ICS using short term T cell lines from a MLC stimulated to produce cytokines. Secondly, the frequency of IFNγ producing alloreactive T lymphocytes was assessed by the ELISPOT assay.

For the first part of this chapter (Section 7.3.5) pairs of PBMC broadly mismatched in HLA-A, B and DR Ag were used. The samples were selected randomly and their tissue type was done at low or medium resolution (see Table 7.1 and 7.2). The class I and class II typing performed in all these samples (BC1, BC2 and BC3) is enough to show a broad mismatch.

ICS was not able to resolve differences in frequencies of cytokine production of allogeneic stimulated cells. This was due to the mitogenic stimulus (anti-CD3 plus PMA) used to stimulate the production of cytokines able to be detected intracellularly. However, anti-CD3 plus PMA stimulation was used because ICS requires a strong re-stimulation to induce detectable intracellular cytokines (Section 7.3.3 and 7.3.4). Thus, using ICS, it was not possible to detect the frequency of cytokine producing cells from allogeneic cultures using alloantigen as re-stimulation, whereas mitogens induced cytokine production to detectable levels (Figure 7.2). However and in contrast with the results shown in Figure 7.2, intracellular IFNγ has been detected in allogeneic T cell clones under re-stimulation with alloantigen using ICS (Heeger, Greenspan et al. 1999). In this study, Heeger et al found that 36.5% cells from a T cell clone produced detectable intracellular IFNγ after 2 hrs of allogenic stimulation. The ability to detect intracellular IFNγ in these cells may be because memory cells have a low threshold of stimulation, but also because clones can be selected for their capacity to produce high and detectable amounts of IFNγ. Whereas
the frequency of IFNγ producing cells measured by the ELISPOT or ICS was similarly high in Heeger's study (38.7% and 36.5% respectively) the frequency of IL-5 spots was above 1 in 300 allostimulated cells but the intracellular frequency of IL-5 producing cells not shown nor mentioned. Similarly, the frequency of IL-4 spots (zero) was only shown with ELISPOT.

Mitogens have been used in the literature to increase the frequency of cytokine producing alloreactive cells. In mice with an experimental aGvHD, the frequency of IFNγ secreting lymphocytes was less that 0.1% even when harvested at the peak of the GvHD, but the frequency was augmented to 30.0% by mitogenic stimulation (Kelso 1990). Nevertheless and as discussed in Chapter 6 Section 6.4, mitogens abrogate differences in the frequencies of cytokine producing alloreactive T cells and control cells (Kelso and Gough 1988; Tary-Lehmann, Hricik et al. 1998). In this Chapter, anti-CD3 plus PMA stimulation induced the production of IFNγ (the type 1 cytokine used) by a high frequency of cells, whereas less cells produced IL-4 (Figure 7.5 and 7.6). This was regardless of culture with allogeneic stimulation or not. Thus, ICS indicates the capacity of the live cells to produce cytokine but the re-stimulation required to produce enough cytokine is so strong that it annuls differences between frequencies of cells stimulated or non stimulated with alloantigens.

Although there was not clear difference between cytokine producing cells from the different MLC, the results from Figures 7.5 and 7.6 show that there was a higher frequency of type 1 IFNγ producing cells within the CD8+ compartment when compared to the CD3+ compartment (that includes both CD4+ and CD8+ cells), confirming that CD8+ cells have a high capacity to produce IFNγ. In contrast, the frequency of type 2 cells (IL-4 positive) was similar between the CD3+ and the CD8+ compartments in the experiments performed (Figures 7.5 and 7.6). Cytotoxic T cells produce large amounts of IFNγ on stimulation (Pestka, Langer et al. 1987), and it has been shown that an inoculum of CD8+ cells is enough to induce GvHD in mice (Korngold 1992), indicating that alloreactive cells CD8+, high IFNγ producers, are central in the development of the disease. The larger capacity of IFNγ production of CD8+ cells maybe due to differences in the regulation of IFNγ gene expression in CD8+ T-cell and other lymphocyte subsets (such as CD4+*) (Aune, Penix et al. 1997) as a more stringent signalling requirements for IFNγ production by CD4+ cells: only CD4+ cells require Stat4 for induction of IFNγ secretion via TCR stimulation and require IL-12 and Stat4 activation to produce IFNγ in response to antigen, whereas IFNγ production by TCR-stimulated CD8+ cells does not require STAT4 and their response to antigen is independent of IL-12 (Carter and Murphy 1999).
In these experiments, PBMC cultured without stimulation survived the 6 days culture. The FSC and SSC of these populations revealed that mainly lymphocytes survive at this time; although some macrophages were present at this point, most of them die of spontaneous apoptosis after 24 hours of in vitro culture in the absence of stimulation (Um, Orenstein et al. 1996). The live cells from these cultures retained the capacity to produce cytokines on stimulation, as seen in Figures 7.5 and 7.5, but they did not proliferate, as seen in the MLRs shown in Figure 7.4 panel A and B ("cells alone").

It has been shown that allogeneic stimulation induces IFNγ secretion in lymphocytes (Velardy, Varese et al. 1989; Tanaka, Imamura et al. 1995) and the amount of IFNγ secreted has been correlated with the degree of alloreactive reactions in BMT: high titres of IFNγ produced in MLC corresponds to the higher grades of aGvHD (Niederwieser, Herold et al. 1990; Tanaka, Imamura et al. 1994). Similarly, high titres of secretion of IFNγ by alloreactive and primed lymphocytes in vitro has been correlated with aGvHD grades II-IV after BMT (Dickinson, Sviland et al. 1994). The measurement of the secretion of IFNγ by alloreactive and primed lymphocytes, as well as the histological damage caused in patient skin explants, has been proposed as a parameter predictive of GvHD after BMT (Dickinson, Sviland et al. 1988; Dickinson, Sviland et al. 1991).

Because the frequency of alloreactive IFNγ producing cells in single HLA mismatched pair has not been investigated, it was proposed in the second phase of this study to define the frequency alloreactive IFNγ producing cells in HLA-DR mismatched unrelated pairs. To do this, Ag specific re-stimulation was used to determine frequencies of IFNγ producing alloreactive lymphocytes in a single mismatch condition using the IFNγ ELISPOT assay.

To find samples mismatched at a single HLA locus, panels of unrelated bone marrow donors were analysed to select volunteers on basis of a common caucasian type. From this group, matched or HLA-DR mismatched pairs were chosen. This was because whereas HLA-DR mismatches favours the development of type 1 CD4+ T alloresponsive cells in MLC (Pawelec, Rehbein et al. 1996), in fully HLA-mismatch pairs, similar frequencies of type 1 and 2 cytokine producing alloreactive lymphocytes were found (Matesic, Valujskikh et al. 1998). HLA-DR mismatches induce the secretion of IFNγ, which is correlated with the degree of mismatch (Toungouz, Denys et al. 1996).

It has been reported that the use of PBMC as stimulator cells for the IL-2 secreting precursor assay of alloreactive T lymphocytes faces the problem of IL-2 produced by the stimulator cells (Reisaeter, Thorsby et al. 1996). Although high doses of irradiation abrogate IL-2 production by stimulators, high doses also reduce the capability of allo-
restimulation by PBMC (Clouse, Bach et al. 1984; Schanz, Roelen et al. 1994). Whereas some studies have tried to avoid this problem using EVB transformed cells, (Deacock and Lechler 1992; Theobald and Bunjes 1993; Zanker, Jooss-Rudiger et al. 1993) it has been seen that EBV transformed cells derived peptides can be presented by HLA molecules shared between stimulator and responder (Wallace, Rickinson et al. 1982; Misko, Pope et al. 1984). Thus, PBMC and not EVB transformed cells were used here as stimulators to perform the IFNγ ELISPOT assay.

The results of the IFNγ ELISPOT show that HLA matched pairs and autologous cultures had low frequency of IFNγ producing cells (below 9.4 spots/5x10^5 cells for responder A in all control except in one case, the matched pair A vs Gr with 20 spots/5x10^5 cells, and below 10 spots/5x10^5 cells for responder W, except in non re-stimulated cells from the W vs BC3 MLC where there was a frequency of 38 and 25 spots per 5x10^5/cells in two experiments, as described in Section 7.3.7). In contrast, the HLA-DR mismatched pairs had high frequencies of IFNγ producing lymphocytes: A vs Gu had 70 and 55 spots/5x10^5 responder cells in two experiments and W vs Witt: 42 and 69 spots/5x10^5 responder cells in two experiments. When 2x10^5 responder cells were used, high frequencies of IFNγ spots were also obtained in these mismatched pairs (Tables 7.6 and 7.7 and Figures 7.9 and 7.10). Although the frequency of spots was not always higher when 5x10^5 responder cells were/well used in comparison with 2x10^5 responders, the frequency of IFNγ spots was only high in the mismatched pairs used. The finding that these HLA-DRB1 mismatched pairs produce IFNγ disagrees with previous reports that propose that only class I disparities induces IFNγ secretion in a 2-5 days MLR (Tourougouz, Denys et al. 1995). The disparities in these results may be because in this chapter, the culture with the allogeneic stimulus is longer (6 days), followed by 24 hr re-stimulation before the detection of IFNγ and because different assays are used for the detection of cytokine: ELISA and ELISPOT. Additionally cells can consume the cytokines secreted to the supernatant during 2-5 days culture but in a ELISPOT assay, the cytokines are more likely to be trapped by the capture Ab.

The results of Section 7.3.7 indicating that HLA-DRB1 disparities induces a high frequency of IFNγ producing cells, also disagree with a study published by Heeger et al (Heeger, Greenspan et al. 1999). Using an IFNγ ELISPOT, these authors found high frequencies of IFNγ producing alloreactive T cells and concluded that there is not correlation between the mismatches and the frequencies of IFNγ spots. This was because in two fully match pairs Heeger et al found both high (more than 30/3x10^5 cells) and low (less than 5/3x10^5 cells) frequencies of IFNγ spots, whereas in a HLA-A, B and DR mismatched pairs they also found high (over 100/3x10^5 cells) a low
(below $5/3 \times 10^5$ cells) frequencies of spots. However, the tissue typing in Heeger's work was done at low resolution: serology typing was used for class I alleles (HLA-C typing was not included), and low resolution SSP was used to type HLA-DRB1 and HLA-DQB1 alleles. The serologically defined HLA-A2 antigenic determinant comprises 30 different alleles and there are 221 alleles in the HLA-DRB1 gene (Marsh, Parham et al. 2000). Therefore, the authors can not assure HLA matching in these samples and the "matched" sample with high frequency of spots is probably a mismatched sample. In contrast, in this chapter the samples used for the ELISPOT were typed at the allelic level (Section 7.2.2). However, low resolution-typing does not explain why Heeger et al found a very low frequency of IFN$\gamma$ spots in one of the HLA-A, B and DR mismatched samples, but the authors did not perform any other functional assay to determine alloreactivity of the samples. In comparison, in this chapter a MLR was done for every experiment, which informs about of the viability of the samples and their proliferation to class II disparities (Figures 7.7 and 7.8). Unexpected results from this Chapter were that, in a broad mismatch (such as responders A/W vs BC3) the frequency of IFN$\gamma$ spots was not necessarily higher than the frequency found in response to a single allele mismatched (Figures 7.9 and 7.10, samples A vs BC3 and W vs BC3 respectively). In these experiments, this may be due to the strong stimulus received in the first hours of re-stimulation in these broadly mismatched samples. Thus, the peak of the response may have been missed in these samples.

Although similar frequencies of IFN$\gamma$ producing cells were found in two responders (A and W) towards their HLA-DRB1 mismatched stimulators, the proliferative response of A toward its stimulator was always higher than the proliferative response of W toward it stimulator. The maximum proliferative response of A was seen when 52,793$\pm$2472 cpm were obtained in the MLR, whereas the maximum proliferative response of W was seen with 7,072$\pm$954 cpm (Figures 7.7 and 7.8). An explanation for this may be related with the number, location and type of amino acid differences that comprise these HLA mismatches. Of the 6 exons of the HLA-DRB1 gene, exons 2 and 3 encode for the extracellular domains with exon 2 encodes for the $\beta1$ domain which forms the peptide-TCR binding region (Stern, Brown et al. 1994). Typing by molecular methods focuses in the HLA-DRB1 exon 2, which is amplified by PCR in SSO, SSP or RSCA (Marsh, Parham et al. 2000). As discussed in Section 7.3.6 (see Tables 7.2, 7.3 and 7.5), the responder A and the stimulator G are probably HLA-DRB1*0401, *1501 and HLA-DRB1*0403, *1501 respectively. The differences in exon 2 between HLA-DRB1*0401 and -DRB1*0403 alleles result in three amino acid disparity at positions 71, 74 and 86 of the protein sequence (peptide binding sites in
the β1 domain) which is shown in Table 7.8. A model of a HLA-DR molecule based on crystallographic data (Stern, Brown et al. 1994) indicating the location of positions 71, 74 and 86 of the β chain is shown in Figure 7.11 panel A. All three of these variable positions lie within an alpha-helical segment and their side-chains project into the peptide-binding groove where they may influence the peptide-binding specificities of these allotypes. The substitution at position 71 of Arginine in HLA-DRβ1*0401 for lysine in HLA-DRβ1*0403 is a conservative difference that maintains a positively charged basic side chain. This difference is unlikely to alter peptide-binding specificity. However, the substitutions at positions 74 and 86 involve more radical differences. The small neutral hydrophobic Alanine at position 74 in HLA-DRβ*0401 is replaced by the large negatively charged acidic side chain Glutamic acid in HLA-DRβ1*0403 and the small Glycine residue at position 86 in HLA-DRβ*0401 is replaced by the larger aliphatic side chain of Valine in HLA-DRβ*0403. (Table 7.8). The responder W and stimulator Witt combination are mismatched for HLA-DRB1*0404 and HLA-DRB1*0401 alleles respectively. These β chains differ at only two positions; the lysine to Arginine substitution at 71 and the Valine to Glycine substitution at position 86 (Table 7.9 and Figure 7.11 B). The presence of two rather than three residue differences in this responder and stimulator combination may account for the lower proliferative response observed across this HLA mismatch.
Table 7.8. Amino acid differences between HLA-DRB1*0401 and *0403. The one-letter code for amino acids is used and their properties are described.

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<th>HLA-DRB1 allele</th>
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<th>*0403</th>
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<tr>
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<td>amino acid</td>
<td>charge</td>
</tr>
<tr>
<td>71</td>
<td>K</td>
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</tr>
<tr>
<td>74</td>
<td>A</td>
<td>Neutral Small Aliphatic Hydrophobic</td>
</tr>
<tr>
<td>86</td>
<td>G</td>
<td>Neutral Very small Aliphatic</td>
</tr>
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</table>

Table 7.9. Amino acid differences in the HLA-DRB1*0404 and *0401. The one-letter code for amino acids is used and their properties are described.

<table>
<thead>
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<th>HLA-DRB1 allele</th>
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<td>71</td>
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<td>Positive charge Basic</td>
</tr>
<tr>
<td>86</td>
<td>G</td>
<td>Neutral Very small Aliphatic</td>
</tr>
</tbody>
</table>

The residue differences that stimulate an alloresponse across the A and Gu and W and Witt responder and stimulator combinations line the peptide-binding groove and are not directly accessible for recognition by TCR. They are likely to exert their effect by altering the repertoire of peptides bound by the HLA-DR molecules and these differences in bound peptide are detected by alloreactive T cells. The Glycine/Valine difference at position 86 may be particularly important. Previous studies have demonstrated that the Glycine/Valine dimorphism at position 86 of the HLA-DR β chain alone can have a significant impact on recognition by alloreactive T cells (Lang, Navarrete et al. 1990; Johnson, Tang et al. 1991; de Koster, van Rood et al. 1992). HLA-DR allotypes that differ only at this dimorphism possess divergent peptide-binding specificities (Davenport, Quinn et al. 1995; Verreck, van de Poel et al. 1996). The presence of Glycine at position 86 of the HLA-DRβ chain creates a pocket within the class II peptide-binding groove that selectively binds peptides possessing a bulky
aromatic or aliphatic residue. In contrast, presence of the larger Valine at position 86 of the HLA-DR β chain limits the size of this pocket and therefore selects smaller aliphatic residues. This radical difference in the repertoire of bound peptides creates many immunogenic epitopes that may be recognised by alloreactive T cells.

In conclusion, ICS was not able to identify distinct frequencies between different cytokine producing alloreactive T cell lines. In contrast, in the IFNγ ELISPOT assay low numbers of IFNγ spots were found in matched pairs whereas higher frequencies of IFNγ spots were found in response to a single HLA-DRB1 allele mismatch. These results are encouraging to develop this assay further to establish the frequency of IFNγ producing cells in HLA-mismatched combinations.
Figure 7.11. Amino acid differences in the β1 domain between two pairs of HLA-DR molecules. Ribbon diagrams of HLA-DR molecules, showing the amino acid residues 71 and 86 (A) or 71, 74 and 86 (B) in fill space highlighted in colours: residue 71 in blue, residue 774 in yellow and residue 86 in red. These residues are located at polymorphic peptide-binding sites. This figure shows an irrelevant bound peptide.
7.5 Future experiments

As two different techniques were used in this chapter to analyse frequencies of alloreactive T cells, future work comprises experiments with ICS and with ELISPOT.

To assay frequency of alloreactive T cells by ICS, staining for the CD3 and CD8 antigens was performed (sections 7.3.2 and 7.3.3). This was done to identify the frequencies of cytokine producing cells in both the T cells (CD3⁺) and cytotoxic T cell (CD8⁺) population. The separate staining provided information of a higher capacity of CD8⁺ cells to produce IFNγ (Figure 7.5 compared with Figure 7.6). However, the CD8 antigen is expressed not only by cytotoxic T lymphocytes but also by some NK cells (Mansour, Doinel et al. 1990; Albi, Ruggeri et al. 1996; Kieffer, Bennett et al. 1996; Lucia, Froio et al. 1997) and the staining for the CD8⁺ antigen only does not allow differentiation between cytotoxic T lymphocytes and NK cells (Ohteki and MacDonald 1994). Four-colours flow cytometry would have solved this problem, by using an additional marker (CD3 plus CD8 for T cells and CD56 and CD8 for NK cells) having two fluorochromes in the cell surface. This can discriminate CD3⁺ cells (NK) and cytotoxic T cells (CD3⁺CD8⁺), and ICS for two cytokines (two fluorochromes) would determine frequencies of both populations of cytokine producing cells.

In contrast with the ICS, in the ELISPOT assay it is not possible to determine the type of cytokine producing cells (CD3⁺, CD4⁺ or CD8⁺ or NK lymphocytes) unless the responder cells are purified by negative depletion using magnetic beads of sorting by flow cytometry before culture in the MLC. This protocol would not include autologous APC the responder population of cells. Using an animal model of skin transplant, Benichou et al found that few days after the transplant, the frequency of IFNγ producing cells stimulated with intact MHC was higher that the frequency of cells stimulated with cells sonicates. Nevertheless, Benichou et al found that the frequency of cells recognising intact MHC molecules decreased with time. Thus, additional modifications to the experiments performed in this chapter can include purified APC (such as monocytes) as stimulators of pure T cells. Whiting the PBMC, monocytes can be separated because of their adherence to plastic, although this results in a poor purity. Alternatively, monocytes (which are CD14⁺ cells in peripheral blood (Simmons, Tan et al. 1989) can be separated by magnetic sorting. Monocytes can be further cultured to develop into dendritic cells. B cells can also be used as APC but, although EBV transformed cells have been used to stimulate
alloreactive T cell lines, antigens of EBV origin can influence the specificity of the response (Burrows, Khanna et al. 1994).

In comparison with LDA, the ELISPOT assays are not performed with dilutions of allogeneic responder cells or their products and give a direct measurement of frequencies of positive cells. The results are read fast and the reading can be assisted by computerised analysis. Although a dilution of responder cells is not necessary for every ELISPOT assay, a more comprehensive number of responder cells/well and number of stimulators/well should be explored for the assay used in this chapter in the future; this is because in the ELISPOT assays performed here, 2 x 10^5 and 5 x 10^5 responder cells/well were used and stimulated with the same number of irradiated stimulators (Section 7.2.6.2). The most consistent results were obtained using 5 x 10^5 responder cells/well compared with 2 x 10^5 cells/well. This may be due to an increased noise/signal ratio when less signal is obtained, as the noise does not decreases using less cells for the assay (i.e., number of IFNγ spots in the absence of stimulation). In this work, the small number of cells limited a broader titration of responder and stimulator cells for the assay. The frequency of IFNγ producing cells was not determined in these experiments because different frequencies were obtained using 2 x 10^5 or 5 x 10^5 responder cells/well. Therefore, this work can be extended to establish the number of cells necessary to determine the frequency of IFNγ spots in a mismatched pair.

Although several negative controls were included in the ELISPOT assay (such as non-restimulated cells and irradiated stimulators, which had low frequency of IFNγ spot, Figures 7.9 and 7.10), 3rd party-irradiated allogeneic PBMC were not included as a control for the specificity of the alloresponse. This was because the limited amount of responder cells available. Nevertheless, the responder cells had different frequencies of IFNγ spots according to the HLA of their stimulators, which suggests that the frequency of IFNγ production may be dependent on the mismatch. Nevertheless, a negative control consisting 3rd party-irradiated allogeneic PBMC should be included in the future for this assay.

The study of frequencies of cytokine producing alloreactive T cells by the ELISPOT assay can be expanded in the future to confirm the results obtained, using a larger number of samples mismatched in one HLA-DRB1 allele. Additionally, mismatches in other class II loci, such as HLA-DP, that in some transplant centres is not matched (Pawelec, Ehninger et al. 1986; Petersdorf, Smith et al. 1993; Santamaria, Reinsmoen et al. 1994; Hansen, Petersdorf et al. 1997), can be included. This work can likewise be extended by including pairs of single class I allele mismatches (were IFNγ
production can be due to the stimulation of alloreactive CD8+ T cells). This is because although BMT are performed with the better match possible, due to the lack of perfectly matched donors, BMT are performed using mismatched donors (Kernan, Bartsch et al. 1993; Sierra, Storer et al. 1997). In those cases, the IFNγ ELISPOT could provide the frequency of IFNγ producing cells before performing the transplant.

Not all the patients receiving BMT develop acute GvHD, and the reason why this happens is unknown. This may be due to the development of different frequencies of cells producing type 1 or type 2 cytokines. Whereas T cell cytokines are involved in the development of the different phases of disease, type 2 cells are correlated with a less aggressive disease (Fowler, Kurasawa et al. 1993; Krenger, Snyder et al. 1995) and are related with chronic GvHD (Rus, Svetic et al. 1995). Recently, frequencies of IL-4 producing cells have been measured by an ELISPOT assay in patient-donor pairs post BMT: a correlation of low frequency of IL-4 producing cells was correlated with bad prognosis whereas high IL-4 was correlated with less aggressive acute GvHD (Imami, Brookes et al. 1998). Furthermore, a negative relation of the frequencies of IL-4 producing cells and CTLp was also found. Thus, the estimation of both type 1 and type 2 cytokines can be combined to provide more extensive information of the transplant outcome. The possibility that different mismatches induce the development of different T helper profiles (type 1 or 2) can also be explored using a two colours-ELISPOT which as been used by Czerkiny et al (Czerkinsky, Moldoveanu et al. 1988). Although classically IL-4 is considered a classical type 2 cytokine, higher frequencies of IL-5 compared with IL-4 have been found in allogeneic reactions with restricted mismatches (Benichou, Valujsikh et al. 1999), which may indicate that IL-5 can be a better cytokine to look for.

The final aim of the development of this IFNγ ELISPOT assay was to correlate the frequencies of IFNγ secreting cells and the development of different grades of acute GvHD, performing the ELISPOT assay with donor-patient pairs programmed for a BMT. Thus, the ELISPOT can be considered as a pretransplant assay that can be used to chose between different donors when more that one is available. This could be done running the ELISPOT assay in parallel using lymphocytes from all the donors as responder cells and the patients cells as stimulators. In bringing this assay to the clinical scenario, it would be important to make it easy and fast to perform in a routinely manner, by reducing the MLC duration to less than 6 days and by offering computerised analysis software to estimate the frequencies of spots. A high frequency of type 1 over type 2 cytokine secreting cells, determined by two colours
ELISPOT may be correlated with aggressive alloreactivity and a stronger profilaxis can then be recommended by the physician.
8. Chapter 8. Expression of the chemokine receptors CCR3 in polarized T cell lines

8.1 Introduction

The expression of receptors for chemoattractants, permits the detection of signals of inflammation, i.e., interleukins, chemokines, or monokines secreted by activated cells (Butcher 1991; Butcher and Picker 1996; Baggio, Dewald et al. 1997). Chemokines are low molecular weight proteins with chemoattractant properties (Baggio, Dewald et al. 1994; Jose, Griffiths-Johnson et al. 1994; Rollins 1997) characterised by a conserved cystein (Cys) residues covalently linked (Rollins 1997). They bind to chemokine receptors with overlapping specificities (Comandiere, Ahuja et al. 1995). For instance, the eotaxin receptor, also know as the chemokine receptor (CCR) 3, expressed on eosinophils, also binds the chemokines RANTES, monocyte chemoattractant protein (MCP)-3 and MCP-2 (Neote, DiGregorio et al. 1993; Daugherty, Siciliano et al. 1996). T lymphocytes activated by chemokines acquire migratory capacity (Tanaka, Adams et al. 1993) due to the expression of adhesion molecules on the T cell surface (such as selectins and integrins) and chemokines receptors. Thus, adhesion molecules and chemokines are able to indicate the route of leukocyte migration (Butcher 1991; Springer 1994).

The expression of different chemokines receptors and adhesion molecules on Th1 or Th2 cells has been described. For example, a ligand for E and P-selectines has been described as exclusively expressed on Th1 cells (Borges, Tietz et al. 1997) and correlated with the exclusive infiltration of Th1 cells to sites of acute inflammation in skin (Astrup, Vestweber et al. 1997). Similarly, Sallusto et al. (Sallusto, Mackay et al. 1997) described the selective expression of the receptor for eotaxin, the CCR3 in Th2 cells derived from CB (Sallusto, Mackay et al. 1997). Other chemokine receptors such as CXCR3, have been found to be preferentially expressed on Th1 and Th0 cells (Sallusto, Lenig et al. 1998). Eotaxin, a chemokine produced by epithelial cells and macrophages (Ganzalo, Jia et al. 1996), was originally described for its attraction properties to eosinophiles and basophiles (Jose, Griffiths-Johnson et al. 1994; Garcia-Zepeda, Rothenberg et al. 1996; Ponath, Qin et al. 1996) which express CCR3. Eotaxin binds to CCR3 with high specificity, inducing cell activation events such as raising the levels of intracellular Ca²⁺ (Kitaura, Nakajima et al. 1996). CCR3 is also expressed in a low proportion of T cells from the periphery. CCR3 on Th cells is
functional, since eotaxin exposure to CCR3+ T cells induces an increase in intracellular Ca2+ levels (Sallusto, Mackay et al. 1997). Furthermore, CCR3 expression enhances T cell migration to eotaxin (Bonecchi, Bianchi et al. 1998), which has been correlated with the type 2 T cell phenotype, since Th2 cells have greater \textit{in vitro} migratory capacities in a eotaxin gradient when compared to Th1 cells (Bonecchi, Bianchi et al. 1998). \textit{In vivo}, eosinophils and T cells (predominantly CCR3+) have been isolated from mice epithelium with inflammatory type 2 processes such as contact dermatitis and ulcerative colitis (Gerber, Zanni et al. 1997). Furthermore, the expression of CCR3 in Th2 cells has been correlated \textit{in vivo} with the ability of T cell migration to the site of inflammation (Loyd, Delaney et al. 2000).

The aim of this Chapter was to generate T cell lines in type 1 or 2 polarizing conditions and to detect the expression of CCR3 on them. This marker could then be used as an additional tool to identify type 2 T cells.
8.2 Material and Methods

8.2.1 Type 1 and type 2 T cell lines

Type 1 and type 2 T cell lines were generated as described in Chapter 2, Section 2.3.2. Non specific PHA lines were generated from PBMC and cultured in complete medium with 20 ng/ml IL-2 and a predetermined optimal concentration of PHA in type 1 (with 2ng/ml hrIL-12) or type 2 (200 U/ml hr IL-4 plus 2 µg/ml anti-IL-12 neutralizing Ab as described in Table 2.2 in Chapter 2) polarising conditions for one week (one cycle). At the end of the first cycle (time 0), the cells were washed in cultured in the same conditions for another cycle. Completion of consecutive cycles were done at time 1, 2 and 3.

8.2.2 Detection of cytokine secretion by polarised T cell lines.

To asses the cytokine profile of the T cell lines which had type 1 or type 2 polarization, their profile of cytokine production was assessed at time 1.

The cells were stimulated for cytokine production with overnight plate immobilized anti-CD3 (PharMingen), plus anti-CD28 Ab (PharMingen) (10µg/ml each) (see Chapter 2, Table 2.3) for 6 hrs cultured in the presence of 3µM monensin. Then the cells were harvested, washed twice in serum free medium and stained extracellularly for CD3 (PerCp) as described in Chapter 2 Section 2.7.1 and intracellularly with anti-IFNγ and IL-4 Ab or isotype matched control Ab for both IFNγ and IL-4 Ab, as described in Chapter 2 Section 2.7.5. Antibodies used to stain for intracellular cytokines (and their isotype matched controls) were all obtained from Pharmingen. The cells were acquired within 24 hrs of staining (40,000 events/sample) and analysed using Cell Quest Software (Becton and Dickinson).

8.2.3 Detection of the expression of CCR3 in polarised T cell lines.

The expression of CCR3 in T cells before and after type 1 or type 2 polarisation was assessed by flow cytometry, by the staining of the cells with the anti-CCR3 rat anti-human non-conjugated Ab (see Chapter 2 Table 2.4) plus a secondary mouse anti-rat FITC Ab (see Chapter 2 Table 2.4). The cells were stained at time 0 (before polarisation), time 1, time 2, and time 3. Serial dilutions of these Ab were used during the course of polarisation.
8.3 Results

8.3.1 Cytokine profile of cells after one week of polarising conditions.

Stimulation through the CD3 and the CD28 co-receptors by immobilised Ab is able to induce IFNγ and IL-4 production in T cell lines detectable by ICS (Chapter 6 Figures 6.1 and 6.2). Thus, this stimulation was chosen to determine the cytokine profile of the T cell lines in this Chapter. PHA cell lines from adult PBMC were cultured for one week as described in Section 7.2.2 and their cytokine profile was determined by ICS. Figure 8.1 shows the cytokine profile of two cell lines grown in type 1 or 2 polarising conditions and stimulated with anti-CD3 plus anti-CD28 Ab for cytokine production. Thus, type 1 conditions induced cells that produce large amounts of IFNγ and low amounts of IL-4 (Figure 8.1, panel A type 1 culture). When PBMC were cultured in type 2 conditions, a lower frequency of cells produced IFNγ and a higher frequency of cells produced IL-4 (Figure 8.1, panel A type 2 culture).

A second pair of lines were grown from another PBMC. After one cycle, a high frequency of the cells cultured in type 1 conditions produced IFNγ but a low frequency of cells produced IL-4 and the cells cultured in type 2 conditions has low frequencies of IFNγ and IL-4 producing cells (Figure 8.1 panel B). The frequency of IL-4 producing cells was always below 3.0% for type 2 cells on both panels A and B. Thus, at this time points the highest frequency of cells grown in any condition were predominantly type 1 and not type 2.

The amount of cytokine produced per cell was determined by the IFNγ and IL-4 MFI (Figure 8.2). The cells grown in type 1 conditions produced more IFNγ compared with cells grown in type 2 conditions, except in the first experiment (Figure 8.2, panel A type 2 conditions) where the cells produced similar amounts of IFNγ and IL-4 upon stimulation. All cells cultures in type 1 conditions produced more IFNγ and IL-4 upon stimulation. In contrast, cells cultured on type 2 polarising conditions did not produce more IL-4 than IFNγ upon stimulation.
Figure 8.1. Cytokine production of T cells grown in type 1 or type 2 polarising conditions. PBMC from healthy donors were cultured with 20ng/ml hr IL-2 plus 2ng/ml hrIL-12 (type 1) or 200 U/ml hrIL-4 plus 2μg/ml anti-IL-12 neutralising Ab for (type 2) for one week. The cells were then harvested and stimulated with overnight plate immobilised anti-CD3 (10μg/ml) plus anti-CD28 Ab (10μg/ml) for 6 hrs in the presence of monensin (3μM). The cells were harvested and stained extracellularly with anti-CD3 AB (PerCP). The production of IFNγ and IL-4 in CD3+ lymphocytes was determined by ICS (as described in Chapter 2 Section 2.7.5). The results represent frequencies of cytokine producing cells T for two pairs of cell lines (Panel A and B) grown in type 1 or 2 cultures. The frequency of IFNγ producing cells is represented as dark bars and the frequency of IL-4 producing cells is represented as light bars.
Figure 8.2. Mean fluorescence intensity of IFNγ and IL-4 produced by T cell lines. Two pairs of T cell lines were grown in type 1 or type 2 polarising conditions for one week as described in Figure 8.1. The cells were then stimulated for cytokine production, stained for cytokines and acquired in a flow cytometer as described in Figure 8.1. The results represent the amount of cytokine produced per cell after stimulation determined as the IFNγ and IL-4 MFI for both pair of lines (Panel A and B). The MFI values were obtained from the mean fluorescence value in the IFNγ or IL-4 quadrants in quadrant plots. In the Figure, the IFNγ MFI is represented as dark bars and the IL-4 MFI is represented as light bars.
8.3.2 *CCR3* expression by T cells under polarising conditions

The CCR3 expressed on T cells is down-regulated shortly after activation (Sallusto, Mackay et al. 1997). Thus, T cells cultures in both polarising conditions were stained for CCR3 before polarisation and at the end of the cycles. Before polarisation, CCR3 expression on T cells from PBMC was less than 0.2%.

Because the available anti-CCR3 Ab (rat anti-human) was not fluorochrome conjugated, a second Ab was used (mouse anti-rat FITC). The T cell lines were stained with dilutions of rat anti-human CCR3 Ab and mouse anti-rat-FITC Ab at the end of each cycle to determine the appropriate Ab dilution, the kinetics of expression and the best time for CCR3 detection.

At the end of the first cycle (time 0), less than 98.0% of cells in the lymphocyte gate were CD3⁺. The cells were stained for CCR3 and it was found that T cells grown in both type 1 and type 2 polarising conditions had a high frequency of CCR3 positive cells (Figure 8.3). The background noise was higher in the type 1 cells (Figure 8.3, type 1 culture, 1st cycle panel). However, for all the Ab dilutions, the frequency of cells positive for CCR3 was higher in type 2 T cells at the end of the 1st cycle.

This pattern of expression was different in the subsequent cycles, where the expression of CCR3 was dramatically reduced in the type 2 culture (Figure 8.3, times 1, 2 and 3), whereas CCR3 was always detected in T cells grown in type 1 conditions (Figure 8.3, times 0, 1 and 2). Furthermore, the frequency of CCR3⁺ T cells in type 1 cultures was higher than in T cells grown in type 2 conditions.
Figure 8.3. Expression of CCR3 on T cell lines grown in type 1 or type 2 polarising conditions. Serial dilutions (µg/ml) of the rat anti-human CCR3 Ab and mouse anti-rat (FITC) were used for the detection of CCR3 expression in T cell lines during type 1 or type 2 differentiation (as described in Figure 8.1). The cells were grown for 3 consecutive cycles and stained at the end of each cycle (time 0, 1, 2, 3) with anti CD3 (PerCp) and dilutions of the anti-CCR3 Ab and anti-rat (FITC) Ab as described in Chapter 2 Section 2.7.1. 10,000 events were acquired for analysis. The results represent the frequency of CD3+ lymphocytes positive for FL-1 fluorescence.
8.3.3 CCR3 expression on cells grown in type 1 and type 2 cultures over-time

Using 20μg/ml anti-CCR3 Ab plus 1μg/ml of mouse anti-rat (FITC) Ab, the expression of CCR3 in two T cell lines was compared and was found that shows that CCR3 is expressed at high frequency in cells from type 1 and type 2 cultures (Figure 8.4). Its expression decreases with time, as shown in Figure 8.3. The expression of CCR3 in T cells from type 2 cultures also decreased with time. This parallel decrease in expression may reflect the fact that both cultures have predominantly type 1 T cells. However, the conditions for the type 2 cultures influenced the behavior of the cells. These cultures always grew faster and the number of cells exceeded the number of cells obtained from type 1 cultures. All cells were healthy as determined by analysis under the microscope and in the FSS and SSC in the flow cytometer.

In conclusion, because CCR3 was detected on type 1 T cells at high frequency, it was not used as an extracellular marker for type 2 cells in this thesis.
Figure 8.4. Kinetics of CCR3 expression on type 1 and type 2 cultures. T cells were grown in type 1 or type 2 polarising conditions in consecutive cycles, as described in Figure 8.2. The cells were stained at the end of each cycle with anti-CCR3 Ab (20μg/ml) plus 1μg/ml of mouse anti-rat (FITC) Ab, acquired and analysed in a flow cytometer as described in Figure 8.2. The same number of cells was always acquired for analysis. The figure represents the frequency of CCR3 expressing T lymphocytes, for cells grown in type 1 (dark bars) or type 2 (light bars) conditions. In the 4th cycle only cells from type 2 cultures were analysed.
8.4 Discussion and conclusions

Several chemokine receptors have been found to be expressed selectively in Th1 or Th2 cells. Thus, whereas the CCR5 and CXCR3 chemokine receptors mRNA has been found in Th1 and not Th2 cells (Bonecchi, Bianchi et al. 1998), the CCR3 chemokine receptor has been found to be preferably expressed in Th2 cells derived from CB (Sallusto, Mackay et al. 1997) or adult blood (Jinquan, Quan et al. 1999). The expression of both CCR3 and eotaxin is associated with type 2 inflammatory processes: the mRNA expression of CCR3 in mature leukocytes from bone marrow aspirates from asthmatic patients (Ziebecoglu, Ying et al. 1999) and the mRNA expression of both CCR3 and eotaxin in atopic dermatitis (Yawalkar, Uguccioni et al. 1999) is elevated compared with levels in normal individuals. Furthermore, the adoptive transfer of Th2 (but not Th1) Ag specific lines into mice was correlated with the elevated mRNA expression of CCR3 and CCR4 in the animals lungs (Lloyd, Delaney et al. 2000). Those Th2 cells were able to induce allergic reactions upon Ag exposition (Lloyd, Delaney et al. 2000). The expression of different chemokine receptors in Th1 or Th2 cells allows selective migration in T cells (Bonecchi, Bianchi et al. 1998).

Beyond the physiological significance of the expression of exclusive surface markers on type 1 and type 2 cells, the establishment of a reliable surface marker as been pursued by many research groups (Del Prete, de Carli et al. 1995; Pernis, Gupta et al. 1995; Scheel-Toellner, Richter et al. 1995; Annunziato, Manetti et al. 1996; Kanegane, Kasahara et al. 1996; Rogge, Barberis-Maino et al. 1997; Sallusto, Mackay et al. 1997; Sallusto, Lenig et al. 1998; Seitzer, Scheel-Toellner et al. 1998; Hariharan, Douglas et al. 1999). However, there has been controversy concerning the relevance of expression of the molecules reported. In humans, CD30 is expressed on Th2 and Th0 clones and not Th1 clones. However, its expression is transient (between 6-12 hrs after mitogenic stimulation) (Del Prete, de Carli et al. 1995). Thus, this marker is helpful to exclude Th1 cells but not to identify Th2 cells. Furthermore, the detection of CD30 on T cells must be done within the limited time window or expression. The human IL-12 receptor β2 subunit (IL-12R β2) was found to be expressed only on Th1 cell lines and clones. Its expression is also transient, but the time required for expression is long (IL-12R β2 mRNA is found after 8 days of stimulation) (Rogge, Barberis-Maino et al. 1997). In mouse, the P-selectine glycoptrotein ligand (PSGL-1), has been reported to be expressed on most of the cells from a Th1 cell line and not expressed in Th2 cells (Borges, Tietz et al. 1997). Similarly, a murine Th2 exclusive surface marker, ST2L, was reported by Xu et al (Xu, Chan et al. 1998). It is
transcribed shortly after stimulation (3 hrs) in Th2 cells lines and clones (Xu, Chan et al. 1998). However, the relevance of the expression of ST2L is still controversial, since it function on hematopoietic cells has not yet been fully identified.

In this Chapter, T cells lines were grown in type 1 or type 2 polarising conditions to detect the proposed Th2 cell marker, CCR3. The culture conditions include hrIL-12 for type 1 cultures and hrIL-4 plus anti-IL-12 neutralizing Ab for type 2 cultures. However, type 1 cells, defined by their cytokine production at the single cell level, were obtained with both conditioning and type 2 cells were not successfully obtained. A high frequency of T cells grown in type 1 conditions secreted IFNγ (Figure 8.1) and few of these cells (less than 3.0%) secreted IL-4, but the amount of IFNγ produced was higher than the amount of IL-4 produced per cell (Figure 8.2). Thus, T cells from these cultures were considered type 1. In contrast, cells grown in type 2 conditions did not have a predominant frequency of cells producing IL-4 and did not produce more IL-4 than IFNγ per cell (Figures 8.1 and 8.2). However, T cells from type 2 cultures had lower frequencies of IFNγ producing cells compared with type 1 cultures. The T cells from type 2 cultures produced less IFNγ compared with the type 1 cultures (Figure 8.2), but the IL-4 production was also low in these T cells, with an IFNγ/IL-4 ratio of 4.25/2.04 and 9.80/0.17 in the two different lines.

Therefore, it was not possible to asses the expression of CCR3 on type 2 T cells. Nevertheless, expression of CCR3 was detected on all the type 1 cell lines. Moreover, these T cells expressed CCR3 with a particular kinetics. Furthermore, the expression of CCR3 was always higher in type 1 cells that in T cells from type 2 cultures.

The expression of CCR3 on T cells from the periphery has been reported to be 1-10.0% and in CB T cells (Sallusto, Mackay et al. 1997; Jinquan, Quan et al. 1999). The frequency of CCR3* cells can increase to 50% in T cell lines derived from CB and polarised in type 2 conditions for two cycles (Sallusto, Lenig et al. 1998) and to 85.0% in T cells from PBMC cultured in IL-4 and IL-2 for 24 hrs (Jinquan, Quan et al. 1999). Here, the maximum expression of CCR3 here was 37.3% in the type 1 T cells (Figure 8.3).

The results from this Chapter contradict earlier reports in the literature regarding CCR3 expression in differentiated Th populations (Sallusto, Mackay et al. 1997). This may be because in Sallusto's work, the T cells were sorted on bases of their expression of CCR3 and then studied for cytokine production, whereas in this Chapter the cells were first polarised and then the expression of CCR3 was determined. Indeed, when Sallusto et al sorted CCR3* T cells from PBMC (1.0% of
cells) into CCR3+ and CCR3-, and then cultured the cells with PHA and IL-2, the T cell lines derived from CCR3+ were found to secrete IL-4 and IL-5. Thus, selecting T cells on the basis of CCR3 expression, they found a correlation with expression of this marker and production of IL-4 in 17% of cells (Sallusto, Mackay et al. 1997). T cell clones consist of an homogeneous population of differentiated cells. Thus, in Sallusto's work, Th1 clones do not express CCR3, whereas CCR3 is expressed in almost all the cells from Th2 clones (Sallusto, Lenig et al. 1998).

Some reports have indicated that CCR3 is preferentially, and not exclusively, expressed on Th2 cells: Gerber et al reported that only 9 out of 13 CCR3+ clones were of the Th2 phenotype (Gerber, Zanni et al. 1997). Thus, the expression of CCR3 on IL-4 secreting cells may be due to the over-selection of these cells in a heterogeneous population. Accordingly, Bonecchi et al (Bonecchi, Bianchi et al. 1998) showed that the mRNA expression of CCR3 in Th2 cells from CB was low and almost indistinguishable from the CCR3 mRNA expressed in Th1 cells, under the same stimulatory conditions that Sallusto et al used (Sallusto, Mackay et al. 1997). Additionally, T cells derived from CB and cultured for 2 consecutive cycles of 10 days under type 1 polarising conditions (2ng/ml IL-12 plus 200 ng/ml anti-IL-4 Ab), did express CCR3 (Bonecchi, Bianchi et al. 1998). In Bonecchi's study, CCR3 expression was lower in Th1 cells when compared with cells cultured under type 2 conditions (200U/ml hrIL-4 plus 2μg/ml anti-IL-12 Ab). However, CCR3+ was not expressed in all the T cells from the Th2 culture, which may reflect that at this state, et al were using T cell lines and no T cell clones. In this Chapter, the frequency of CCR3 positive cells was consistently higher on time on T cells grown on type 1 conditions (Figure 8.4). The discrepancies with the literature can be that only T cell lines were studied here (which are not as homogeneous as T cell clones. Although this may imply that the cells have not been differentiated to the extent to express CCR3 accordingly to their cytokine phenotype, the results here indicate that CCR3 can be found at a high frequency in predominantly type 1 T cell lines.

In conclusion, CCR3 is not exclusively expressed in Th2 type cells but may be associated with them. Because CCR3 was found to be expressed in type 1 T cells, it can not be used as a type 2 T cell marker.

8.5 Future experiments

Although CCR3 was found on the Th1 cell lines used here, a Th2 cell line was not obtained. This was in spite of the protocol of differentiation, which included IL-4 and anti-IL-12 Ab and proliferation mediated by CD3 and CD28 stimulation. The
reason for this result may be that peripheral cells were used to obtain T cell lines and as seen in Chapter 6, a percentage of the T cells from periphery have been already primed, express the CD45RO Ag, and may have been already committed to a cytokine profile. In spite of this, culturing peripheral T cells with IL-12, Th1 cell lines were obtained. Thus, it may also be that the amount of Ab used to stimulate proliferation favoured the development of Th1 cells. Further work should include Th1 and Th2 cells which can be derived from CB cells. Although CD45RA cells from periphery could also be used, it has been proposed that CB derived cells become efficient cytokine producers faster than cells from periphery (Early and Reen 1999).

Future work should also include the detection of the CCR3 expression on PBMC, CB cells and lines at earlier time points after contact with stimulus (in this case, cytokines). This is because here, CCR3 expression was looked at the end of the cycles, based on published results indicating that the CCR3 expression on T cells is down-regulated after stimulation (Sallusto, Mackay et al. 1997). However, CCR3 expression was detected by Bonecchi et al within 24 hrs of stimulation (Bonecchi, Bianchi et al. 1998). T cell clones should be included in future work to ascertain that the cells have completed a Th differentiation program and conform a homogeneous cell population.

Additionally, another markers for Th1 or Th2 cells can be studied. This can include the IL-18R, which has been found in Th1 and not Th2 cells (Xu, Chan et al. 1998) or proteins involved in Th1 or Th2 signaling such as STAT4 and STAT6 (Kotanides and Reich 1996; Takeda, Tanaka et al. 1996; Naeger, McKinney et al. 1999). The detection of these markers can be done by staining when the proteins are expressed on the cells surface (the case of receptors) of Western blot (the case of STAT proteins).

GvHD is one of the most important post-BMT complications. Although HLA-matching is the single most important factor that determines the development of GvHD (Madrigal, Scott et al. 1997), BMT with mismatched HLA are performed because of the lack of more suitable donors. Several strategies have been pursued in the attempt to predict GvHD, one being the direct and indirect detection of cytokines produced in allogeneic reactions *in vitro*. During the course of acute GvHD, there is an activation of Th1 cells and a dominance of type 1 cytokines (Niederwieser, Herold et al. 1990; Imamura, Hashino et al. 1994; Nestel, Kichian et al. 1997). However, the relationship between the frequency of cytokine production in Th1 and Th2 cells and the development of GvHD is unknown. This thesis investigated the frequencies of cytokine producing alloreactive T cells by developing an assay to detect the cytokines produced with stimulation by specific HLA mismatches.

This work was divided by five stages. The capability to produce type 1 and type 2 cytokines by naive T cells from CB was explored in the first stage. This was because CB transplantation induces less severe GvHD and at lower frequency compared with BMT. It has been considered that T cells from CB can not develop a strong allogeneic reaction and have been classified as defective (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999). However, the capability of CB cells to produce type 1 and type 2 cytokines had been underestimated. In the second stage, the influence of soluble factors of CB on alloreactivity was studied to determine if the environment of the cells may influence their capacity to response to stimulus, since CB cells have a high threshold of stimulation. The following stage of this work was the search for a suitable form to obtain frequencies of antigen specific cytokine producing cells. Thus, the effect of different mitogenic stimulation on the cytokine production by memory T cells was studied. It was found that the mitogen used determines the frequency of cytokine producing cells. Therefore, in the 4th stage of this study the frequencies of cytokine producing cells were determined using a sensitive assay that allows for the use of antigenic stimulation. In an additional stage, the expression of the eotaxin receptor CCR3 on T helper cell lines, was explored. This was because it has been proposed that Th1 and Th2 cells can also be classified on basis on the surface antigens they express and CCR3 has been associated with Th2 cells (Sallusto, Mackay et al. 1997). Thus, CCR3 could be utilised as a extracellular marker for Th2 cells.
Frequencies of cytokine production by naïve cells derived from CB.

The results presented in Chapters 3 and 4 shown that naïve CD45RA⁺ cells derived from CB have the capability to produce cytokines at high frequencies when stimulated optimally. CD45RA⁺ T cells from CB produced IL-2 (Chapter 3 Figure 3.4), IFNγ (Chapter 4 Figure 4.1) and IL-4 (Chapter 4 Figure 4.2) at higher frequencies than those previously reported in the literature (Chalmers, Janossy et al. 1998; Schultz, Rott et al. 1999; Krampera, Tavecchia et al. 2000). This was because high concentrations of PMA stimulated the cells optimally. These results indicate that naïve T cells from CB are not defective in their signalling or in their capability to produce these cytokines. In Chapter 3, it was seen that a strong stimulation of PKC with 50 ng/ml PMA enabled CD45RA⁺ T cells to produce IL-2 at high frequencies (Chapter 3 Figure 3.4). It has been reported that with concentrations of PMA higher than 5 ng/ml, the frequencies of IL-2 producing cells (mononuclear and CD45RA⁺) from cord and from adult blood are similar (Anderson, Anderson et al. 1990; Chipeta, Komeda et al. 1999). Nevertheless, CD3 stimulus alone, induces a lower level of IL-2 production by CB T cells compared with adult T cells (Splawski, Jelinek et al. 1991; Watson, Oen et al. 1991). The results in Chapter 3 show that stimulation of CB naïve T cells via CD3 and CD28 induced a low frequency of IL-2 producing cells and low levels of cell proliferation (Chapter 3 Figures 3.4 and 3.8). In contrast to CD3 signalling, CD2 plus CD28 signalling induces optimal stimulation in naïve cells from CB, generating levels of proliferation similar to those obtained in adult cells (Gerli, Bertotto et al. 1989; Hassan, O'Neill et al. 1995). The CD2 plus CD28 induced proliferation is independent of IL-2 production, which is lower in naïve cells from CB compared with their adult counterparts (Hassan, O'Neill et al. 1995). The lower levels of IL-2 mRNA produced by CB samples obtained with CD2 plus CD28 stimulation can be enhanced to adult levels with exogenous IL-1β (Hassan and Reen 1994). This suggests that appropriate APC stimulation can enhance the levels of IL-2 production by naïve cells. Thus, the high threshold of stimulation observed in naïve T cells from CB does not imply defects in the cells. As discussed in Chapters 3 and 4 of this thesis, these cells are functional and has been shown that they have the capability to develop into efficient cytokine producing cells (Early and Reen 1999; Chipeta, Komeda et al. 2000).

The low responsiveness obtained from CB T cells has been proposed as the reason for the lower and milder GvHD incidence using CB for transplantation (Han, Hodge et al. 1995; Hassan and Reen 1997; Chalmers, Janossy et al. 1998; Krampera, Tavecchia et al. 2000). However, the proliferation of CB cells stimulated with allogeneic cells from
cord or adults is higher or similar compared to that of cells from adult periphery (Deacock, Schwarer et al. 1992; Risdon, Gaddy et al. 1994; Keever, Abu-Hajir et al. 1995). The frequencies of alloreactive HTLP are higher or similar in CB than in adult blood (Deacock, Schwarer et al. 1992; Wang, Sviland et al. 1998), although the frequencies of CTLp are lower in CB (Risdon, Gaddy et al. 1994; Keever, Abu-Hajir et al. 1995; Wang, Sviland et al. 1998). The reason for the similar HTLP frequencies in both populations may be due to the capability of CB cells to produce the same levels of IL-2 than adult cells with certain stimulation (Chapter 3 Figure 3.4 and (Wilson 1986; Fairfax and Borzy 1988). It has been reported that CD8+ lymphocytes from CB are less capable of performing some functions, such as cytokine production, compared with CB CD4+ cells after identical stimulation (Chalmers, Janossy et al. 1998). Therefore, within the CD3+ compartment, CD8+ T cells from CB may have even higher requirements for stimulation. In this work only the CD3 or CD8 markers were considered because the expression of the CD8 and CD4 Ag in the T cell surface is differently influenced by the state of T cell stimulation. Stimulation with PMA and ionomycin induces a sustained down-regulation of the CD4 but not the CD8 Ag (Anderson and Coleclough 1983; Hoxie, Matthews et al. 1986; Sleckman, Bigby et al. 1989). Thus, the studies that stimulate with PMA and ionomycin to asses cytokine production by CD4+ T cells (Anderson, Anderson et al. 1990; Jung, Schauer et al. 1993; Picker, Singh et al. 1995; Prussin and Metcalfe 1995; North, Ivory et al. 1996; Fukui, Katamura et al. 1997; Sewell, North et al. 1997; Chalmers, Janossy et al. 1998; Ito, Koide et al. 1998; Sommer, Meixner et al. 1998) may be underestimating the proportion of CD4+ cells in the samples and do not offer an accurate frequency of CD4+ cytokine producing cells.

The differences between naïve T cells from cord and adult blood, which accounts for the former high requirements of stimulation of CB cells (Chapters 3 and 4), may be located upstream of PCK. This is suggested because high concentrations of PMA enabled cytokine production at high frequencies in naïve T cells and because PCK is the target for PMA stimulation (Niedel, Khun et al. 1983). The activation of PCLy1 links CD3/TCR receptor stimulation to the calcium-calceurin pathway, ZAP-70 phosphorylation and NF-AT activation (Guse 1998; Weber, Bell et al. 1992; McCaffrey, Luo et al. 1993; Shibasaki, Price et al. 1996). It has been described that PCLy1 is expressed in different amounts and localisation in mononuclear cells from CB adult blood, proposing this as the reason for a reduced production of cytokines by CB cells (Miscia, Di Baldassarre et al. 1999). However, PKCβ, an obligatory isofrom for IL-2 production (Kelleher and Long 1992; Szamel, Bartels et al. 1993; Aggarwal, Lee et al. 1994) is expressed at similar levels in CD45RA+ CD4+ T cells from both CB
and adult blood (Hassan, Rainsford et al. 1997). Other differences between naïve cells from cord and adult blood have been found at the levels of expression of early-signalling proteins. There is a lower expression of CD3 in CB cells compared with adult cells (Gerli, Bertotto et al. 1989) and it has been reported that whereas Fyn was expressed at normal levels in CB samples, Lck was under-expressed (Miscia, Di Baldassarre et al. 1999). Because Lck and Fyn activate the CD3 (Chan, Iwashima et al. 1992), this was proposed by Miscia et al to be a decisive factor for the reduced responsiveness of CB T cells to CD3 stimulation. Nevertheless, after stimulation with PHA, the expression of Lck in mononuclear cells from CB and adults was up-regulated to similar levels, although the cells from CB produced less cytokine (Pirenné-Ansart, Paillard et al. 1995). Thus, it is unlikely the low level of IL-2 production in CB cells is due to the low level of Lck expression.

Recent publications have demonstrated that under appropriate primary stimulation, naïve T cells can produce IL-2, IFNγ and IL-4 at high frequencies (Iezzi, Scotet et al. 1999; Bogunia-Kubik, Perez-Cruz et al. 2000; Ise, Totsuka et al. 2000; Perez-Cruz, Fallen et al. 2000). Indeed, in the experiments performed in Chapter 4, freshly isolated naïve cells produced IFNγ and IL-4 within 6 hrs of mitogenic stimulation (Chapter 4 Figures 4.1 and 4.2). However, it has been proposed that IFNγ and IL-4 expression is repressed in naïve cells and that only entrance to the cell cycle allows gene expression (Bird, Brown et al. 1998). Nevertheless, it was recently found that the expression of IFNγ in naïve T cells can be independent of cell cycle entry (Laouar and Crispe 2000). Furthermore, under histone hyperacetylation or DNA demethylation, naïve T cells can express the IFNγ and IL-4 genes before their cell cycle completion (Bird, Brown et al. 1998). Thus, naïve T cells have the capability of expressing the IFNγ and IL-4 genes. In contrast with these cytokines, there is consensus that cell division is not necessary for IL-2 expression in naïve cells (Bird, Brown et al. 1998; Laouar and Crispe 2000). The production of IFNγ and IL-4 by CD45RA+ T cells that is shown in Chapter 4 was possible only with strong stimulation (Figures 4.2 and 4.3). This may be the reason why previous studies have not detected or have detected very low frequencies of type 1 and type 2 cells in naïve T cells from CB (Chalmers, Janossy et al. 1998; Chipeta, Komeda et al. 1999; Krampera, Tavecchia et al. 2000). In addition to this, the frequencies of cytokine producing cells have been determined in these studies and in Chapters 3 and 4, by ICS. However, as seen in Chapter 3, this technique has low sensitivity: stimulation by anti-CD3 and anti-CD28 Ab induced IL-2 dependent proliferation in peripheral T cells (Chapter 3 Figures 3.8 and 3.9). However, using ICS, only a very low frequency of IL-2 producing cells were detected in these T cells (Figure 3.3). Anti-CD3 plus anti-CD28 Ab stimulation also induced
proliferation in naïve cells (Figure 3.8) but IL-2 was not detected by ICS (Figure 3.4). Therefore this technique requires a strong stimulation to detect cytokine and some of the cytokine produced by stimulus such as CD3 plus CD28 antibodies may not be detected.

If cells from CB can produce type 1 and type 2 cytokines, can they induce GvHD after transplantation? Since the capability of IL-2 production and proliferation upon allogeneic stimulation is not impaired in CB naïve T cells, the milder GvHD observed after CB transplantation may be due to the balance of type 1 and type 2 cytokines produced. A high capability of IL-4 production has been proposed as a characteristic of unprimed populations, such as those derived from neonatal animals (Adkins and Hamilton 1992; Adkins, Ghanei et al. 1993). Additionally, naïve T cells can develop into efficient IL-4 producers (Adkins and Hamilton 1992; Byun, Demeure et al. 1994; Demeure, Yang et al. 1995). In support of this, the results from Chapter 4 indicate that the production of IL-4 was more frequent that the production of IFNγ under several stimulus (Figures 4.2 and 4.3). However, under mitogenic stimulation, CD45RA+ T cells from CB cells can efficiently become either Th1 or Th2 cytokine producing cells (Early and Reen 1999). Interestingly, CB mononuclear cells can produce more IL-6 than adult cells in response to allostimulation (Milosevits, Pócsik et al. 1995). As IL-6 can induce the development of Th2 cells (Rincon, Anguita et al. 1997), this suggests that CB APC may favour the development of a Th2 response towards alloantigens. Thus, the profile of cytokine production by naïve cells will depend on the stimulatory conditions such as encounter with different APC and the interaction with stimulatory molecules and their capability of cytokine production.

Cytokines in adult and CB serum.

In addition to the characteristics of CB derived cells discussed above, the maintenance of the low responsive state in cells from CB may be regulated by soluble factors present in the serum, as discussed in Chapter 5. These soluble factors present in the cord and the mother blood may contribute to a successful pregnancy. It has been observed that CB serum is qualitatively different from adult serum (Chapter 5 Figure 5.8). As a result of incubation in CB serum but not in adult serum, the proliferation of T cells stimulated by the CD3 was reduced, indicating a lack of stimulus in the CB serum or the presence of an inhibitor (Chapter 5 Figure 5.2). Similarly, the proliferation of T cells to alloantigen was reduced in the presence of CB serum compared with adult serum, suggesting that CB serum can induce a state of tolerance to alloantigens. MCSF was present in serum from cord but not from adult blood (Figure 5.5.9) and the addition of MCSF reduced the proliferative response to
allostimulation (Chapter 5 Figure 5.10). Similarly, the addition of anti-MCSF Ab to MLR performed in CB sera increased the proliferation to alloantigens. Therefore, it was proposed that MCSF can contribute to maintain an allogeneic tolerant state. In addition, adult cells stimulated with alloantigen in the presence of CB sera had a reduced capability of IFNγ and IL-4 production compared with cells stimulated in adult sera (Figure 5.8). Nevertheless, CB did not reduce the ability of the cells to produce IL-2 (Figure 5.7), supporting the observation that the production of IL-2 is not reduced cells that have been in the CB environment (Figure 3.4 in Chapter 3).

In pregnancy, a tolerant state to alloantigens may be related with the fact that the foetus is developed within a foreign body without eliciting an allograft rejection. The placenta represents an allogeneic tissue graft that is intimately exposed to maternal immunocompetent cells, but does not elicit an effective allograft reaction. The mechanisms regulating the immunological acceptance of the antigenic foreign embryo leading to successful establishment of pregnancy are not well understood. Although it is generally accepted that the survival of the foetus is dependant on an active suppression of the maternal immune responses in pregnancy (Suciu-Foca, Reed et al. 1983; Tafuri, Alferink et al. 1995), if this were absolute it would be expected that pregnant women would be more susceptible to infection, a scenario which is not beneficial to either mother or foetus. There is therefore more likely to be a local suppression by the mother (Munn, Zhou et al. 1998) and/or an ongoing suppression of the immune response by the foetus.

There are other cytokines which are found in the placenta and CB which may play a role in inhibiting “foetal rejection” such as IFNα, which is known to suppress T and B cell function and PGE2 production, which levels are high in CB serum and in trophoblast cultures (Aboagye-Mathiesen, Toth et al. 1994; Zdravkovic, Knudsen et al. 1997). IFNα may contribute to the local maternal immunosuppression. As IFNα has been demonstrated to be involved in prolongation of allograft survival (Mobraithen, De Maeyer et al. 1973; Hirsch, Ellis et al. 1974) a tolerant state to alloantigens can also be due to the presence of type 2 cytokines preferentially to type 1 cytokines in maternal blood. Accordingly, high levels of IFNγ and IL-2 are more related with recurrent abortions and the presence of type 2 cytokines in maternal serum have been related with successful pregnancies (Wegmann, Lin et al. 1993; Raghupathy 1997). The production of type 1 cytokine upon antigenic challenge during gestation has been correlated with impaired pregnancies and foetal loss whereas successful pregnancies occurred in those animals that develop responses dominated by type 2 cytokines (Krishnan, Guilbert et al. 1996; Piccirinni, Beloni et al. 1998). Supernatants from placental cells of normal murine gestation contain type 2 cytokines (IL-4, IL-5, and IL-
10) and although IFN\(\gamma\) is also present, it levels decrease with time (Lin, Mosmann et al. 1993). A suppressive environment, containing cytokines such as IL-10, may contribute to create an immuno-tolerant state that may be needed in a successful pregnancy (Chaouat, Assal Meliani et al. 1995).

The methodology used determines the profile of cytokines produced by the T cell.

In Chapters 3, 4 and 6 of this thesis, naïve T cells from CB (CD45RA\(^+\) CD45RO\(^-\)), resting T cells from periphery (CD45RA\(^-\) and CD45RO\(^+\)) and memory T cells (CD45RA\(^-\) CD45RO\(^-\)), were stimulated with various mitogens to induce cytokine production. Whereas CD3 plus CD28 induced cytokine production mainly in the memory population, PMA and ionomycin was a strong stimulus that induced cytokine production in T cells at these differentiation stages (Chapter 3, 4 and 6).

It was discussed in Chapter 6 that the mode of stimulation determines the pattern of cytokines production in memory T cells and clones. This was seen comparing the stimulation provided by PMA and ionomycin, PMA plus anti-CD3 Ab or anti-CD3 plus anti-CD28 antibodies. The results from Chapter 6 show that high frequencies of IFN\(\gamma\) and IL-4 producing memory T cells can be induced with CD3 plus CD28 and CD3 plus PMA stimulation (Chapter 6 Figures 6.1 and 6.3). The highest frequency of type 1 and type 2 cells was obtained with PMA and ionomycin stimulation. However, PMA and ionomycin also induced the detection of the high frequency of Th0 cells in these memory populations of T cells (Chapter 6 Figure 6.5). This indicated that different methods of stimulation of memory T cells induce different frequencies of cytokine producing cells. In support of these results, it has been reported that different mitogens induce production of different cytokines. The stimulation of murine cells with different mitogens (Con A or anti-CD3 Ab plus PMA) induced different patterns of IFN\(\gamma\) and IL-4 production (Sander, Cardell et al. 1991). In memory CD4\(^+\) and CD8\(^+\) cells, different mitogens induce a different kinetic of IFN\(\gamma\) and IL-4 production (Sander, Cardell et al. 1991). Different effects of stimuli on other cell responses have also been reported. Whereas short-term activation of PKC by synthetic diacylglycerol induces only the expression of IL-2R, sustained PKC activation mediated by PMA results also in IL-2 secretion (Szamel, Kracht et al. 1990). Similarly, PHA stimulation but not exogenous IL-2 augmented PKC\(\alpha\) and PKC\(\beta\) mRNA levels on peripheral T cells (Isakov, Mally et al. 1992).

The remarkable ability of PMA and ionomycin to induce IFN\(\gamma\) and IL-4 production in a large proportion of cells is because the expression of both the IFN\(\gamma\) and the IL-4 genes is dependent on elevated calcium levels (Kaldy and Schmitt-Verhulst 1991;
Kubo, Kincaid et al. 1994). In T cells, ionomycin or PMA alone can induce IFNγ transcription and expression (Hardy, Manger et al. 1987; Young and Ortaldo 1987) but the combination of both mitogens synergise for IFNγ expression (Kaldy and Schmitt-Verhulst 1991). This was seen in Chapter 6 Figure 6.1, where PMA or ionomycin alone induced IFNγ production in memory T cells, but at low frequencies. Ionomycin stimulation alone can induce IL-4 production because intracellular Ca²⁺ elevation induces IL-4 transcription and secretion (Todd, Grusby et al. 1993; Kubo, Kincaid et al. 1994). However, PMA alone is not able to induce IL-4 expression (Dokter, Esselink et al. 1993) as seen in Figure 6.3, where IL-4 production was dependent on ionomycin but not PMA. Thus, PMA and ionomycin induces the expression of IFNγ and IL-4 more effectively than other stimulus, such as CD3 or CD28 stimulation because they induce a sustained stimulation of the cells. Nevertheless, the Ca influx induced by ionomycin can be toxic for the cells, compared with the flux induced by anti-CD3 Ab stimulation (Donnadieu, Bismuth et al. 1995). This toxic effect of ionomycin was seen only in CD45RA⁺ cells from CB stimulated with high concentrations (Chapter 4, Figure 4.5). The 6 hrs of incubation in ionomycin were not toxic for other cells, that may be because naïve T cells have a lower capacity to regulate the levels of intracellular calcium compared with other T cell populations.

Studies of the cytokine expression at the single cell level indicate a considerable segregation in the pattern of cytokine expression (Bucy, Panoskaltsis-Mortari et al. 1994; Bucy, Karr et al. 1995; Kelso, Groves et al. 1995). Thus, the co-expression of Th1 and Th2 cytokines is a relatively rare event. However, in Chapter 6 PMA and ionomycin induced the highest frequencies of Th0 cells in T cells from periphery compared with other stimulus (Figure 6.7). This effect was also seen in CD45RO peripheral T cells and in T cell lines (Figures 6.5 and 6.8). Since PMA and ionomycin induce a sustained stimulation that is optimal for IFNγ and IL-4 gene expression, the presence of both cytokines at high frequencies in memory T cells may be due to the strength of this stimulation. The information provided from TCR triggering and ICS studies has shown that the Ag dose and the strength of the TCR/MHC-peptide interaction determine the expression of cytokines at the single cell level. Thus strong stimulation is able to induce the production of IFNγ and IL-4 by T cells at high frequencies (Hosken, Shibuya et al. 1995; Itoh and Germain 1997; Tao, Grant et al. 1997). Nevertheless, this stimulation was used in this thesis because, as mentioned above, the technique used to detect intracellular cytokines (ICS) in Chapters 3, 4 and 6 requires a powerful stimulation to the cells. Only this stimulation revealed the ability of CB CD45RA⁺ cells to produce IL-2, IFNγ and IL-4 at high frequencies.
The type 1 or type 2 cytokine phenotype, but not the type 0 phenotype are expressed in differentiated memory cells and are correlated with distinct immune conditions (Abbas, Kennet et al. 1996; Mosmann and Sad 1996). Thus, the high frequency of Th0 memory cells present in periphery (Chapter 6) or in alloreactive T cell clones (Kelso and Gough 1988) may be due to the type of stimulus used for cytokine production. Even though stable Th0 cells can be developed under certain conditions in vitro (Miner and Croft 1998), chronic stimulation in vitro induces Th differentiation, which correlates with the profile of cytokines developed in vivo after chronic stimulation (Doncarli, Stasiuk et al. 1997). Furthermore, using Ag specific stimulation in vitro, Th1 or Th2 cells are found at high frequencies whereas Th0 cells are rarely found (de Boer, Fillie et al. 1998). Thus the high frequency of memory type 0 cells found in Chapter 6 with the ICS technique may be due to the strength of the stimulation used (such as stimulation with PMA and ionomycin).

The strength of PMA and ionomycin stimulation resulted in the simultaneous production of IFNγ and IL-4 both in Th1 and Th2 clones (Figure 6.10). Cells from these clones also produced IFNγ or IL-4 alone. A change in the profile of cytokine production in established T helper cells has been obtained by altering the strength of the stimulus varying the Ag concentration (Hosken, Shibuya et al. 1995; Itoh and Germain 1997; Tao, Grant et al. 1997). Altered peptide ligands can actually modify the cytokine production pattern in established memory T cells, inducing the novel expression of some cytokines (Windhagen, Scholz et al. 1995; Ausubel, Krieger et al. 1997) and abrogating the expression of others (Kumar, Bhardwaj et al. 1995; Chaturvedi, Yu et al. 1996; Boutin, Leitenberg et al. 1997; Janssen, van Oosterhout et al. 2000). Nevertheless, the expression of type 1 and type 2 genes should be theoretically repressed in cells of an opposite phenotype, but certain stimulation may bypass the mechanisms of control: in differentiated T cells, high levels of cAMP inhibit the production of IFNγ induced by TCR but not by ionomycin stimulation (Kaldy and Schmitt-Verhulst 1991). The exclusion of cytokine expression and manifestation of a Th1 or Th2 phenotype has been suggested to be due to a selective expression of proteins rather than the expression of a repressor. This is because a Th1-Th2 chimera cell (a fusion from a Th1 and a Th2 clone) simultaneously expressed IL-4 and IL-2 mRNA (Ho, Hodge et al. 1996). It has been recently proposed that the status of the chromatin structure either facilitates or blocks gene expression. In cells polarised in Th2 conditions, the IL-4 locus acquires a particular pattern of locus accessibility (Agarwal and Rao 1998). An open and transcriptionally competent chromatic configuration of the IL-4 locus, not found in naïve cells, was observed in Th2 differentiating T cells and was dependent on the presence of STAT6 (Agarwal
and Rao 1998). Thus, chromatin remodelling was proposed to be a mechanism involved in the exclusive expression of IFNγ or IL-4, accompanied by DNA demethylation. During Th1 cell development, the chromatin structure of Th2 genes might become resistant to chromatin remodelling and more inaccessible to transcription factors, concluding in a Th1 irreversible phenotype (Agarwal and Rao 1998). However, it has been reported that the ectopic expression of the transcription factors c-Maf and T-bet can induce the production of cytokines of an opposite phenotype in differentiating or fully differentiated Th cells (Zheng and Flavell 1997; Szabo, Kim et al. 2000) indicating that the genes of the “opposite” phenotype are accessible to transcription when the pertinent transcription factor is present. Furthermore, the production of cytokines of the opposite phenotype obtained with APL contradicts the proposition that during Th cell development, the chromatin structure acquired would produce irreversible Th phenotypes as suggested by Agarwal et al. It may also be possible that under weak stimulation (with mild mitogens or Ag) only some cytokines are expressed but strong stimulation reveals that the cells are not fully differentiated.

In conclusion, mitogenic stimulation can reveal the capability of a T cell to produce cytokines and facilitate the detection of their cytokines production. The ICS technique used to detect cytokine production required strong stimulation, most effectively provided by PMA and ionomycin. However, each mitogen induces a different frequency of cytokine producing cells due to their signalling. The results from Chapter 6 suggested that a more accurate determination of the frequencies of cytokine producing T cells require the use Ag specific stimulation and presentation in APC, as discussed below.

Detection of frequencies of alloreactive T cells in vitro.

In Chapter 7 section 7.3.6 it was shown that it was not possible to assay the frequencies of cytokine producing alloreactive T cells by ICS. This was because this technique required mitogenic stimulation to induce detectable levels of cytokine production (Figure 7.2). However, this technique evaluates the potential of the cells to produce cytokine but, as concluded in Chapter 6, the use of mitogens does not induce the stimulation provided by Ag. The results in Figure 7.2 indicated that Ag re-stimulation and not mitogenic stimulation is required to estimate frequencies of cytokine producing allostimulated cells. It was found that the IFNγ ELISPOT (Section 7.3.7) was sensitive and able to detect IFNγ producing cells when a single mismatch in HLA class II (HLA-DRB1 allele) was used to stimulate the cells. The HLA match pairs induced low proliferation in MLR assays and the lowest frequencies of IFNγ producing cells (in both cases, the values were comparable to the
negative controls). In contrast, the mismatched pairs induced high proliferative responses and the highest frequencies of IFNγ spots (Figures 7.9 and 7.10). The frequencies provided by the ELISPOT were Ag specific, because cells without re-challenge had low frequencies of IFNγ spots, similar to those of irradiated cells (the background of the assay). The determination of the frequencies of IFNγ was done because the development of alloreactive reactions are closely related with the production of IFNγ. Indeed, it has been observed that an increase in levels of IFNγ production post-BMT are associated with the development of acute GvHD (Niederwieser, Herold et al. 1990; Nestel, Kichian et al. 1997) and a marked increase in serum IFNγ levels preceded or coincided with the appearance of clinical symptoms of acute GvHD (Niederwieser, Herold et al. 1990; Imamura, Hashino et al. 1994). IFNγ is also produced by alloreactive lymphocytes ex vivo post-BMT (Velardy, Varese et al. 1989; Cooley, Wright et al. 1994) and during the course of experimental acute GvHD, the percentage of lymphoid cells expressing IFNγ mRNA is significantly increased (Allen, Staley et al. 1993).

Clinically, the most successful way to prevent GvHD is to obtain a close HLA-match between donor and recipient (Madrigal, Scott et al. 1997). However, this may not be achieved due to a lack of suitable donors. Therefore, the prediction of GvHD, based on in vitro alloreactive tests, is crucial to select the more appropriate donor. Using an MLC in unrelated class II mismatched PBMC pairs, the detection of increased levels of cytokine mRNA or secreted cytokine has been correlated with post-BMT complications (Bishara, Kedar et al. 1991; Bonnotte 1991; Theobald, Nierle et al. 1992; Danzer, A Campo et al. 1994; Tanaka, Imamura et al. 1994; Toungouz, Denys et al. 1994; Tanaka, Imamura et al. 1995). Analysis of these MLC supernatants shown that HLA-DR mismatches correlate with the production of IL-2, IFNγ, TNF, IL-6 and IL-12 (Danzer, Kirchner et al. 1994; Toungouz, Denys et al. 1994; Toungouz, Denys et al. 1995; Toungouz, Denys et al. 1995; Toungouz, Denys et al. 1996). Nevertheless, the measurement of mRNA or cytokines in the supernatant of the MLC has not provided reliable information to predict the development of GvHD.

The CTLp and HTLp assays have been used to predict the grade of GvHD developed after a BMT. High frequencies of HLTLp are associated with the development of acute GvHD and maintenance of chronic GvHD (Theobald, Nierle et al. 1992; Bunjes, Theobald et al. 1995) and high frequencies of CTLp correlate with the development of acute GvHD (Spencer, Brookes et al. 1995). The information provided by these assays help in the selection of donors (Spencer, Brookes et al. 1995). Another strategy to predict GvHD determined that the production of IFNγ and TNFα by CD4 T cells in HLA-A, B and DR matched pairs can be correlated with
the grade of GvHD developed post-transplant (Dickinson, Sviland et al. 1988). The skin explant assay developed by Dickinson et al (Dickinson, Sviland et al. 1991) predicts the development of GvHD based in tissue damage seen in the skin explants (Sviland, Dickinson et al. 1990) and has been applied to modify the prophylaxis received by the patients (Dickinson, Hromadnikova et al. 1999). However, this assay requires a histopathologist to read the skin explants and skin biopsies from patients. Furthermore, these assays do not determine the frequencies of type 1 and type 2 cells, which may determine the outcome of the transplant. In comparative studies, some centres have found no correlation between the CTLp and HTLp assay (Bunjes, Theobald et al. 1995) or the skin explant assay and the CTLp or HLTP assays (Dickinson, Sviland et al. 1998) in predicting GvHD. However, a IL-4 and IFNγ ELISPOT assay can be able to both provide information of the frequencies of cytokine producing alloreactive cells and the profile of cytokine production in a determined donor-patient pair.

The ELISPOT assay is technically less laborious compared with other assays that provide frequencies, such as limiting dilution assays. In a multicenter comparative assay, the frequencies of virus specific cytotoxic cells provided by the IFNγ ELISPOT and the CTLp assay showed a good correlation (Scheibenbogen, Romero et al. 2000). These features suggest that the IFNγ ELISPOT has the potential to be incorporated in the clinic to assess frequencies of alloreactive T cells in the context of BMT. Indeed, it has been used in models of renal transplantation where the frequency of IFNγ producing alloreactive (mainly T) lymphocytes correlated with transplant rejection (Matesic, Lehmann et al. 1998; Heeger, Greenspan et al. 1999). These studies suggest that the determination of frequencies of IFNγ producing cells in donor-patient pairs could be adopted as a strategy to detect alloreactivity due to HLA mismatches and to predict the risk of developing high grades of GvHD. Since the production of IFNγ is related to the development of acute disease and probably aggressive GvHD, the determination of the frequency of IFNγ producing cells may provide useful information for the physician before BMT.

It has been proposed that IL-4 is necessary for the production of IFNγ and IL-4 by alloreactive T cells because IL-4 induces the expression of CD80 and CD86 on allogeneic APC (Bagley, Sawada et al. 2000). Thus, type 2 cells are not simply tolerant to alloantigen but mediate a different type of response, as histological damage can be observed in mice transplanted with type 2 cells incapable of inducing lethal GvHD (Krenger, Cooke et al. 1996). However, there is a correlation of the production of type 2 cytokines with a less aggressive acute GvHD leading to development of chronic GvHD (Fowler, Kurasawa et al. 1993; Rus, Svetic et al.
1995). This observation has now been reported in the context of BMT by Imami et al, who studied the frequency of IL-4 producing cells in patients receiving BMT by an ELISPOT (Imami, Brookes et al. 1998). In that study, it was found that a high frequency of IL-4 producing cells are correlated with a better prognosis (Imami, Brookes et al. 1998). Furthermore, high frequencies of IL-4 producing cells were correlated with low frequencies of CTLp.

The results obtained in Chapter 7 encourage the further development of the IFNγ ELISPOT assay aiming to determine frequencies of IFNγ producing cells in mismatched pairs to finally, help in the prediction of GvHD. This strategy to manage GvHD is used at the Royal Victoria Infirmary at Newcastle, UK, where information provided by their assay guides in the selection of the most suitable donor and in the assignment of stronger or weaker immunosuppression to the patient (Dickinson, Hromadnikova et al. 1999).

Expression of CCR3 in type 1 cell lines.

The eotaxin receptor CCR3 has been reported to be preferentially expressed on Th2 cells (Sallusto, Mackay et al. 1997). In the present study, the detection of CCR3 was considered as a means to detect Th2 cells, in addition to the determination of the cytokine profile in this T cell subset (Chapter 8). However, CCR3 was found on type 1 T cells (Chapter 8 Figure 8.3), which suggested that it is not an exclusive Th2 marker. The cells used in Chapter 8 were obtained from peripheral blood and were polarised using type 1 and type 2 conditions under polyclonal stimulation. Since peripheral blood contains both primed and unprimed cells (Figure 3.1 in Chapter 3), Th1 and Th2 cells may have been present in the samples used in Chapter 8. This did not affect the development of type 1 T cells lines, although a cell line with a Th2 cytokine secretion profile was not obtained. Furthermore, the cells used by Sallusto and those used in Chapter 8 were polarised with the same conditions (see Chapter 8 Section 8.2.1), although Sallusto et al used polarised CB cells. CCR3 was found in the type 2 cultures only at the beginning of the cultures, but in all cell cultures, the frequency of expression declined with time (Figure 8.4). The expression of CCR3 correlates with the presence of Th2 cells and with immune reactions dominated by Th2 cytokines (Gerber, Zanni et al. 1997; Yawalkar, Ugucioni et al. 1999). However, the correlation found on CCR3 expression and Th2 cells derived from immunised organisms may not be always reflected in the expression of CCR3 on in vitro generated Th2 cells. Indeed, there has been controversy on the expression of CCR3 on
Th2 cells. CCR3 expression may be limited to T cells that have been primed in the presence of both IL-2 and IL-4 (Jinquan, Quan et al. 1999), although Th2 cells can arise in the absence of IL-4 (see Section 1.8 Chapter 1). This suggests that CCR3 may not be found in all Th2 cells and the results from Chapter 8 indicate that CCR3 is not an exclusive Th2 marker. Thus, the production of cytokines were used to classify the type 1 or type 2 T cells.

In conclusion, this thesis shows that, contrary to previously reported work, naïve T cells from CB are capable of producing IL-2 and IFNγ and IL-4 at high frequencies when their stimulation threshold has been reached. However, with low levels of stimulation, CB naïve cells produce less cytokine than cells at other stages of differentiation. This indicates that the threshold of stimulation of CB T cells is higher than other T cells, which may be influenced by the environment the cells live in. Indeed, the response to alloantigens in vitro was reduced in this study by incubation of cells in CB serum. This was partially due to MCF5 present in the CB serum. This work also shows that ICS, the technique used to determine frequencies of cytokine producing type 1 and type 2 T cells, has low sensitivity and require high stimulation. Thus, when various mitogenic stimulation were used to detect cytokines by ICS in memory T cells, it was shown that the frequency of IFNγ and IL-4 producing cells depended on the stimulus. These results indicated that mitogenic stimulation may not reflect the frequencies of cytokine producing Ag specific cells. Since it was not possible to determine the frequencies of type 1 and type 2 cells in short-term alloreactive T cell lines using ICS, a sensitive assay was adapted to the detection of frequencies of IFNγ producing alloreactive cells. The ELISpot assay detected the presence of cells that respond to a single HLA-DRB1 allele mismatch by the production of IFNγ. Because the frequency of IFNγ producing cells is related with aggressive allogenic reactions, the results obtained with the IFNγ ELISpot in this thesis, are encouraging to be further explored. The detection of high frequencies of IFNγ producing alloreactive T cells could be applied to select donors and to decide modifications in the prophylaxis for alloreactivity, such as GVHD.

The CCR3 marker, which is proposed to be a Th2 marker, was considered to be used in this thesis to detect Th2 cells in addition to the determination of the profile of cytokine production. However, CCR3 was found in Th1 cells and was not adopted to define Th2 populations. Finally, it was shown that CCR3, which is proposed to be a Th2 marker, is also present in type 1 T cell lines and therefore does not define type 2 T cells.
10. Appendixes

10.1 Buffers

10.1.1 Phosphate buffered saline (PBS)

A 10 fold (10X) solution of PBS was made with the following reagents in 1 litre distilled water (H₂O) (pH 7.2-7.4) and sterilised in an autoclave.

NaCl (BDH, Poole, Dorset, UK) 80.0 g.
Na₂HPO₄ (BDH), 11.6 g.
KH₂PO₄ (BDH), 2.0g
KCL (BDH), 2.0g

10X PBS commercially available (Biowittaker, Walkersville, Maryland, USA) was also used diluted in distilled water.

10.1.2 Buffers for OKT3 Ab purification by Protein-A columns.

The Ab purification was done using protein A columns (see section 10.3) with the following buffers.

PBS (pH 7.4).
100 mM Tris (Sigma, St Louis, Missouri, USA), pH 8.0.
10 mM Tris, pH 8.0
0.2 mM glycine (BDH), pH 3.0.
1M Tris pH 8.0
Filtered Bradford solution (Sigma)
20% ethanol (BDH).
10 mM sodium azide (BDH).

All the buffers were filtered after preparation.
10.1.3 Buffers used in flow cytometry.

10.1.3.1 Staining buffer for extracellular staining

The buffer used for staining for flow cytometry (Chapter 2 section 2.7) was made with 1% heat-inactivated FCS (BioWhittaker, Maryland, USA) and 0.1% (w/v) sodium azide (BDH Laboratory Supplies, Poole, UK) in PBS (pH 7.4 - 7.6)

10.1.3.2 Permeabilisation buffer for intracellular cytokine staining (ICS)

The buffer used for ICS (Chapter 2 section 2.7.5) was staining buffer (see above) plus 0.1% saponin (Sigma).

10.1.4 Buffers for ELISPOT:

The following buffers were used to perform the ELISPOT assay (Chapter 2 Section 2.9):

0.1 M NaHCO3 (BDH), pH 8.2
PBS + 5% bovine serum albumin (BSA) (Sigma)
PBST: PBS + 0.1% Tween 20 (Sigma)
PBST + 1% BSA.
TBS: 50mM Tris (Sigma) 150mM NaCl pH 8.0
TBST: TBS + 0.5% Tween 20
TBS + 1% BSA

Alkaline phosphatase buffer (APB):
0.1M NaCl, 0.05M MgCl, 0.1M TRIS pH 9.5

The alkaline phosphatase substrate solution to detect the secondary Ab in the ELISPOT reaction was made with:

10 ml APB
32 µl of BCIP (Gibco, Gaithesburg, MD, USA)
44 µl NBT (Gibco)

10.2 Non specific T cell lines generated by polyclonal stimulation with Phytohaemagglutinin (PHA).

PHA is a lectine (protein produced by animals, plants and slime moulds) that bind non-covalently to specific sacchararides residues in glycoproteins, as membrane receptor proteins in mammalian cells (Wilson 1995). PHA binds to the co-receptor CD2 and is
used to stimulate T cells in a non-antigen (polyclonal) specific manner when is presented by APC.

To determine the optimal concentration of PHA to be used to generate the T cell lines, PBMC were cultured with serial dilutions of PHA (Murex, Kent, UK) (100, 10, 1.0, 0.1, 0.001, μg/ml) in 96 round bottom well plates (Falcon, Becton and Dickinson, New Jersey, USA), in triplicate wells. Proliferation was measured as the amount of \(^3\)H Thymidine incorporated by the cells (as described in Chapter 2 Section 2.4.) and measured as count per minute (cpm). A serial dilution of was used to determine the optimal concentration of PHA to be used. Figure 10.1 shows a typical titration of PHA. A titration curve was always performed for each batch of PHA.

![Graph showing PHA titration curve](image)

**Figure 10.1. PHA titration curve.** PBMC were cultured (1x10^5 cells/ml) in 96 well plates with 200μl/well, with triplicates of serial dilutions of PHA. Cells were cultured for three days and proliferation was estimated by \(^3\)H Thymidine incorporation as described in Chapter 2 Section 2.4. The graph shows the mean of counts after \(^3\)H Thymidine incorporation per minute ± SD of three wells for the different dilutions. This figure is a representative PHA titration curve.

### 10.3 Purification of the anti CD3 antibody OKT3 by Protein A.

The OKT3 mAb reacts with 90% of normal peripheral T lymphocytes, by recognition of a 20kD cell surface molecule expressed in mature immunocompetent peripheral T lymphocytes, the CD3 complex of proteins expressed with the TCR in the T cell surface (Reinherz, Meuer et al. 1982). The OKT3 hybridoma is a hybridoma (Kung, Talle et al. 1980). This Ab was used in this thesis to stimulate T cells, through the TCR pathway, because it cross-binds the CD3 co-receptor molecule on the surface of
the cells (Tax, Willems et al. 1983; Weiss, Imboden et al. 1986; Leo, Foo et al. 1987).
It can be purified using Protein A (a Staphylococcus aureus cell wall polypeptide
product) because of its affinity to the Fc Ab region. It is used for IgG2a murine Mo
Ab purification (Harlow and Lane 1988).

The OKT3 hybryddoma was grown to obtain the OKT3 Ab. The hybridoma cell line
(ATCC, Maryland, USA) was grown in 10% FCS RPMI (hybridoma medium), until the
cells reached 10^6 cells/ml, when they were divided 1:1 in fresh hybridoma medium.
Cell-free supernatants were collected for Ab purification. For supernatant collection, the
cells were centrifuged at 1000g for 10 minutes, the supernatant recovered and filtered in
45μm Minisart filters (Sartorius, Gottingen, Germany) (Harlow and Lane 1995). This
Ab suspension was stored at -20°C and only thawed for Ab purification.

Purification of Ab by affinity columns using protein A (HighTrap-protein A, Amersham,
Pharmacia Biotech, Little Chalfont, UK) and a G10X250 borosilicate glass column
(Amicon, Stonehouse, UK). The buffers used were brought to room temperature,
filtered using a 0.2μm Minisart filter (Sartorius) and degas using a pump by inverse air
pressure, for 10 minutes. To set up the glass column, it was filled with the degassed
Protein-A suspension.

The column was then washed with 10 volumes PBS at 1ml/minute rate. The
supernatant was then applied at the same rate flow or overnight at the lowest rate flow.
Then, the column was washed with 10 volumes of 100 mM Tris pH 8.0 and
subsequently 10 volumes of 10 mM Tris pH 8.0, to wash all the proteins bound non
specifically to the protein A. The sample was then eluted with 0.2 M glycine pH 3.0.
The eluted Ab was collected in small tubes where 20μl of 1M Tris pH 8.0 had been
added to restart pH. The Protein-A column was stored after washing it with 100 mM
Tris pH 8.0 to neutralise pH.

To detect the fraction where the Ab was contained, a crude determination with
Bradford test was done, by mixing 40 μl of the Bradford solution plus 10μl of the
sample for each fraction. Protein containing fractions turn bright blue (Harlow and Lane
1988). Once determined the Ab containing fractions, they passed trough PD-10 PBS
Columns (Amersham, Pharmacia Biotech). To do this, 2.5 ml of sample were applied in
a dry PD-10 column, allowing all the sample to flow. Then 5.3 ml PBS were applied to
elute the sample. The eluted was collected in clean 5 ml tubes

The PD-10 column was washed with PBS. Sterility was maintained by storing the
column with 10 mM sodium azide and keeping it at 4°C.
The Ab solution was quantified by concentration of protein/volume. This was done using a UV light spectrophotometer (Shimadzu, Kyoto, Japan) measuring the sample at 460 nm.

10.4 OKT3 Ab titration

Plate immobilised and soluble purified or commercial OKT3 Ab (see Chapter 2 Table 2.3) was titrated to determine the optimal concentration of every batch of Ab used (Figure 10.2).

10.4.1 Plate immobilised OKT3 Ab titration.

To titrate plate immobilised OKT3 Ab, serial dilutions of the Ab in serum-free medium were added in triplicate to a 96 flat bottom well plate (100µl/well) and incubated at 37°C for 4-8 hr. The plates were washed twice with serum free-medium, keeping sterile conditions by flicking out the plate content whiting the laminar flow hood.

The plates were then blocked with 200µl/well of complete medium and incubated at 37°C for 30 minutes. Then, the medium was discarded and add the cells were added (0.2x10^7 cells/well). The cells were incubated for three days and cell proliferation measure by thymidine incorporation.

![Graph showing the titration of immobilised OKT3 Ab](image)

Figure 10.2. Titration of immobilised OKT3 Ab. Serial dilutions of the Ab were immobilised in the plates as explained in the text before the addition of the cell-suspension (1x10^5 PBMC/well). The plates were then incubated for three days in a humidified atmosphere at 37°C and CO₂. 1 µCi [³H] thymidine/well was added 18 hours before harvesting the wells on glass-fibre filters. Radioactivity was counted with a liquid scintillation counter. Results are expressed as mean of triplicate counts
per minute (cpm) ± SD, and represent [³H] Thymidine intake by dividing cells. The optimal concentration of this batch is 50μg/ml. This figure is a representative immobilised OKT3 titration curve.

10.4.2 Soluble OKT3 Ab titration

In a PBMC microenviroment, the anti CD3 Ab can provided in a soluble instead of immobilised. To titrate soluble OKT3, triplicates of a serial dilution of the Ab solution were added to 1x10⁵ PBMC/well in 96 round bottom well plate in complete medium. The plates were incubated for three days in a humidified atmosphere at 37°C and CO₂. 1 μCi [³H] thymidine/well was added 18 hours before harvesting as described above.

![Graph showing thymidine incorporation](image)

**Figure 10.3.** Titration of soluble OKT3. 1X10⁵ PBMC/well were cultured in a serial dilution (ng/ml) of the purified OKT3 Ab in triplicates. The cells were incubated for three days as described in Figure A.1. 1 μCi [³H] thymidine/well was added 18 hours before harvesting the wells on glass-fibre filters. Radioactivity was counted with a liquid scintillation counter. Results are expressed as mean of triplicate counts per minute (cpm) ± SD. This figure is a representative soluble OKT3 titration curve.
10.5 Letter to request blood from donors of the panel of Anthony Nolan bone marrow donors and consent form

THIS IS NOT A REQUEST FOR BONE MARROW

Dear (name)

I would like to thank you very much for being part of our donor list. In the Anthony Nolan Bone Marrow Trust, our aim is to help people that need a bone marrow transplant. This wouldn't be possible without the altruistic help of our donors and all the people who support the Trust. One of the problems of the bone marrow transplantation is the graft rejection to the patient (Graft versus Host Disease). At the Anthony Nolan Research Institute, we are working to find solutions to this problem. Some successfully results have been already published in international journals to the use of hospitals and clinics in all over the world.

We are inviting you to participate in a project for the in vitro study of the white cells' reaction from to determined donors. All the study is carried out at the Anthony Nolan Laboratories and if you wish you can be informed of the results of the study.

This is a letter sent to specific donors asking for your participation into a research program for the Anthony Nolan Research Institute. We just need 50 ml of your blood, taken by your GP, and sent to us in the parcel provided with this letter. The blood can be taken by you GP on a Monday, Tuesday or Wednesday and sent immediately please, so it doesn't get lost in the post! We will be grateful for your contribution, since this will provide more understanding of the bone marrow transplantation and the problems that the patients can face.

Your participation in this research program doesn't mean that you will be called for bone marrow donation.

We are sending a consent form to be fill out by you if you wish to accept. Please inform us if you don't wish to take part in the program. Do not hesitate to contact me if you have any questions, at any time. My contact number is (0171) 2 84 83 27.

Sincerely,

Isabel Perez-Cruz
CONSENT FORM TO TAKE PART IN THE RESEARCH PROGRAM "IMMUNOLOGICAL MECHANISMS INVOLVED IN THE GRAFT VERSUS HOST DISEASE."

I__________________________________ have read the above letter and would like/would not like to participate in the study.

(delete as appropriate)

Name:

Address:

Telephone:

Name of your GP:

Address and telephone number of your GP:

Have you have any serious illnesses recently? Y_______ N_______

Sex:     F_______ M_______

Age:

Signature____________

Date______/______/_______

The information above is confidential. No more than one sample is needed.

Please return this form and the blood sample in the envelope provided, to Isabel Perez Cruz, at The Anthony Nolan Research Institute, Research Department, The Royal Free Hospital, Fleet Road, Hampstead, London NW3 2QG.
10.6 Concentrations of PMA and Ionomycin used in ICS studies.

A. Concentrations of PMA used for ICS.

<table>
<thead>
<tr>
<th>PMA (ng/ml)</th>
<th>References</th>
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<tbody>
<tr>
<td>5</td>
<td>(Jung, Schauer et al. 1993; Assenmacher, Schimtz et al. 1994; Picker, Singh et al. 1995; North, Ivory et al. 1996; Ferry, Antrobus et al. 1997; Chalmers, Janossy et al. 1998)</td>
</tr>
<tr>
<td>10</td>
<td>(Sewell, North et al. 1997; Woodside, Long et al. 1999)</td>
</tr>
<tr>
<td>20</td>
<td>(Prussin and Metcalfe 1995)</td>
</tr>
<tr>
<td>50</td>
<td>(Elson, Nutman et al. 1995; Openshaw, Murphy et al. 1995; Prussin and Metcalfe 1995; Jason and Larned 1997; Kaplan, Wurster et al. 1999)</td>
</tr>
<tr>
<td>1000</td>
<td>(Anderson, Anderson et al. 1990)</td>
</tr>
</tbody>
</table>

B. Concentrations of ionomycin used for ICS.

<table>
<thead>
<tr>
<th>Ionomycin (μM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>(Anderson, Anderson et al. 1990; Openshaw, Murphy et al. 1995; Kaplan, Wurster et al. 1999)</td>
</tr>
<tr>
<td>1 (700 ng/ml)</td>
<td>(Jung, Schauer et al. 1993; Elson, Nutman et al. 1995; Picker, Singh et al. 1995; Prussin and Metcalfe 1995; Prussin and Metcalfe 1995; North, Ivory et al. 1996; Ferry, Antrobus et al. 1997; Chalmers, Janossy et al. 1998; Woodside, Long et al. 1999)</td>
</tr>
<tr>
<td>&gt;1 (1000 ng/ml)</td>
<td>(Jason and Larned 1997)</td>
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<tr>
<td>3</td>
<td>(Sewell, North et al. 1997)</td>
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</table>

Table 10.1. Commonly used concentrations of PMA (A) and ionomycin (B). The concentration listed are commonly used in the literature to induce the detection of IL-2, IFNγ, IL-4 and IL-5 by ICS.
### 10.7 Protein Sequence Alignments for exon 2 HLA-DRB1 alleles.

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<th>1</th>
<th>50</th>
<th>71</th>
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<th>86</th>
<th>100</th>
<th>150</th>
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<td>DRB1*0101</td>
<td>GDTRPRFLWQLKFECFFNGTERVRLLERCIYNQIESVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQR</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0401</td>
<td>-----E-V-H-----F-D-YF-H-----Y------K</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0403</td>
<td>-----E-V-H-----F-D-YF-H-----Y------</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0404</td>
<td>-----E-V-H-----F-D-YF-H-----Y------</td>
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<td></td>
<td></td>
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<tr>
<td>DRB1*0101</td>
<td>-----RAAVDTYCRHNYGVGESFTVQRRVEPKVTYPSTQPLQHNLVCSVSGFYPGSIEVRWFRNGQEEKAGVSTGLIQNGDWTFQTLVMLETVFRSGEY</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0401</td>
<td>-----Y-E-----A-----N-----T-----</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0403</td>
<td>-----E-----V-----Y-E-----A-----N-----T-----</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DRB1*0404</td>
<td>-----E-----V-----Y-E-----A-----N-----T-----</td>
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<tr>
<td>DRB1*0101</td>
<td>-----TCQVEHPSVTSPLTVEWRARSEQKMLSGVGFLGLFLGAGLFIYFRNQKHSGLQPTGFLS</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1*0401</td>
<td>-----L------</td>
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<td></td>
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<tr>
<td>DRB1*0403</td>
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</tr>
<tr>
<td>DRB1*0404</td>
<td>-----L------</td>
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</tbody>
</table>

Table 10.2 HLA-DRB1*0101, 0401, 0403 and 0404 protein sequence alignments for exon 2.

Sequence comparison between the DRB1 alleles in exon 2. The numbers refer to amino acid positions and dashed lines represent conserved residues. The one-letter code for amino acids was used.
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Bilbiography


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