INTRACELLULAR CYTOKINES AND THEIR THERAPEUTIC MODULATION IN IMMUNOLOGICAL DISORDERS

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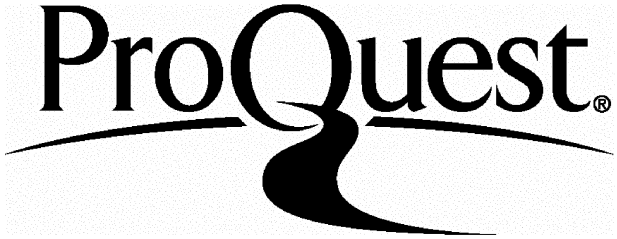

Abstract

This study investigated abnormalities of cytokines principally in common variable immunodeficiency (CVID), and the effects of the immunomodulatory agent intravenous immunoglobulin (IVIG) on cytokine regulation. To this end, a rapid, small-volume assay of intracellular cytokine synthesis was established for lymphocytes. For 50 μl aliquots of heparinised blood diluted 1:2, the optimum culture conditions were: PMA (10 ng/ml), ionomycin (2 μmol/l), and monensin (3 μmol/l) cultured for 2 h for assessment of IFN-γ, TNF-α, IL-4 and CD69 expression, and 4 h culture for IL-2 expression.

Validation was performed in a variety of settings where abnormalities of cytokine regulation are known to occur, and then extended to investigate additional situations where cytokine irregularities have not been formally established. The high IFN-γ expression by $CD8^+$ T cells in CVID was confirmed, γδ T cell IFN-γ expression in sarcoidosis was similar to that of $CD8^+$ cells. A Th2-bias was confirmed in hyper-IgE syndrome.

The immunomodulatory effects of drugs on cytokine regulation in CVID and atopic eczema, were studied. IVIG, *in vitro* and at two different dose ranges *in vivo*, and ciprofloxacin *in vitro* , were studied. Both these diseases are treated with IVIG. The *in vitro* studies of IVIG showed a small but significant reduction in IFN-γ expression in CVID $CD4^+$ cells with increasing concentrations of IVIG from 15±1.87% with no IgG down to 10.5±1.7% with 10 mg/ml IgG $p=0.006$) and 8.8±2.6% with 20 mg/ml IgG ($p=0.04$). The replacement dose *in vivo* study revealed IVIG could increase the potential of $CD8^+28^-$ cells to make TNF-α, and $CD4^+$ lymphocytes to make IL-2 in patients with CVID. NK cell IFN-γ and monocyte IL-12 expression was not affected by IVIG *in vivo*. High dose IVIG in eczema patients resulted in CD69 expression in both $CD4^+$ and $CD8^+$ cells declining during the six months of hdIVIG therapy to approximately 60% of baseline values. Ciprofloxacin *in vitro* had no discernible effect on cytokine expression.

High T cell IFN-γ levels in CVID may be caused by abnormal monocytes. Overnight incubation of lymphocytes with monocytes from CVID patients increased IFN-γ expression, yet this effect was not totally abrogated by anti-IL-12, suggesting that additional cytokines may be involved in the high IFN-γ expression.

Since different T lymphocyte populations responded differently to therapeutic immunomodulation, 4-colour flow cytometry was used to examine activation markers in T lymphocyte subsets in CVID and normal subjects. Both absolute counts of various subsets of T cells, and the expression of the activation markers CD25 and HLA-DR were examined in peripheral blood. Although serum CD25 levels are elevated in CVID, an increase in $CD25^+$ cells was not detected. $HLA\text{-}DR^+$ cells were increased however, particularly in $CD4^+28^-$ cells. DR expression was lower in XLA than in normal controls. The proportions of CD28+ and CD45RA+ cells were decreased in CVID.

These findings were used to construct a model for cytokine dysregulation in CVID, and to highlight potential new areas for therapeutic immunomodulation of CVID, including the use of high-dose IVIG, and co-administration of dopamine receptor modulating drugs.

Contents

Copyright declaration

Declaration

I designed the study reported in this thesis and performed all the experiments by myself, except for the kind assistance given for some tests, acknowledged in the appropriate places. I performed all the statistical analyses myself. The whole thesis and all figures and tables were prepared and typed by myself.

Blood donations were taken by informed consent, and were used with the approval of the Ethics Committee of the Royal Free Hospital.

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Margaret North and Charo Cambronero were each kind enough to do one of the intracellular cytokine assays on the high dose patients whilst I was on holiday, and my grateful thanks go to them. Liz King provided extensive help with the hundreds of tubes required for the lymphocyte activation and subsetting experiment, and I am extremely grateful for her assistance. Sisters Cilla Freud and Annie Ryan were invaluable in helping to recruit patients to the study and providing samples of blood whilst also running an extremely busy clinical service, and I am indebted to them.

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Abbreviations

BSA	Bovine serum albumin
Con A	Concanavalin A
CoV	Coefficient of variation
CVID	Common variable immunodeficiency
ECD	Energy-coupled dye
EDTA	Ethylene-diamine tetra acetic acid
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
IgA, IgG, IgM, IgE	Immunoglobulin A, G, M, E
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
IFN-γ	Interferon gamma
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
p	Probability of result occurring by chance
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBS/A/A	Phosphate-buffered saline containing azide and albumin
PC5	Phycoerythrin/cyanine 5 conjugate dye
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PMA	Phorbol-12,13-myristate acetate
PWM	Pokeweed mitogen
RPMI1640	Roswell Park Memorial Institute medium 1640
SAC	*Staphylococcus aureus* Cowan strain I
SD	Standard deviation
SEM	Standard error of the mean
TNF-α	Tumour necrosis factor alpha
TGF-β	Transforming growth factor beta
XLA	X-linked agammaglobulinaemia

List of Figures

All figures **follow** the page number listed

List of Tables

1 General Introduction

1.1 Aims of the study

It is now axiomatic that abnormalities of cytokine regulation underlie the pathogenesis of many disorders. A principal interest of the present project was to study cytokine regulation in the primary immunodeficiency disease common variable immunodeficiency (CVID). However, diseases as diverse as asthma and inflammatory bowel disease can be said to be 'immunoregulatory' disorders, involving altered cytokine patterns. One fundamental aspect of understanding the mechanisms of disease therefore becomes the ability to measure cytokines in the clinical setting. Currently, this is problematic, and satisfactory assays are not in general use in clinical practice.

The first aim of this study was therefore to develop a technique with which to measure lymphocyte cytokines in cells within the peripheral blood, in a manner which could eventually be developed for use in the routine clinical immunology laboratory. The assay would have to be relatively straightforward to perform, be rapid and robust, and ideally use relatively small blood samples. This work is described in Chapter 3.

Having developed such a whole blood assay, the second aim was to validate the new technique in a variety of settings where abnormalities of cytokine regulation are known to occur, and then to extend its use to investigate additional situations where cytokine irregularities have not been formally established. As mentioned above, the principle disease studied was CVID, a primary antibody deficiency with some inflammatory manifestations. This work is outlined in Chapter 4.

The finding of disease mechanisms in medical research leads to attempts to interfere with the process therapeutically. The third aim, described in Chapter 5, was to examine the immunomodulatory effects of drugs on cytokine regulation in two diseases, these were CVID, and severe atopic eczema. Two agents were examined in detail: intravenous immunoglobulin (IVIG), *in vitro* and at two different dose ranges *in vivo*, and ciprofloxacin, a 4-fluoroquinolone antibiotic said to have immunomodulatory properties. Both the diseases studied are treated with IVIG.

As the work progressed, it became clear that some of the abnormalities in cytokine regulation found in CVID could be attributable to abnormalities of monocyte cytokine regulation. The fourth aim was therefore to examine the role that monocytes play in the cytokine abnormalities of CVID, by means of depletion and blocking experiments. This is outlined in Chapter 6.

The finding that different T lymphocyte populations respond to therapeutic immunomodulation differently in different diseases lead to the fifth aim of the study, which was to use the latest flow cytometric technology to examine activation markers in T lymphocyte subsets in CVID and normal subjects. Both absolute counts of various subsets of T cells, and the expression of two activation markers were examined in peripheral blood, and are described in Chapter 7.

Finally, the overall aim of this work was to use the data obtained with the new method to construct a hypothesis for the dysregulation of cytokines in CVID and to attempt to explain how this immunoregulation is altered by the therapeutic interventions.

1.2 The role of cytokines in the regulation of the immune system

1.2.1 Cytokines

Cells of the immune system can communicate by means of transfer of molecules, from one cell to a receptor on another cell, which goes on to signal an effect. The broad term used to describe one class of these molecules is cytokines (for discussion see Klein & Horejsí, 1997). Cytokines are distinguished from hormones, growth factors and chemokines by their action over short distances, often in a paracrine or even an autocrine manner. The majority of cytokines are glycoproteins in the range 10-20 kDa molecular mass, which combine with high affinity to their receptors. Cytokines can be divided into several families on the basis of their structure, although their range of activities and target cells may be extremely diverse.

Representatives of several cytokine families were studied here because of their fundamental role in the operation of the normal immune system, and the range of diseases that can result when their regulation becomes abnormal. These include the interferon family (e.g. interferon-γ), the haemopoietin family (e.g. interleukin-2, interleukin-4), the TNF family (e.g. tumour necrosis factor-α), and a separate family of miscellaneous cytokines (e.g. interleukin-12).

1.2.1.1 Interferon-gamma

Interferon-γ (IFN-γ) is important in this study, as it is a key cytokine in the regulation of the immune system (see below), and abnormalities of IFN-γ have been noted in the diseases studied here. For example abnormally high IFN-γ expression has been reported in CVID (North *et al*, 1996). IFN-γ is a homodimeric glycoprotein containing six alpha helices in each subunit, each made up of 9 to 12 residues (de Maeyer & de Maeyer-Guignard, 1998). The human IFN-γ gene is on chromosome 12, region p12.05, consisting of four exons, resulting in a 166-amino acid polypeptide including a 23-residue signal sequence. Interferon-γ is only produced by T cells and NK cells. T cells producing IFN-γ can be $CD8^+$ (Celis *et al*, 1986) or $CD4^+$ of the Th1 subset (see below). The recognised activities of IFN-γ include: (1) stimulation of MHC class II (Blanar *et al*, 1988) on B cells and macrophages. (2) activation of macrophages and induction of macrophage intracellular killing mechanisms such as production of reactive oxygen intermediates and the secretion of hydrogen peroxide (Flesch & Kaufmann, 1991). (3) Induction of indoleamine 2,3-dioxygenase, which converts tryptophan to kynurenine. This kills intracellular parasites by tryptophan starvation (Taylor & Feng, 1991). (4) Activation of NK cells (Koh *et al*, 1997). (5) Regulation of IgG subclasses (Snapper & Paul, 1987).(6) Antiviral activity by stimulation of oligoadenylate synthetase, which leads to the activation of a latent endoribonuclease. (7) Induction of nitric oxide synthase in macrophages. This mechanism is thought to be one of the key mechanisms by which IFN-γ inhibits viral replication (Campbell *et al*, 1994). (8) Induction of interleukin-12 by monocytes (Kubin *et al*, 1994).

1.2.1.2 Interleukin-2

Interleukin-2 (IL-2) is essential for normal T cell growth and development. A growth factor for cytotoxic T cells present in lymphocyte-conditioned medium was identified in 1976, and was termed 'blastogenic factor', 'costimulator activity', and 'thymocyte differentiation factor' and is now termed IL-2 (Morgan *et al*, 1976). This potent growth and differentiation factor stimulates both T cells and NK cells, is a growth factor for B cells and can stimulate monocytes (Thorpe, 1998).

IL-2 in humans is encoded as a single gene on chromosome 4q26-28, consisting of three introns and four exons. There is a promoter region 75 bp upstream from the translation unit site, which shares some homology with the interferon-γ gene (Fujita *et al*, 1983). Stimulation of T cells by antigen or mitogen results in production and secretion of IL-2. Maximal production occurs when dual signalling is employed, for example *in vitro* via the T cell receptor and by activation of protein kinase C with phorbol esters. This results in maximal production of the proteins AP-1 and NFkB (McGuire & Iacobelli, 1997) which along with other proteins (NFAT-1, Oct-1/NF-IL-2-A) bind enhancer elements 5' of the IL-2 gene. IL-2 gene expression is also controlled by extensive post-transcriptional modification of mRNA, by motifs 3' of the untranslated region of the mRNA. Additional regulation is mediated by a negative response element and a labile repressor which reduces processing of IL-2 mRNA precursors (Kaempfer *et al*, 1987).

Only T lymphocytes can produce IL-2, and both $CD4^+$ and $CD8^+$ T cells can do this. In the 'Th1/Th2' scheme described below, initially only Th1 cells were thought to secrete IL-2, but it is now known that Th2 cells can also make IL-2. IL-2 production is maximal following T cell activation through multiple signalling pathways, and following mitogen-driven activation, IL-2 production peaks at 40-48 hours (Thorpe, 1998).

The effects of IL-2 are greatest on T cells, resulting in dramatic proliferation of activated cells. All subpopulations of T cells are affected, but resting cells require unphysiologically more IL-2 due to lower expression of IL-2R alpha chain (CD25). In addition to proliferation, IL-2 causes T cell differentiation, enhancing cytotoxic activity, increasing T cell motility and induction of the cytokines interferon-γ, IL-4 and tumour necrosis factor-alpha (TNF-α), activates B cells to produce immunoglobulin (Ralph *et al*, 1984), increases the cytotoxic activity of large granular lymphocytes (LGL) and natural killer (NK) cells

(Ortaldo *et al*, 1984) and causes myelocytic lineage cells to proliferate and undergo differentiation (Baccarini *et al*, 1989).

1.2.1.3 Interleukin-4

Interleukin-4 (IL-4) is the archetypal Th2 cytokine, and therefore was of interest in this study in terms of its effects on immunoregulation of Th1/Th2 balance. IL-4 is a 129 amino acid protein of molecular mass 18-19 kDa, which is N-glycosylated and contains three internal disulphide bonds between cysteine residues (Mire-Sluis, 1998). This gives the molecule the overall shape of a four-helix bundle. The IL-4 gene is located on chromosome 5q23.3-31.2, and contains four exons.

Originally identified as a B cell growth factor (BCGF), IL-4 was also previously known as B cell differentiation factor (BCDF), and B cell stimulatory factor-1 (BCSF-1) (see Mire-Sluis, 1998). IL-4 is produced mainly by T cells, particularly $CD4^+$ $CD45RA^-$ cells (deKruyff *et al*, 1995), and little is expressed by $CD8^+$ T cells (Seder *et al*, 1992). Ligation of the T cell receptor, or of CD3 or CD2 mediates induction of IL-4. Mitogens can also induce IL-4 though with differing kinetics, for example Concanavalin A (ConA) induces IL-4 but with a low transcription rate, and PMA increases ConA mediated IL-4 expression subsequent to mRNA stabilisation (Dokter *et al*, 1993). Mast cells, basophils and eosinophils (Nonaka *et al*, 1995) also make IL-4 .

IL-4 has numerous biological effects including activation of B cells, proliferation of B cells (with costimulation), isotype switching of B cells to produce IgE (in the presence of $CD4^+$ T cells) (Pene *et al*, 1988) and IgG4 (Lundgren *et al*, 1989. Monocytes express MHC Class II, CD13, CD23, CD18, CD11b and CD11c in the presence of IL-4 (reviewed in Mire-Sluis, 1998).

IL-4 promotes the growth of activated T cells (both $CD4^+$ and $CD8^+$) and makes T cells more responsive to IL-2 (Mitchell *et al*, 1989). It regulates the induction of the cytolytic activity of T cells, and is inhibited by IL-12 (Abdi & Herrmann, 1997), thus providing reciprocal inhibition of T-helper-1 (Th1) responses once a T-helper-2 (Th2) response is underway (see section 1.2.2.1 below for a discussion of Th1/Th2).

1.2.1.4 Tumour necrosis factor-alpha

Tumour necrosis factor-α (TNF-α) is an important cytokine in immunodeficiency studies, since an association between a polymorphism of TNF-α (+488A), and the granulomatous form of common variable immunodeficiency has been found (Mullighan *et al*, 1997). TNF-α is a 157 amino acid with very diverse properties (Beyaert & Fiers, 1998). Monomeric TNF-α (17 kDa) forms homotrimers; each monomer is very strongly linked by non-covalent forces, such that no dissociation occurs, and the trimer is the biologically active moiety (Wingfield *et al*, 1987). The larger (26 kDa) integral transmembrane protein is released into the surrounding environment by proteolytic cleavage. The 3 kb TNF-α gene is located on human chromosome 6, close to the major histocompatibility (MHC) locus (Spies *et al*, 1991). Many cell types synthesise TNF-α, particularly monocytes and macrophages (reviewed in Beyaert & Fiers, 1998). Of interest in this study, Th1-like T cells are producers of TNF-α, and the exclusive producers of lymphotoxin (previously known as TNF-β), a structurally related TNF-family member.

TNF-α has many biological activities, including: cytotoxicity, especially to tumour cells, an effect enhanced by the presence of other Th1-like cytokines such as IFN-γ (Williamson *et al*, 1983), induction of monocyte differentiation, and it is mitogenic to many cells (fibroblasts, T cells and B cells). TNF-α is also antiviral (Wong & Goeddel, 1986). A key role is immunomodulation, since TNF-α is proinflammatory, causing the secretion of IL-1, IL-6 and IL-8 by macrophages (Beyaert & Fiers, 1998). This effect is opposed by transforming growth factor-β (TGF-β) (Flynn & Palladino, 1992).

1.2.1.5 Interleukin-12

The finding of elevated interleukin-12 (IL-12) production by monocytes from patients with CVID (Cambronero, Sewell, *et al*, manuscript submitted) made IL-12 an important cytokine to include in this study. IL-12 is a cytokine linking the innate and adaptive immune systems (Locksley, 1993), and was originally termed natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF). IL-12 is a potent modulator of NK and T cell functions, and a key cytokine in the development of a Th1-like immune response (Chizzonite *et al*, 1998). This heterodimer is composed of two subunits, p35 and p40, and is only biologically active in this form. Subunit p40 is

encoded on human chromosome 5q31-33 and p35 resides on chromosome 3p12-3q13.2 (Sieburth *et al*, 1992). The p40 subunit is composed of 306 amino acids, and the p35 of 197 amino acids. Both are disulphide-linked, and also have substantial degrees of internal disulphide linking. Neither subunit alone has significant biological activity, except that p40 monomer or homodimer can inhibit IL-12 activity (Mattner *et al*, 1993). IL-12 binds a multiple subunit receptor, formed from IL-12Rβ_1 and IL-12Rβ_2 chains, which is expressed on resting NK cells (Desai *et al*, 1992) and activated NK cells (Naume *et al*, 1993), as well as on activated T cells (both $CD4^+$ and $CD8^+$).

The primary source of IL-12 is monocytes/macrophages, although B cells can also synthesise IL-12 in small amounts (d'Andrea *et al*, 1992). Stimulants that lead to IL-12 expression include bacterial lipopolysaccharide (LPS) and *Staphylococcus aureus* Cown Strain I (SAC), but not phorbol esters (except in cell lines) (d'Andrea *et al*, 1992). IL-12 has numerous effects including upregulation of NK cytotoxicity, and proliferation of activated (but not resting) T cells (Kobayashi *et al*, 1989).

IL-12 has a significant effect on cytokine expression by T and NK cells, inducing IFN-γ in a dose-dependent manner (Chan *et al*, 1991). This IFN-γ induction can be enhanced synergistically *in vitro* by addition of IL-2, IL-1, TNF-α, PMA or anti-CD3, and inhibited by IL-4, IL-10 or TGF-β (reviewed in Chizzonite *et al*, 1998). Expression of TNF-α, GM-CSF, and IL-8 can also be induced in NK cells by IL-12 (Perussia *et al*, 1992). IL-12 regulates the induction of Th1 cells in both mice and humans. Human $CD4^+$ T cell lines cultured with the allergen *Der p* I to develop a Th2 response, exhibit a Th0 (IFN-γ and IL-4 producing) or Th1 (IFN-γ but not IL-4) phenotype when cultured with IL-12 (Manetti *et al*, 1994). $CD4^+$ T cell lines stimulated with purified protein derivative from *M tuberculosis* (PPD) normally develop a Th1 phenotype; blocking IL-12 with monoclonal antibodies results in the development of a Th0 phenotype (Manetti *et al* 1993). This effect on Th1/2 phenotypes is evident in IgE production experiments, where IL-12 has been shown to inhibit IL-4 stimulated IgE production (but not pokeweed stimulated IgG, IgM or IgA production) from cultured cells (Kiniwa *et al*, 1992).

1.2.2 Patterns of cytokine secretion

The way in which cytokines regulate each other, and the means by which this mechanism can go wrong, are fundamental issues addressed by this study. Although some cytokines share a broad range of overlapping functions, others may have opposing activities. The opposition is often reciprocal, so that upregulation of one arm of the response downregulates the other. Several schemes exist to describe these actions, the key features of which are described below.

1.2.2.1 The 'Th1/Th2 Paradigm'

The production of cytokines by T cells depends on the type of T cell. Apart from their classification into $CD4^+$ and $CD8^+$ T cells, it is now recognised that T cells can be subdivided according to their cytokine secretion profile. Initial work by Mosmann and Coffman (1989) proposed the division of murine T helper ($CD4^+$) cells in to Th1 and Th2 subsets. Following antigenic stimulation, Th1 cells produce IFN-γ and TNF-β and IL-2, Th2 cells produce IL-4, IL-5 and IL-13. Th1 cells activate macrophages via IFN-γ, and so are the principle effectors of cell-mediated immunity. They also are cytotoxic and stimulate production of antibodies. In contrast, Th2 cells stimulate IgE production, stimulate mast cells and activate eosinophils. A key feature of the 'Th1, Th2 paradigm' is that the products of one arm of the system inhibit development of the opposite arm, so that IFN-γ inhibits the development of a Th2 response, and IL-4 inhibits development of a Th1 response (Romagnani, 1996). The picture is not entirely clear however, since it is now apparent that there can be differential effects of the counter-regulatory cytokines, for example under different conditions IL-4 can both enhance IFN-γ synthesis, *yet also* inhibit the development of IFN-γ-producing cells (Noble & Kemeny, 1995).

The Th1/Th2 model has been extended to the human immune system (Romagnani, 1994) and further subsets of cells have been included including Th0 cells (which secrete both patterns of cytokines). There is also evidence of polarised cytokine responses occurring in CD8 T cells, leading to so-called Tc1 and Tc2 responses (Romagnani, 1997). A third population of cells, sometimes termed Th3 or Tr1 (regulatory T cells) have been proposed, which secrete TGF-β and/or IL-10 (O'Garra *et al*, 1997). It is clear that

although initially described in mice, the human Th1/2 model is different, with many more Th0 cells (Katsikis *et al*, 1995). It is also clear that T cell clones, derived from human cells, behave very differently to normal T cells in terms of their cytokine production profiles (van der Pouw-Kraan *et al*, 1993). There are no specific surface markers that clearly distinguish Th1 from Th2 cells. CD30 (Del Prete *et al*, 1995) and eotaxin receptor/CCR3 (Sallusto *et al*, 1997) have been proposed as Th2 markers, although the CD30/Th2 concept has been contested (Hamann *et al*, 1996). The lymphocyte activation gene-3 product (LAG-3) has been proposed as a Th1 marker (Annunziato *et al*, 1996). Different developmental stages of T cells are also associated with different cytokine regulatory patterns, for example, in terms of 'memory' (CD45RO+) and 'naive' (CD45RA+) T cells: both can produce IL-13, yet only CD45RO cells produce IL-4 (Jung *et al*, 1996), yet both are Th2 cytokines.

1.2.2.2 Cytokine-biased populations of lymphocytes

In addition to the Th1/Th2 paradigm to describe cytokine polarisation in T cells, other schemes to distinguish functional subsets have also been put forward. One such scheme is based on expression of CD7 by $CD4^+$ lymphocytes.

1.2.2.2.1 CD7 Subpopulations of T lymphocytes

T Lymphocytes can be divided into two populations on the basis of their CD7 expression. CD7 is a 40 kDa glycoprotein expressed on the majority of peripheral blood T cells, NK cells and all thymocytes (Lobach *et al*, 1985). CD7 is involved in signal transduction and regulation of cell proliferation, although the natural ligand for CD7 has not yet been identified. Blocking CD7 can disrupt T cell proliferation (Lazarovitz & Karsh, 1988), and cross-linking CD7 results in T cell proliferation. Certain forms of cutaneous T cell lymphoma (CTCL) lack CD7 expression (Haynes *et al*, 1981). The majority of CD4+ CD7- cells are CD45RO+ (Kukel *et al*, 1994). Expansion of this subset has been associated with diseases that involve chronic T cell stimulation, and CD7- T cells have been suggested to represent a separate population of memory T cells (Reinhold & Abken, 1997), since they can form a self-renewing population (Reinhold *et al*, 1996a). They also preferentially express cutaneous lymphocyte antigen-1 (CLA), and home to skin, comprising ~70% of epidermal T cells in normal human skin (Berg *et al*,

1991). Of note in this context, CD7- T cells appear to be Th2-biased, as the majority produce high levels of IL-5 following stimulation *in vitro* with PMA and ionomycin, compared with CD7+ cells. Interestingly, this cytokine polarisation in CD7 subsets was not seen with IFN-γ or IL-4 expression (Reinhold *et al*, 1996b). CD4+CD7- cell clones have been found to secrete IL-4 and IL-10, but not IL-2 following stimulation with anti-CD3 and anti-CD28 (Autran *et al*, 1995). CD7- and CD7+ subsets of CD8 cells do not behave in this way.

1.2.2.3 Cytokine production by non-T cells

Many cells apart from T lymphocytes are significant sources of cytokines. Intracellular cytokines within two cell types were examined in this study: monocytes, since they are a primary influence on Th1-cytokines by means of IL-12, and NK cells, since they are potent producers of IFN-γ.

1.2.2.3.1 Monocytes

Monocytes are a potent source of production of many cytokines including IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α, and GM-CSF (Mire-Sluis & Thorpe, 1998). Cytokines are involved in bilateral signalling between lymphocytes and monocytes, for example the production of IL-12 by monocytes results in IFN-γ production by NK cells and T lymphocytes (Chan *et al*, 1991). IL-10 produced by lymphocytes is inhibitory to monocytes, and IFN-γ leads to monocyte activation and further secretion of IL-12. Extensive feedback loops exert control, so lymphocyte IL-10 induction by monocyte IL-12 results in down-regulation of IL-12 production (reviewed in de Waal Malefyt, 1998).

1.2.2.3.2 NK cells

NK cells are lymphoid cells that are usually defined as expressing CD16 and CD56, but not CD3 or B cell markers (Trinchieri, 1989). They are potent sources of IFN-γ and can also produce IL-3, and TNF-α (Mire-Sluis & Thorpe, 1998). Numerous cytokines act on NK cells including IL-2, which activates them, and which is opposed by IL-4. IL-6 augments NK cell responses. Both IL-12 and IL-18 promote NK cell IFN-γ induction (Okamura, 1998).

1.2.3 The assessment of intracellular cytokines

The ability to assess cytokine profiles in different immune cells is fundamental to understanding both normal immunoregulation and its dysfunction in disease. Traditionally, cytokines have been measured in supernatants from lymphocyte cultures by enzyme-linked immunosorbent assay (ELISA) or bioassays (Thorpe *et al*, 1992). This provides a useful but incomplete picture, since the relative contribution of individual cell subsets to cytokine production is not determined. With advances in cell fixation and permeabilisation it became possible to identify individual cytokine producing cells by indirect immunofluorescence microscopy; initially by two-layer staining (Sander *et al*, 1991) and more recently with directly conjugated anti-cytokine antibodies. Such methods are useful but laborious and have only a limited ability to distinguish the lymphocyte's phenotype as well as cytokine production. However, the advent of intracellular cytokine assessment by flow cytometry, staining permeabilised cells with fluorochrome-conjugated anti-cytokine antibodies, permitted large numbers of cells of known phenotype to be examined(Picker *et al* 1995; Prussin & Metcalf, 1995; Schauer *et al* 1996; North *et al*, 1996 and summarised by Carter & Swain, 1997).

Various techniques to measure intracellular cytokines have been reported for peripheral blood mononuclear cells isolated by density gradient centrifugation (Prussin & Metcalfe, 1995; North *et al*, 1996). Although these are useful, they need comparatively large volumes of blood (10-20 ml) which is disadvantageous in the paediatric studies, or when serial testing is required. Also, the process of cell separation may affect cell activation status (Maino *et al*, 1995) and perturb cytokine production (Maino *et al* 1996).

To assess potential cytokine production, various culture conditions (involving changes in culture time, type and dosage of stimulants) have been proposed (Picker *et al* 1995; Prussin & Metcalf, 1995; Schauer *et al* 1996; North *et al*, 1996). Since cytokine production by normal resting cells is minimal, most published techniques use a strong supra-physiological stimulus in culture to demonstrate the potential of each cell to synthesise cytokines. Many of these methods use culture for 10-12 h with stimulants such as phorbol myristate acetate (PMA) that activates cells via the protein kinase C (PKC) pathway, together with ionomycin that mobilises intracellular calcium stores. This PKC activation down-regulates cell surface CD4 expression on T cells (Petersen *et al*, 1992),

making identification of $CD4^+$ T cells and subsequent assessment of cytokines within the T helper subset very difficult.

1.3 Immunological disorders

1.3.1 Common variable immunodeficiency

1.3.1.1 Clinical background

Common variable immunodeficiency (CVID) is the most common primary antibody deficiency of adults (see review by Cunningham-Rundles & Bodian, 1999). The majority of patients develop symptoms either in early childhood or late adolescence, and present most commonly with recurrent sinopulmonary infections. *Haemophilus influenzae, Moraxella catarrhalis,* and *Streptococcus pneumoniae* are the commonest pathogens, yet other organisms infect other systems more commonly than in normal subjects, for example gut infections with *Giardia lamblia* and joint infections with *Mycoplasma sp.* In addition to the infectious manifestations, CVID is distinct from other immunodeficiency disorders in being associated with chronic unexplained inflammatory disease of the lungs, gut, liver spleen and skin, often with granuloma formation (Hermaszewski & Webster, 1993).

Although X-linked agammaglobulinaemia (XLA) was the first antibody deficiency syndrome described (Bruton, 1952) the classification of antibody deficiency syndromes (including CVID) has been hampered by lack of appropriate investigative techniques in the past. Following Bruton's description of XLA, immunoglobulin levels were examined in a variety of other patients suffering from recurrent infections, and resulted in the recognition of adult-onset antibody deficiency in males (discussed in Hammerstrom & Smith, 1999) and females (Pearce & Perinpanayagam, 1957) - which became known as CVID. The inability to determine B cell numbers and cell protein expression in the past has almost certainly lead to the misclassification of some cases of CVID. Examples include males with XLA but low numbers of B cells being misclassified as CVID. Additionally, females with immunoglobulin deficiency may have been labelled as CVID when in fact they may have had mutations of the mu-heavy chain gene (Yel *et al*, 1996) or mutations in surrogate light chain (Minegishi *et al*, 1998).

The mechanism of the immunodeficiency in CVID has not yet been totally established, although numerous proposals have been put forward (reviewed by Webster *et al*, 1997).

Initial work focussed on B cell abnormalities, since the disease is characterised by antibody deficiency. Stimulation of CVID B cells *in vitro* with combinations of CD40-ligand, IL-10 and other cytokines showed that it is possible to stimulate CVID B cells to secrete all main immunoglobulin classes (Eisenstein *et al*, 1994). Bryant *et al* (1990) classified CVID into three main types based on the patient's B cell responses to anti-immunoglobulin μ-chain and IL-2 (discussed below). These findings suggested that the primary abnormality in CVID lay not in the B cell, but in other populations of cells. Many patients with CVID have a T cell lymphopoenia. This is associated with a failure of T cell sensitisation to either tetanus toxoid (Stagg *et al*, 1994) or keyhole limpet haemocyanin (Kondratenko *et al*, 1997).

Genetic explanations for CVID have long been sought. Association has been made between selective IgA deficiency and CVID with the MHC Class II/III region, particularly HLA DR3, C4, DQβ (codon 57) (Schaffer *et al*, 1991; Howe *et al*, 1991; Olerup *et al*, 1992). In addition, certain polymorphisms (+448) of the TNF-α gene have been associated with the granulomatous form (Type A) of CVID (Mullighan *et al*, 1997). A large microsatellite linkage study of 85 families with selective IgA deficiency or CVID is currently underway, yet preliminary studies have discounted formal genetic linkage between MHC genes and IgA deficiency or CVID (Vorechovsky *et al*, 1995).

1.3.1.2 Cell activation in CVID

1.3.1.2.1 B cells

Some indication that cell-cell interactions are abnormal in CVID was gleaned as early as 1976, when Siegal *et al* reported a series of lymphocyte mixing experiments, demonstrating a reduction in immunoglobulin production by cultured normal B cells stimulated with PMA, to which lymphocytes from immunodeficient patients were added.

The production of hybridomas from patients with CVID was noted to be difficult following stimulation with PMA or SAC alone, yielding no viable hybrids from 600 million cells (Denis *et al*, 1983). The combination of both mitogens however, led to successful hybridoma production, yielding production of 15 IgM secreting cell lines from five CVID patients, but no IgG lines were induced.

B cell differentiation was studied in CVID (Saxon *et al*, 1989) and showed a reduction in in B cells expressing the surface marker CD21. Early B cell markers were not enhanced, and activation markers were absent. B cells from 14 /17 patients failed to proliferate or secrete Ig in response to 'T cell replacing factor' (IL-2) and/or PMA. This study was interpreted as showing a B cell defect in the transition between proliferation and differentiation.

B cell activation defects were also studied by classifying CVID into three groups: those with no B cells, those with low B cells and those with normal B cell numbers (Matsuoka *et al*, 1986). However, only ten patients were included. The patients in the normal B cell number group had varying responses to SAC in co-cultures with allogenic T or B cells, demonstrating some B cell defects, and some T cell defects. T cell help was described as normal in the first two groups of patients with low or absent B cells. The possibility of defects in the PKC pathway in CVID B cells was supported by Kaneko *et al* (1996) who demonstrated failure of PKC activation and translocation to the plasma membrane following stimulation with phorbol ester or anti-μ.

1.3.1.2.2 Monocytes

The removal of monocytes from cultures of peripheral blood mononuclear cells in two patients with CVID resulted in increased proliferative responses to PHA and ConA, but not to PPD (Arala-Chaves, *et al*, 1982). Similar mixing experiments by Siegal *et al* (1976) showed that monocytes from CVID patients were responsible for the reduction in both IgG production and PWM-induced proliferation. Supernatants from monocyte cultures showed similar inhibitory properties.

Impaired proliferative responses to a variety of polyclonal activators were confirmed in a series of 39 patients with CVID by Cunningham-Rundles *et al* (1982). The depressed B cell reactivity did not correlate with B cell numbers, suggesting an intrinsic B cell abnormality. Similar results were obtained by Pollack *et al* (1985), demonstrating impaired immunoglobulin production by peripheral blood mononuclear cells from CVID patients, that was corrected by removal of monocytes in some patients, and CD8+ T cells in others. CD8+ T cell numbers did not correlate with the degree of inhibition. In this

study, normal serum (but not CVID serum) contained both inhibitory and stimulatory factors, depending on the timing of administration.

T and B cell responses were dissected in fifty patients with CVID (Farrant, 1991) showing a mixture of defects in both cell types. Patients with low B cell numbers also had low proliferative T cell responses to mitogens. The proliferative response to PHA was found to be inadequate in 5/9 patients with CVID (North *et al*, 1991a) with additional studies demonstrating a defect in the PKC activation pathway of T cells. This defect was not corrected by the addition of IL-2.

1.3.1.2.3 T cells

T cell activation in CVID was found to be deficient in terms of IL-2 production and T cell proliferation. Additional stimulation with PMA and the anti-CD3 monoclonal antibody, OKT3, was able to overcome this defect. Additional studies confirmed that the defect was in the CD3 activation sequence (Fiedler *et al*, 1987) but not in the PMA-induced pathway. IL-2 and IL-2 receptor gene expression was reported as normal, suggesting a post-translational defect. It was shown that monocytes were responsible for this decreased IL-2 expression, and that the defect was not corrected by blocking the prostaglandin synthetase pathway with indomethacin.

A letter in the New England Journal of Medicine (Lebranchu *et al*, 1990) also reported the CD4+45RA+ deficit in CVID. The proliferative responses to mitogens in CVID patients with low CD4 counts were further investigated by Lebranchu *et al* (1991), revealing that the major defect was in CD4+45RA+ cells. The proliferative response to ConA or PHA was proportional to the CD4 count.

Other pathways have been implicated in the defective proliferative response of CVID T cells to mitogens: anti-CD28 stimulation was shown to enhance mitogenic stimulation of CVID T cells in a normal manner (North *et al*, 1994). The costimulatory effects of monocytes on CD2-triggered T cell proliferation was found to be impaired in CVID, and the mitogenic response of anti-CD2 plus anti-CD45 was also impaired (Zielen *et al*, 1994a).

The signalling systems in CVID T cells were further explored by Fischer *et al* (1996), who showed that CVID cells failed to make second messenger inositol (Ins (1,4,5)P3) after

superantigen or anti-TCR MAb. Neither IL-2 nor anti-CD28 were able to overcome this deficit, but stimulation with PHA or aluminium fluoride was. This suggested a defect in TCR-signalling. Intracellular tyrosine kinase expression was not activated in 3/6 CVID patients following TCR/CD3 stimulation, suggesting a defect in the earliest step of TCR signal transduction (Majolini *et al*, 1997). Studies of sodium-proton antiport in the red cells, as a read-out of protein kinase C (PKC) stimulation was decreased in a patient with CVID (Gur *et al*, 1997). The defect was corrected by insulin, which is a tyrosine kinase activator. The authors suggested that an alteration in serine-threonine phosphorylation may have been responsible for the defect.

The finding of differential TCR Vβ gene use in CVID (particularly in those patients with increased numbers of CD8+ T cells) suggests an antigen (or superantigen) driven response (Duchmann *et al*, 1996).

1.3.1.3 Cytokines in CVID

Numerous abnormalities of cytokine regulation have been demonstrated in CVID. In the following section, previous work describing abnormalities of the cytokines examined in this study (IL-2, IFN-γ, and TNF-α) are briefly reviewed. In addition, work on other cytokines are also described for completeness.

1.3.1.3.1 IL-2

A reduction in IL-2 production CVID following PHA and/or ConA activation was noted by Gronewold *et al* (1984). Studies of IL-2 and IL-4 showed impaired IL-2 production following ConA stimulation of cells from one patient with CVID (Saiki *et al*, 1984). IL-2 responses and IL-2Rα expression on activated T cells were normal in this patient.

Studies by Ariga *et al* (1987) examined the responses of CVID B cells to combinations of *Staphylococcus aureus* Cowan strain I (SAC) and exogenous IL-2. The responses could be divided into four groups: no response to either, a response to SAC but not SAC+IL-2, normal proliferation to either but no Ig production, and normal proliferation and Ig production to either stimulant. Similar classification schemes were introduced by Bryant

et al (1990), classifying the response of CVID B cells to anti-IgM and recombinant IL-2; Group A made no IgM or IgG response; Group B secreted IgM alone; Group C made both IgG and IgM; a fourth group did not have peripheral B cells. The effect of PMA on T cells from CVID patients was unable to influence Ig secretion in group A or B patients, even with the addition of IL-2 or IL-4 (Franz *et al*, 1992), implying that the protein kinase C pathway is not involved in the CVID activation defect.

Sneller & Strober (1990) demonstrated low levels of IL-2 gene expression following PHA stimulation of CVID PBMCs, but normal levels of IL-2Rα chain (CD25), and normal levels of c-*myc*. The inability of lymphocytes from some CVID patients to secrete normal levels of IL-2 but to express normal levels of IL-2Rα following PHA stimulation was confirmed by Vukmanovic *et al* (1992), *In vitro* T cell proliferation could be restored in these patients by the addition of exogenous IL-2. Accessory cell defects were excluded on the grounds of normal IL-1 and normal IL-6 production, and stimulation of T cells with PMA and ionomycin to bypass the PKC pathway resulted in normal proliferation and IL-2 production.

In addition to studies with polyclonal activators (eg Hauber *et al*, 1993), inadequate IL-2 production (but normal IL-2Rα expression) was found following antigenic stimulation and superantigen stimulation (Fischer *et al*, 1993a).

In contrast to studies using mitogens and antigens, anti-CD3 stimulated T cells from CVID patients were found to produce similar IL-2 levels to T cells from normal controls (Fischer *et al*, 1993b). The defect in IL-2 production was further dissected by Eisenstein *et al* (1993) who found that this is a property of CD4+ T cells, with accessory cells from CVID patients capable of supporting normal proliferation and IL-2 production. The CD4+ cells in CVID respond poorly to anti-CD2, staphylococcal enterotoxin B (SEB) or PHA, but with minimal impairment to anti-CD3. PMA and ionomycin, or anti-CD3 plus anti-CD28 resulted in normal IL-2 production. The addition of IL-2 to SAC-stimulated cells from CVID patients was shown to restore in vitro immunoglobulin production (Rump *et al*, 1990). PWM, a T cell stimulator, was only able to restore Ig synthesis in 1 of 5 patients.

Therapeutic intervention with subcutaneous human IL-2 was examined in a placebo-controlled double-blind trial (Rump *et al*, 1993). There were no significant side effects reported, but *in vivo* IgG synthesis was not stimulated, although a few patients had an *in vitro* increase in IgG and/or IgM production following PWM stimulation (which stimulates both B and T cells). There was however, a significant reduction in infections six months after (but not during) the study. Cunningham-Rundles *et al* (1994) reported on the use of subcutaneous IL-2 conjugated to polyethylene glycol (PEG/IL-2) in CVID and demonstrated an increase in antibody responses (but not total immunoglobulin levels). A larger study of PEG/IL-2, given weekly subcutaneously, demonstrated increased antibody to keyhole limpet haemocyanin (KLH) in 4/5 patients, and 1 subject had increased T cell proliferation and normal IL-2 production (Cunningham-Rundles *et al*, 1995).

IL-2 production from cultured PBMCs was reported as normal in 6/6 patients with CVID (Inoue *et al*, 1993).The authors concluded that B cell dysfunction was the cause of CVID, and that Th1 function was normal. But a different study confirmed low IL-2 and high IL-4 production, by CVID cells stimulated by PWM (Ferrer *et al*, 1995).

Typifying the problems in dissecting the abnormalities of CVID, using intracellular cytokine detection techniques instead of measuring production of IL-2 in culture supernatants, North *et al* (1996) demonstrated normal levels of IL-2 production post stimulation with PMA and ionomycin in CVID.

In summary, various studies have demonstrated inadequate production of IL-2 by T cells from CVID, depending on the stimulus used. However, intracellular techniques have shown that the production of IL-2 per T cell is normal in CVID, yet the number of $CD4^+$ cells is lower.

1.3.1.3.2 IL-3

Normal or raised levels of IL-3 expression, despite abnormally low IL-2 and IFN-γ expression, were found in CVID (Hauber *et al*, 1995).

1.3.1.3.3 IL-4

Elevated serum IL-4 levels were reported in 36% of CVID patients (n=25), and in none of the controls. Furthermore, 75% of those subjects with high IL-4 had high IL-6 as well (Aukrust *et al*, 1994a).

Production of IL-4 by CVID lymphocytes was noted to be deficient following PHA or ConA stimulation (Pastorelli *et al*, 1989). The response of B cells from CVID patients to IL-4 was examined and resulted in IgE production in only 2/8 cases (Pastorelli *et al*, 1990). One IL-4 responding patient also made adequate IL-4 on stimulation. IL-4 production was very low in 5 of the 6 remaining CVID patients, and these all had very low serum IgE levels, One patient with low IgE could make normal IL-4 responses on activation. IL-4 was shown to suppress IgG, IgM and particularly IgA production. T cell culture supernatants from CVID cells also suppressed Ig production in some, but not all, cases (Pastorelli, 1989).

IL-4 mRNA expression when CVID cells were stimulated with PHA was significantly decreased compared with levels expressed by T cells from normal controls (Warren & Strober, 1990).

Stimulation of B cells from patients with CVID using anti-CD40 plus IL-4 showed normal proliferation, and normal levels of IgE production (Nonoyama *et al*, 1993). The same group reported that CD40-ligand expression post-stimulation was lower than normal controls (Farrington *et al*, 1994; Ochs *et al*, 1994). The low levels of CD40L expression correlated with poor IL-2 production. The CD40-Ligand expression was noted to be low on CD4+ T cells, although the extent of the defect varied from patient to patient (Brugnoni *et al*, 1996). One study (Oliva *et al*, 1997) suggested that CD40-ligand expression may be normally expressed in up to two-thirds of CVID patients, but did not assess the functional activity of the protein. Exogenous IL-4 was able to restore Ig synthesis of CVID B cells treated with IL-10 and anti-CD40 (Punnonen *et al*, 1997).

In summary, despite one study claiming elevated serum levels of IL-4 in CVID, the consensus is that IL-4 production *in vitro* by CVID T cells is abnormally low.

1.3.1.3.4 IL-5

An additional 'Th2-like' cytokine, IL-5 has also been studied in CVID, and is discussed here for completeness. Unstimulated lymphocytes rarely make IL-5, as assessed by in situ hybridisation studies. Both PWM and other activators stimulate peripheral blood lymphocytes and spleen cells to produce IL-5 mRNA after 3 days of stimulus, with 1-2% of peripheral blood mononuclear cells becoming IL-5+. CVID patients (n=20) produced similar percentages of IL-5+ cells as normal controls (Smith *et al*, 1990).

Sneller & Strober (1990), in contrast, demonstrated lower levels of IL-5 mRNA expression than normal following stimulation of CVID PBMCs. Only four CVID patients were studied however.

These two studies may have differed in their sensitivity to detect IL-5 mRNA; a consensus is that CVID patients certainly do not make elevated amounts of IL-5, and may produce less than normal controls. Together with the IL-4 findings, this would fit with the clinical picture of CVID, in which eosinophilia and high IgE levels are not a feature.

1.3.1.3.5 IL-6

IL-6 is an important cytokine in the development of the acute phase response (Richards, 1998), and is discussed here for completeness. Studies of IL-6 showed 13/17 CVID patients had elevated serum levels 2-18 fold higher than in normals (Adelman *et al*, 1990) and higher spontaneous IL-6 production by cultured lymphocytes. Stimulated IL-6 production was equivalent to that seen with cells from normal controls. Increased in vitro IL-6 production by CVID lymphocytes was also noted by Pandolfi *et al* (1993a and 1993b), but the increased levels did not correlate with numbers of monocytes, lymphocytes, or lymphocyte subsets. Exogenous IL-6 was unable to increase the production of IgM by PWM-stimulated CVID cells.

In contrast, a study by von der Heyden-Rynsch *et al* (1993) confirmed increased IL-6 production in CVID, but found that it was proportional to increased monocyte numbers. They found that the IL-6 production was normal when corrected for monocyte numbers. They also found IL-6R expression to be normal (Junker *et al*, 1993).

Circulating IL-6 levels in CVID patients were shown to decrease following 32 weeks of therapy with 13-*cis* retinoic acid (Saxon *et al*, 1993). In vitro studies by Zhang *et al* (1997) suggested a better response (in terms of IgM and IgG production) to 9-cis retinal than either 13 -*cis* retinoic acid or all-*trans* retinoic acid.

In summary, IL-6 production is elevated in CVID, most probably because of an on going acute phase response. There does not appear to have been a comparison of IL-6 levels in CVID compared with XLA patients to control for a similar pattern of infections and therapy.

1.3.1.3.6 IL-9

IL-9 is a growth factor for activated T cells, and also increases the immunoglobulin production of stimulated B cells (reviewed in Renauld, 1998). Low levels of in-vitro IL-9 expression were found in CVID patients following stimulation with tetanus toxoid (Hauber *et al*, 1995). Adding exogenous IL-2 to the culture system resulted in restoration of IL-9 secretion (but not of IFN-γ secretion). IL-2 without antigen did not correct the IL-9 deficit.

1.3.1.3.7 IL-10

IL-10 is of interest in CVID, since it is involved in the inhibition of Th1 cytokines (de Waal Malefyt, 1998). IL-10 production by cultured lymphocytes was reported to be normal (Fritsch *et al*, 1994) in a study of 25 CVID patients. It was noted that some subjects did not make immunoglobulin on stimulation with PWM, despite the addition of IL-4.

Stimulation of B cells from patients with CVID with anti-CD40 plus IL-10 showed various patterns of immunoglobulin synthesis (Nonoyama *et al*, 1993). Further studies by Eisenstein *et al*, 1994) demonstrated that IL-10 plus anti-CD40 stimulation can result in eventual Ig synthesis, after seven days of culture, suggesting a B cell anergy problem.

IL-10 induced Ig production was significantly enhanced by the addition of IL-2 (Nonoyama *et al*, 1994). IL-10 was said to be more effective at restoration of Ig production than IL-2 in children with CVID (Zielen *et al*, 1994b). Punnonen *et al* (1997) were unable to make anti-CD40 treated CVID B cells produce IgG in the presence of IL-10, but noted that the addition of IL-4 to the system greatly enhanced Ig synthesis (in both CVID and normal controls).

Using anti-CD40 as a B cell stimulant, IL-10 alone or in combination with IL-2 or IL-4 was unable to restore IgG synthesis in 12/13 CVID patients (Oliva *et al*, 1997). These authors also found IL-10 production by CVID T cells to be normal with both anti-CD3 or anti-CD3 plus PMA as stimulants.

In summary, whilst IL-10 production does not appear to be deficient in CVID, attempts to increase immunoglobulin production by the addition of IL-10 (and/or other cytokines) *in vitro* have met with mixed success.

1.3.1.3.8 IL-12

Interleukin-12 is an important cytokine to consider in CVID, forming a link between the innate and adaptive immune systems, as IL-12 produced by monocytes increases the production of Th1 cytokines (Chizzonite *et al*, 1998). The proportion of monocytes positive for IL-12 following stimulation with LPS has been found to be significantly higher in CVID compared with expression in monocytes from normal controls (Cambronero, Sewell, *et al*, 2000).

1.3.1.3.9 TNF-α

The role of the pro-inflammatory cytokine TNF-α is important to consider in CVID, since TNF-α is important for granuloma formation, a clinical feature of CVID (discussed above). Serum TNF-α levels were found to be the same in CVID as in normals (North *et al*, 1991b) in a study which also demonstrated normal soluble CD4 in CVID, but elevated serum levels of soluble CD8, soluble CD25, and β-2 microglobulin. The extent of this abnormal elevation correlated with the degree of B cell dysfunction and severity of the disease. However, high TNF-α levels together with CMV antigen were found in a CVID patient and this was proposed as a cause of CMV reactivation in this disease (Docke *et al*, 1994). However, in a small series of eight CVID patients treated with IVIG,

elevated serum TNF-α levels were noted in only in the patients who had an adverse reaction to IVIG (Farber, 1994).

Raised serum TNF-α levels were confirmed in CVID (Aukrust *et al*, 1995), and were found to be significantly associated with glutathione depletion in CD4+ cells. Glutathione is important in the protection against intracellular oxidative stress, and exogenous glutathione was able to restore IL-2 expression or proliferation in these experiments (Aukrust *et al*, 1995).

Later, Aukrust *et al* showed not only high serum TNF-α levels in CVID, but also both types of soluble TNF-receptors (Aukrust *et al*, 1996). Both PBMCs and monocytes from CVID patients spontaneously secreted significantly higher than normal amounts of TNF-α, and lower amounts of TNF-α and soluble TNF-receptor (sTNF-R) following LPS stimulation. There was also a significantly higher TNF:TNF-R ratio in CVID serum and culture supernatants. This suggests persistent *in vivo* activation of the TNF system in CVID. Another marker of *in vivo* activation, plasma L-selectin, was found to be increased in CVID (Zhang *et al*, 1996). In addition, surface L-selectin expression was reduced, particularly on B cells and CD4+CD45RO+ cells. Normal levels of expression were found on other cell populations.

North *et al* (1996) demonstrated normal percentages of PBMC capable of making TNF-alpha following stimulation with PMA/ionomcyin in CVID, using intracellular cytokine flow cytometry.

In summary, although serum TNF-α levels in CVID may be elevated compared with TNF-α levels in normal control subjects, particularly in the presence of infection or adverse reaction to therapy, the proportion of lymphocytes capable of making TNF-α following stimulation is similar in CVID and normal controls.

1.3.1.3.10 IFN-γ

The investigation of IFN-γ regulation in CVID highlights the difficulties of interpreting cytokine levels when different techniques, such as culture supernatants and intracellular

methods, are used. Increased IFN-α and IFN-γ production in supernatants was first noted in CVID following PHA and/or ConA activation studies by Gronewold *et al* (1984). Studies by Matricardi *et al* (1984) showed normal IFN-α production in culture supernatants from CVID patients following *in vitro* activation with Newcastle disease virus. Increased IFN-γ levels in culture supernatants were also found in the same study in CVID (but reduced in hyper IgM syndrome, hyper IgE syndrome and ataxia telangectasia).

Stimulation of PBMCs with Sendai virus or PHA produced significantly higher levels of IFN-γ (and IFN-α) in CVID than in PBMCs from normal controls (Strannegard *et al*, 1987). Neither interferon was detectable in the serum of the patients, although antiviral activity of CVID serum was higher than normals, suggesting an increase in some IFN production. Beta-2 microglobulin levels, increased by IFN, were higher in CVID than normals. In contrast, examination of culture supernatants from four patients with CVID following stimulation with PHA *in vitro* showed poor IL-2 and low IFN-γ production (Paganelli *et al*, 1988). The IFN-γ secretion did not increase following the addition of recombinant IL-2.

Other studies have also found low levels of IFN-γ production by CVID cells into culture supernatants after stimulation with PHA or ConA (Pastorelli *et al*, 1989). This defect lasted in several patients for at least five months. There was defective production of other cytokines (IL-2 and IL-4) but proliferation was normal. The defect in the production of these cytokines was hypothesised to contribute to the deficient immunoglobulin production.

Similar cytokine defects were noted in 4 CVID patients (Sneller & Strober, 1990), who in addition to low IL-2, IL-4 and IL-5 mRNA expression, also noted low IFN-γ mRNA levels, and low IFN-γ protein levels. This defect was thought to be dependent on IL-2, since in the initial IL-2 independent phase of T cell activation, the IFN-γ mRNA level was normal. Also, the addition of exogenous IL-2 returned IFN-γ levels to normal.

Although low IFN-γ transcription was also noted in CVID following stimulation of T cells with super-antigens such as SEA (Fischer *et al*, 1993a), normal IFN-γ was produced

following anti-CD3 stimulation, suggesting an intrinsic TCR defect in CVID (Fischer *et al*, 1993b).

Further analysis of subgroups of CVID patients showed that those patients with low CD4 counts and high numbers of CD8+57+ cells made increased amounts of IFN-γ (Jaffe *et al*, 1993). These CD8+ cells were noted to have suppressive activity, reducing IgG (but not IgM) production from SAC+IL-2 stimulated B cells.

Since IFN-γ is known to induce IgG2 expression (Snapper & Paul, 1987), four patients with IgG2 deficiency were studied, and shown to have decreased mitogen-induced IFN-γ expression (Inoue *et al*, 1995). Exogenous IFN-γ resulted in restoration of IgG2 syntheses; in CVID however, exogenous IFN-γ did not result in IgG2 synthesis.

MxA protein, which is induced by interferons and inhibits viral replication (Ronni *et al*, 1993) which is raised in both virally and autoimmune induced interferon expression, was normal in a cohort of CVID patients (Rump *et al*, 1995), arguing against a viral or autoimmune aetiology for this condition.

Of the studies using intracellular cytokine techniques, significantly elevated IFN-γ production was noted at the single cell level following stimulation with PMA and ionomycin (North *et al*, 1996). Percentages of cells producing IL-2 or TNF-alpha were normal.

In summary, IFN-γ is not present in significant levels in the serum of CVID patients (or normal controls). There is no consensus regarding IFN-γ production by cultured CVID lymphocytes, with some studies showing higher, and other studies showing lower levels, although a variety of culture conditions were employed. In contrast, following *in vitro* stimulation the levels of intracellular IFN-γ, particularly within $CD8^+$ cells, is higher in CVID patients than in normal subjects.

1.3.2 X-linked agammaglobulinaemia

1.3.2.1 Clinical background

X-Linked agammaglobulinaemia (XLA) represents a similar antibody deficiency state to CVID, with a similar range of infections, and treated in the same way with IVIG, but without the inflammatory component that is present in CVID. For this reason, patients with XLA were included in appropriate experiments in this study as a control group.

XLA was the first primary antibody deficiency to be discovered (Bruton, 1952a & b), the mechanism for which was simultaneously identified by two groups to be a defect in a cytoplasmic tyrosine kinase, termed Bruton's tyrosine kinase, *Btk* (Vetrie *et al*, 1993; Tsukada *et al*, 1993). The clinical manifestations have been extensively described (for review see Smith & Witte, 1999). In brief, XLA is characterised by severe, recurrent extracellular bacterial infections mainly of the upper respiratory tract, but also including pneumonia, gastrointestinal infections, skin infections, meningitis and septicaemia. In addition there is a profound susceptibility to enteroviral infections which cause a meningoencephalitis, and sometimes a dermatomyositis/fasciitis syndrome. The laboratory findings consist of severely decreased B cell numbers and extremely low levels of serum immunoglobulins. This can result in the clinical absence of lymphoid tissues such as tonsils.

The block in B cell production occurs at the stage between terminal deoxynucleotidyl transferase (TdT^+) $C\mu^-$ pre pre B cells, and $C\mu^+$ pre B cells. *Btk* is a 659 amino acid, 77 kD protein with three domains; mutation analysis of many families with XLA has revealed that mutations are possible in almost any region of the protein (Vihinen *et al*, 1998). The exact function of *Btk* has not yet been established, but the evidence suggests that it is involved in multiple intracellular signalling pathways. In normal B cells, activation of the cell through surface IgM, IL-5R, CD38 or IL-6R leads to detectable phosphotyrosine on *Btk*, but this phosphorylation does not occur next to the specific receptor through which the activation signal has been given, suggesting that intermediate signals are also involved. Events preceeding *Btk* phosphorylation include activation of the *Src* family kinases: *Fyn, Blk, Hck* and *Lyn* which phosphorylate immunoreceptor tyrosine-based motifs (ITAMs) on the Igα and Igβ components of the B cell receptor. These in turn phosphorylate, and hence activate *Syk* (Roth & DeFrance, 1996). A key

role for *Btk* appears to be in the regulation of calcium fluxes subsequent to phospholipase C-γ (PLCγ) phosphorylation which *Btk* dependent (Takata & Kurosaki, 1996). As far as it is known, there is no intrinsic defect within XLA T cells.

1.3.2.2 Cytokines in XLA

There are only two studies which directly examine the role of cytokines in XLA. Aukrust *et al* (1994a) noted that unlike CVID, neither IL-4 nor IL-6 were elevated in seven XLA patients. An examination of cytokines in culture supernatants in five XLA patients showed similar levels in cell culture supernatants from normal controls in response to a variety of stimuli (Plebani *et al*, 1997).

1.3.3 Sarcoidosis

1.3.3.1 Clinical background

Sarcoidosis is a multi-system disorder of unknown aetiology, characterised by granuloma formation in affected organs. The clinical features have been extensively described (e.g. Muller-Quernheim, 1996) and are reviewed briefly here. The lung is the commonest involved organ, and sarcoidosis is the commonest lung interstitial lung disease of unknown aetiology. The prevalence rate ranges between 9 patients per 100 000 in Italy, to 64 per 100 000 in Sweden, with a marked predilection for African Americans. Patients usually present with non-specific constitutional complaints such as fever, malaise, anorexia or respiratory symptoms such as cough, exertional dyspnea or chest pain. The histopathology of lung lesions is characteristic, and consists of non-caseating granulomas within alveolar, bronchial and vascular walls. The lesions are usually scattered throughout the lung, and a range of progression of lesions is often seen from highly cellular lesions through to fibrosis and progressive hyalinisation. There are no pathognomonic criteria for the diagnosis of sarcoidosis. The disease naturally resolves in most patients, although there is an untreated mortality rate of around 5%. There is no specific treatment, although antiinflammatory therapy, in the form of corticosteroids, is usually used for lesions causing dysfunction of vital organs, or disabling or disfiguring lesions .

1.3.3.2 Cytokines in sarcoidosis

Cytokines, in combination with other extracellular signalling proteins, play a key role in the pathophysiology of sarcoidosis (Müller-Quernheim, 1996): regulating the expression of adhesion molecules on vascular endothelium, activating inflammatory cells, and modulating the local survival and proliferation of different types of immune cells in the sarcoid granuloma (Agostini *et al*, 1998). The granuloma in sarcoidosis consists of a central cluster of mononuclear phagocytes surrounded mainly by $CD4^+$ T cells, but also some $CD8^+$ T cells and B cells (Agostini *et al*, 1997). This accumulation of $CD4^+$ T cells can result in a circulating peripheral CD4 lymphopoenia, with accumulation of memory T cells in the lymph nodes, lung, spleen, conjunctivae and skin. Macrophage-derived cytokines (IL-1, IL-15, TNF-alpha) and chemokines such as IP-10, and RANTES are thought to provide both chemotactic gradients for T cells and proliferation signals for T cells and macrophages (as reviewed in Agostini *et al*, 1997).

There is evidence that a Th1-like cytokine profile predominates during the establishment of hypersensitivity granulomas (Kunkel *et al*, 1996). Cytokine profiles biased towards the Th2 pattern result in impaired granuloma formation and reduced resistance to intracellular pathogens, although in murine schistosomiasis granulomatous inflammation can be IL-4 driven (Wynn *et al*, 1993). In patients with active sarcoidosis there is evidence of IL-12 production in bronchioalveolar fluid specimens, which biases the cytokine profile towards Th1 (Moller *et al*, 1996), and this bias has been confirmed by others (reviewed in Moller, 1999) and suggests that a positive-feedback circuit involving IL-12 and IFN-γ becomes established in chronic sarcoidosis. There is speculation that this cycle becomes down-regulated during remissions by TGF-beta, but that if down-regulation fails to occur the TGF-beta can result in fibrosis.

1.3.3.3 Gamma delta T cells in sarcoidosis

T cells expressing the γ/δ T cell receptor (TCR) constitute 10-30% of T cells in the granuloma resulting from murine schistosomiasis , and accumulations of γ/δ T cells have also been found following infections with *Mycobacterium tuberculosis* and *Listeria monocytogenes* (O'Brien *et al*, 1989). In active human sarcoidosis, expansions of TCR $V\delta1^+$ γ/δ T cells were found in the lung (Forrester *et al*, 1993). Interestingly, the same study

found an abnormally high percentage of γ/δT cells (approx. 20-40%) in the blood of some patients with sarcoidosis, but this was secondary to a decrease in TCRα/β bearing T cells. A separate study failed to demonstrate increases in circulating γ/δ cells in the peripheral blood of 21 patients with sarcoidosis (Wilsher *et al*, 1995), and did not find increases in γ/δ cells in either the broncheoalveolar lavage fluid or transbronchial biopsies of patients with sarcoidosis (Wilsher *et al*, 1996). Interestingly, studies of murine γ/δ T cells have shown that depending on the antigen driving the response, peritoneal γ/δT cells can have biased cytokine production like α/βT cells, and produce predominantly IL-4 (with *Nippostrongylus sp*) or IFN-γ (with *Listeria sp*) (Ferrick *et al*, 1995).

1.3.4 Hyper IgE syndrome

Since elevated serum IgE is the result of a Th2-biased immune system, the present study was fortunate to be provided with an example of a putatively Th-2 biased disease in the form of a patient with hyper IgE syndrome (HIGES).

1.3.4.1 Clinical background

The clinical and laboratory features of hyper IgE syndrome (HIGES) have been extensively documented (e.g. Grimbacher *et al*, 1999) and are briefly reviewed here. HIGES (previously known as Job's Syndrome) is characterised by extremely high IgE levels (>20 000 kU/ml), recurrent staphylococcal skin and tissue infections, coarse facies, blood and tissue eosinophilia and intermittent chemotactic defects. HIGES was first described by Rebecca Buckley in 1972, although Job's syndrome of recurrent severe cold staphylococcal abscesses in red-headed girls was reported by Davis in 1966. Both sexes are affected, sometimes with an incomplete autosomal dominant pattern of inheritance in the few familial cases described (Grimbacher *et al*, 1999).

Early onset cutaneous and deep-seated staphylococcal infections, mastoiditis, pneumonia and subsequent pneumatocoele formation, bone joint and visceral infections are less common. The skin looks superficially like eczema, but with less itching and typical. Despite very high IgE levels, classical allergic features are rare. Some patients have osteopenia with recurrent fractures. Recurrent infections occur, predominantly coagulase

positive *staphylococcus* sp, but also *Candida albicans*, *Haemophilus influenzae*, group A *Streptococcus* sp, *Aspergillus* sp and *Trichophyton*, as described by Grimbacher *et al* (1999).

Laboratory abnormalities in HIGES have been extensively reviewed (e.g. Buckley, 1996a & b). As well as the high IgE levels, serum IgD can also be increased, but IgG, IgM and IgA levels are usually normal. There are decreased antibody and T cell responses to pathogens. T cell CD4 and CD8 levels are usually normal, but with lower $CD45RO^+$ cells. B cells from HIGES patients make less IgE in response to IL-4 than normal subjects, suggesting that they have already been excessively stimulated by IL-4 (Classen *et al*, 1991).

1.3.4.2 Cytokines in hyper IgE syndrome

Del Prete *et al* (1989) reported defective IFN-γ and TNF-α production by T cells from four patients with HIGES stimulated by mitogens *in vitro*, and a clonal analysis revealed fewer T cells able to make these Th1 cytokines but no increase in IL-4 precursors. Paganelli (1991) reported markedly low or undetectable IFN-γ in lymphocyte culture supernatants from 5 patients stimulated with PMA and ionomycin. Two studies failed to find deficient IFN-γ production in 18 patients (Buckley, 1996a). Since elevated IL-4 leads to increased IgE production (Bergstedt-Lindqvist *et al*, 1988), and since IFN-γ can inhibit IL-4-induced IgE synthesis in animals (Coffman & Carty, 1986) and humans (Pene *et al*, 1988) IFN-γ has been proposed as therapy for HIGES. King *et al* (1989) gave IFN-γ to 5 patients in doses of 0.05-0.1 mg/m^2 three times weekly for 2 and 6 weeks respectively. At the higher dose, there was a decline in serum IgE in 2/5 subjects. Although there were no adverse effects reported, there was no change in the clinical status of the patients.

1.3.4.3 High-dose IVIG in hyper IgE syndrome

Small numbers of case reports of successful treatment of HIGES with high-dose IVIG (2 g/kg) have been reported. Kimata (1995) reported three patients (two of whom also had Kawasaki disease) successfully managed by a single course of IVIG (400 mg/kg for five days) resulting in reduced serum IgE levels and reduced IgE synthesis by B cells in culture, and near-clearance of skin lesions within 28 days (although no skin scores were reported). In contrast, Wakim and colleagues (1998) included a patient with HIGES in a trial of high-dose IVIG for atopic eczema. There was no significant reduction in serum IgE level (although a downward trend was seen) and no change in skin score.

1.3.5 Atopic eczema

Another immunoregulatory disorder, often described as Th2 biased, is atopic eczema. Blood samples from patients with atopic eczema receiving experimental high-dose intravenous immunoglobulin therapy were available in this study, hence atopic eczema is discussed below.

1.3.5.1 Clinical background

Atopic eczema is a chronic inflammatory skin disease, with the primary manifestations of pruritus, skin lesions in flexures and lichenification (in adults), a chronic relapsing course, and usually either a personal or family history of atopic eczema or respiratory allergy (Holden & Parish, 1998). Several inflammatory skin lesions are seen: acute lesions are very itchy, with papules on erythematous skin, and usually excoriations with erosions and serous exudate. Subacute lesions consist of erythematous scaling papules. As lesions become chronic the skin becomes thickened, lichenified and covered in fibrotic papules. The skin is usually dry, although the distribution of the lesions varies with age (face, scalp and extensor surfaces in infancy, with more lesions in flexural areas in adulthood).

Patients with atopic eczema have elevated serum IgE levels in 80-85% of patients. Approximately 85% have high specific IgE levels to inhalant or food allergens (Jones & Sampson, 1993). For unknown reasons, patients with specific IgE production by radioallergosorbent (RAST) test or skin prick test may not have clinical sensitivity to that allergen, indeed test positivity may outlast the duration of the disease (May, 1976) which is suggestive that the disease is not exclusively mediated by simple IgE-dependent mast cell degranulation.

Patients with atopic eczema have an increased incidence of viral, fungal and bacterial superficial skin infections. In over 90% of cases these are due to *Staphylococcus aureus.* Clinical observation suggests that treatment with anti-staphylococcal antibiotics can result in marked skin improvement, even when there is no overt staphylococcal skin infection. This has been attributed to to the presence of staphylococcal superantigens activating T cells and macrophages (Leung, 1995a & b). Interestingly, patients with atopic eczema produce IgE against staphylococcal superantigens, particularly staphylococcal enterotoxins A and B (SEA, SEB) and toxic shock syndrome toxin-1 (TSST-1), which

may explain how local staphylococcal antigen production may induce IgE-mediated histamine release (Leung *et al*, 1993).

1.3.5.2 Cytokines in atopic eczema

Several lines of investigation have suggested that atopic eczema is the result of an immunoregulatory defect. Allergen-specific T cells from patients with atopic eczema (from both peripheral blood and skin lesions) produce increased amounts of IL-4 and IL-5, and reduced amounts of interferon-γ (Leung, 1997). Not only are the T cells Th2-biased, but monocytes from eczema patients have elevated levels of cAMP phosphodiesterase and secrete increased amounts of IL-10 (Ohman *et al*, 1995) and prostaglandin E2 (Chan *et al*, 1993). These inhibit interferon-γ production and also polarise the cytokine response towards a Th2 pattern.

An understanding of the immunoregulatory abnormality in atopic eczema requires an appreciation of the histological appearances of the skin. Even in non-lesional skin, there are histological abnormalities consisting of mild hyperkeratosis, epidermal hyperplasia and a sparse (mainly T cell) cellular infiltrate of the dermis. In acute lesions there is intracellular oedema with a sparse cellular infiltrate of T cells. In the dermis, the infiltrate is perivenular, and contains T cells and some monocytes but not usually eosinophils, basophils nor neutrophils. As the lesions become chronic, the epidermis becomes hyperplastic and hyperkeratotic, with increased numbers of macrophages, mast cells and eosinophils (Leung, 1997).

At the cellular level, cytokine expression (as determined by *in situ* hybridisation of cytokine mRNA) is abnormal in non-lesional skin with over-expression of IL-4, but interestingly not IL-5 or interferon-γ (Hamid *et al*, 1994). Chronic, as opposed to acute, atopic eczema lesions contain significantly fewer cells containing IL-4 mRNA, but more cells expressing IL-5 mRNA. Eosinophils in chronic lesions also over-express IL-5, IL-10 and GM-CSF mRNA (Hamid *et al*, 1994).

Any similarity of chronic atopic eczema skin lesions to delayed-type hypersensitivity (DTH) reactions is superficial, in that tuberculin skin reactions are Th1-like with IFN-γ

production and increased HLA-DR expression on keratinocytes (Tsicopoulous *et al*, 1992). Yet keratinocytes from atopic dermatitis lesions are not HLA-DR positive (Tsicopoulous *et al*, 1992). Further evidence against a DTH-like picture comes from reports of increased IL-4 and IL-5, and reduced IFN-γ in lesional skin (Hamid *et al*, 1994). In addition, IL-4 itself can induce skin inflammation. Other cells apart from T lymphocytes may contribute to the inflammatory changes in eczema. IgE binds to the low affinity IgE-receptor, CD23 on monocytes; CD23 expression is itself induced by IL-4. Ligation of CD23 by allergen-specific IgE can result in monocytes producing leukotrienes, platelet activating factor, IL-1 and TNF-α. Langerhans cells also express CD23 and the high-affinity IgE receptor ($Fc^{\varepsilon}R$). These $Fc^{\varepsilon}R$-bearing Langerhans cells may play an important role in eczema pathogenesis since they are 1000-fold more efficient at presenting allergen to T cells than Langerhans cells without allergen-specific IgE (Mudde *et al*, 1990). In summary, cell infiltrates in eczema lesions are likely to be the result of both the products of IgE-dependent mast cell degranulation and Th2-like cell mediated responses.

Having developed allergen-specific T lymphocyte responses, these T cells must infiltrate the skin to cause local inflammation. This tissue-selective homing of T cells to skin is mediated by differentially expressed homing receptors and endothelial cell ligands (Picker & Butcher, 1992). One of the homing receptors relevant to skin homing in humans is the cutaneous lymphoid antigen (CLA): memory T cells in eczema lesions express CLA, in contrast to T cells in the lungs of asthmatics, which do not (Berg *et al*, 1991). At the antigen-specific level, T cells specific for casein in milk-induced eczema express higher levels of CLA compared with T cells of irrelevant specificity or T cells from non-atopic control subjects (Abernathy-Carver *et al*, 1995). Interestingly, expression levels of another homing receptor, L-selectin, were not significantly different. Another study compared T cells from patients with house-dust mite sensitive asthma with T cells from patients with house-dust mite sensitive eczema: only the CLA+ T cells proliferated to *Der p* I (the major dust mite allergen) in eczema patients, and there was a preponderance of CLA- cell proliferation in the asthmatics (Babi *et al*, 1995). In the atopic eczema patients, the CLA+ cells demonstrated spontaneous IL-4 (but not interferon-γ) production, emphasising the importance of Th2-type cytokines in the pathogenesis of atopic eczema.

1.3.5.3 High-dose IVIG in atopic eczema

In atopic eczema refractory to steroid-therapy, various treatments have been tried. High-dose intravenous immunoglobulin (hdIVIG) at 2 g/kg has been attempted in a few cases (Kimata, 1994; Gelfand *et al*, 1996) with some reported benefits, particularly with coexisting idiopathic thrombocytopenic purpura or Kawasaki disease, with effects lasting up to six months post treatment. A small open study did not reduce serum IgE levels despite an improvement in the skin score (Gelfand *et al*, 1996). Despite a small trial showing a small improvement in skin lesions following hdIVIG (Pons-Guiraud, 1986), there is insufficient evidence to enable a firm recommendation for hdIVIG to be standard therapy, even for refractory atopic eczema (Jolles *et al*, 1998a).

1.4 Immunomodulatory agents

1.4.1 Intravenous immunoglobulin

One of the primary aims of this study was to examine the immunomodulatory effects of IVIG on intracellular cytokine expression in immunoregulatory disorders. The prime example is of IVIG used to treat patients with CVID, hence this was studied in detail.

1.4.1.1 Background

Intravenous immunoglobulin (IVIG) therapy, is now the treatment of choice for patients with antibody deficiencies. This replacement dose IVIG is typically given at doses of 200 - 400 mg/kg every 3-4 weeks.

1.4.1.2 Source and production methods

Intravenous immunoglobulin (IVIG) is the pooled product of thousands of donations of plasma. A committee of the World Health Organisation established criteria for commercial IVIG production in 1982 (WHO, 1982) establishing that at least 1000 donors are used per batch, but in commercial practice 5000 - 20 000 donations are used. The methods of production of IVIG have been extensively reviewed (Lundblad & Schroeder, 1992) but are outlined briefly below. IgG is initially isolated from plasma by the Cohn alcohol fractionation process, with modifications by Oncley and later Kistler & Nitschmann. This process uses cold ethanol and low ionic concentrations combined with

low pH to isolate IgG. Cohn Fraction II contains 95-99% IgG, but also small amounts of IgA, IgM, IgD and IgE along with other proteins. The IgG also tends to aggregate which can produce severe reactions when infused into patients. Numerous different processes are then used to further purify monomeric IgG including: low pH combined with trace pepsin treatment, polyethylene glycol precipitation, DEAE-Sephadex adsorption and silica gel adsorption. Techniques such as pasturisation, nanofiltration or solvent/detergent treatment are additional steps used to remove enveloped viruses. Stabilising agents such as glycine or sugars such as maltose or sucrose are also added to prevent reaggregation of the IgG during lyophilisation and storage.

1.4.1.3 Mechanisms of action

The mechanisms of action of immunomodulatory doses of IVIG have not been clearly established, but can be divided into four main groups (Kaveri, 1994) which are now discussed in turn.

1.4.1.3.1 Fc dependent mechanisms

IVIG can block Fc receptors and exert an effect. There are four main lines of evidence for this: firstly, IVIG reduces the clearance of autologous erythrocytes coated with anti-D (Fehr *et al*, 1982). Secondly, IVIG treatment of patients with ITP impairs the ability of monocytes to form rosettes with IgG-coated erythrocytes (Kimberly *et al*, 1984). Thirdly, the *in* vivo effects of IVIG in ITP can be simulated by using antibodies to FcγRIII (Clarkson *et al*, 1986). Fourthly, anti-D immunoglobulin can increase platelet counts in patients with ITP who are RhD positive (Salama *et al*, 1983). The beneficial effects of IVIG in immune cytopoenias are likely to be due to the saturation of Fc receptors on splenic macrophages.

IVIG can also bind to other immunologically active compounds, for example C3b and C4b, reducing the formation of immune complexes (Basta *et al*, 1989).

1.4.1.3.2 V region-dependent mechanisms

Alteration of the patient's idiotypic network by anti-idiotypic antibodies in IVIG is also an important mode of action. This was first demonstrated by the fall in anti-factor VIII titre in a patient with anti-factor VIII autoimmune disease treated with IVIG (Sultan *et al*, 1984). It is now known that the $F(ab')_2$ blocks antibody/antigen binding, that $F(ab')_2$ derived from IVIG and bound to a column will remove autoantibodies (Rossi *et al*, 1989), and that IVIG itself bound to a column will remove antibodies (Ronda *et al*, 1994). IVIG does not, however, contain antibodies against common immunoglobulin allotypes (Rossi *et al*, 1988). Interestingly, there are parallels in disease states, when antibodies to autoantibodies are seen in a number of autoimmune diseases during the recovery phase. These examples include: myasthenia gravis (Dwyer *et al*, 1983), Guillian-Barré syndrome (van Doorn *et al*, 1990), ANCA-positive vasculitis (Rossi, 1991), and SLE (Zouali *et al*, 1983).

1.4.1.3.3 Interaction of IVIG with T cells

IVIG contains antibodies against the beta-chain of the T cell receptor (Marchalonis *et al*, 1992), and also against CD5 and CD4 (Vassilev *et al*, 1993; Hurez *et al*, 1994). There are various reports of inhibition of T cell proliferation by IVIG that are outlined below.

1.4.1.3.4 Effects *in vitro*

Various workers have shown that IVIG has immunosuppressive actions. Kawada & Terasaki (1987) demonstrated the effects of IVIG *in vitro*, using cells separated from healthy donors, and intact and Fc preparations of IVIG from 5-20 mg/ml in a 4-day PHA-stimulated culture system. There was a considerable reduction of T cell proliferation when IVIG was added during the culture period. When IVIG was added before the stimulation with PHA, there was no inhibitory effect. In contrast, pre-

treatment with IVIG for 24 hours reduced NK and ADCC activity. These effects were also reported when Fc fragments were used instead of intact Ig, suggesting that the Fc component plays a major role in the immunosuppression.

Similar results were obtained by Klaesson *et al* (1993), who demonstrated a significant reduction by IVIG of mitogenic responses to PHA, concanvalin A and pokeweed mitogen. In contrast to Kawada & Terasaki's work (1987) these effects were greater when IVIG was added more than 12 hours before PHA stimulation, and the effect was dose-dependent. Single-donor preparations of IVIG inhibited proliferation more than commercial multiple-donor preparations, but there was no difference between standard IVIG preparations and cytomegalovirus (CMV) hyper Ig. In contrast to Kawada's studies, intact Ig, and $F(ab')_2$ did not have significantly different effects, but Fc fragments did not inhibit proliferation in these experiments (Klaesson *et al*, 1993).

The inhibitory effects of IVIG on mitogen-induced proliferation was demonstrated *in vitro* by van Schaik *et al* (1992), who showed that both antigen-dependent and -independent responses were inhibited by IVIG in a dose-dependent manner. The range of IVIG used *in vitro* was up to 50 mg/ml, i.e. at the upper end of the high-dose IVIG (2 g/kg) achievable therapeutic range *in vivo*.

Amran *et al*, (1994) showed that IVIG could inhibit T cell proliferation induced by anti-CD3 or tetanus toxoid, but not that induced by PMA & ionomycin over an IVIG concentration range of 0-10 mg/ml.

IVIG was shown to arrest cells at the G_0-/G_1 phase and inhibited cells from entering S-phase (van Schaik *et al*, 1994). This effect was not due to cytotoxicity, since removal of the IVIG by washing and subsequent culture of the cells in fresh medium resulted in the recovery of normal proliferation.

1.4.1.3.5 Effects *in vivo*

Both pooled normal human immunoglobulin, and single donor immunoglobulin were shown to reduce PWM-induced plaque-forming cell (PFC) formation following 300

mg/kg infusion into patients with CVID (Stohl *et al*, 1986). This effect was short-lived, and sera collected ≥24 hours post-IVIG infusion no longer inhibited cell proliferation.

The suppressive effects *in vivo* effects of IVIG when used at low dose (100 - 200 mg/kg) were demonstrated in antibody-deficient children (Ballow *et al*, 1989). In this study, the effects of the children's sera on the immunoglobulin-producing activity of PWM-stimulated normal lymphocytes was assessed. Even low-dose IVIG was shown to enhance the suppressive activity of the patient's lymphocytes, an effect that was reversed on cessation of IVIG therapy.

Although the IgG within IVIG preparations plays a role in its suppressive effect on mitogen-induced cell proliferation, there are also the effects of the stabilising agents in the commercial preparations to consider. Alder *et al*, 1996) reported that both maltose and sucrose, at concentrations present in commercial IVIG preparations, can inhibit PHA, and to a lesser extent, PMA-induced proliferative responses *in vitro*. Maltose, but not sucrose, was able to inhibit the anti-CD3-induced response.

1.4.1.3.6 Modulation of the release and function of cytokines by IVIG

The reported effects of IVIG on various cytokines have been confusing, since various technologies have been employed, with increasing degrees of sophistication in recent years.

1.4.1.3.7 The *in vitro* effects of IVIG on cytokines

IL-1 production by monocytes is reduced *in vitro* by IVIG, yet requires an intact Fc region of the immunoglobulin, which suggests an important role for the macrophage Fc receptor in down-regulating monocyte IL-1 production (Iwata, 1987). These effects were confirmed *in vivo* by the successful inhibition of fever in rabbits treated with LPS and IVIG, compared with rabbits treated with LPS and pespsin-treated IgG.

The *in vitro* effects of IVIG on monokine production were studied at the single-cell level using indirect immunofluorescence microscopy (Andersson & Andersson, 1990) and showed that IVIG significantly decreased IL-6 but not TNF-α production by monocytes stimulated with either LPS or *Borrelia burgdorferi* spirochaetes.

IVIG was cultured with PMBC from normal volunteers and shown to selectively increase gene transcription and secretion of IL-1 antagonist (IL-1ra) and IL-8 (Ruiz de Souza *et al*, 1995). It was demonstrated that both Fc and F(ab')2 regions were required for this effect. On the other hand, expression of IL-1α, IL-1β, TNF-α and IL-6 by monocytes was not affected by co-culture with IVIG. Interestingly, LPS stimulation in the presence of IVIG resulted in more IL-8 secretion and reduced IL-1ra production, suggesting IVIG and LPS stimulation pathways in monocytes are distinct.

TNF-α and IL-1α production by stimulated PMBCs from healthy individuals *in vitro* was inhibited in a dose-dependent manner by both IVIG and IgG obtained from cancer patients (Abe *et al*, 1989). This effect was seen with intact Ig, to a lesser extent with Fc fragments, and not at all with $F(ab')_2$ fragments. The IVIG was shown to inhibit stimulation by PHA, LPS, ConA and IL-2, but not PMA.

IgG, either surface-bound or in solution, can bind to monocytes via FcR and results in enhanced TNF-α and IL-1 production by monocytes (Kuhnert *et al*, 1990). Intact IgG was required, since $F(ab')_2$ did not show this activity. In contrast, lymphocytes (identified only by their non-adherence to plastic) in these experiments did not produce TNF-α following exposure to IVIG.

Assessment of cytokine production by stimulated PBMCs was done following co-culture with IVIG by Andersson's group (1993, 1994). Following stimulation with either anti-CD3, or PMA and ionomycin, PBMCs were examined for intracellular cytokine production using indirect immunofluorescent microscopy. IVIG at a concentration of 6 mg/ml resulted in a delay in cell proliferation with either mitogen. Cytokine production following PMA and ionomycin was unchanged compared with non-IVIG treated cells for IL-2, IL-10, IFN-γ and TNF-α. There was a reduction in the number of cells producing IL-3, IL-4, IL-5, TNF-β, and GM-CSF until approximately 96 hours of

culture with IVIG and stimulant, when generally more of these cytokines was produced. No difference was demonstrated between the response of Th1-like and Th2-like cells. When anti-CD3 was used to activate the cells, IVIG resulted in less IL-2, IL-10, IFN-γ, TNF-α, and TNF-β; IL-8 expression was unchanged. IL-2 receptor expression was down-regulated by IVIG. It is important to point out that the method used by these authors (fluorescence microscopy) only distinguished lymphocytes - not individual lymphocyte subsets, although this is now possible by flow cytometric determination of cytokine production, and was done in the experiments presented in this thesis.

IVIG inhibited secretion IL-2 and IL-4 from PMA and ionomycin stimulated lymphocytes in a dose-dependent manner (Amran *et al*, 1994). The addition of exogenous IL-2 or IL-4 could overcome the inhibitory effects of IVIG on either anti-CD3 or PMA and ionomycin-induced T cell proliferation.

The effect of IVIG on the production of cytokines was also studied at the single-cell level following stimulation with streptococcal pyrogenic exotoxin-A (Skansén-Saphir *et al*, 1993). IVIG resulted in less IFN-γ and TNF-β expression, even after its addition to the culture 24 hours after streptococcal pyrogenic exotoxin-A (SPE-A) stimulation.

1.4.1.3.8 The *in vivo* effects of IVIG on cytokines

In a trial of IVIG in recurrent epilepsy (Ling *et al*, 1993) IVIG was given at 600 mg/kg every four weeks to 18 patients with intractable epilepsy. Plasma levels of IFN-γ, IL-2, IL-4 and IL-6 were measured before and 20 minutes after each IVIG treatment. No change in cytokines in response to IVIG at months 1, 3 or 6 was noted. However, there was a statistically significant increase in both plasma IL-6 and IFN-γ following each infusion. In a few patients, kinetic studies were performed and demonstrated peaks in IFN-γ at various times up to three days post infusion, with a slower peak in IL-6 production. An indirect immunofluorescence microscopy technique was used to identify the cells producing IFN-γ, using the technique of Andersson *et al* (1988). This could only identify that both NK cells and lymphocytes were involved in IFN-γ production. Assessment of Fc receptor expression demonstrated an increase in high-affinity IgG

receptor (CD64) on monocytes following IVIG, and a slight increase in the low-affinity Fc receptor (CD23). There was no increase in activation markers (CD23, CD25, HLA-DR) on T cells, monocytes or B cells following IVIG. These results were interpreted as IVIG causing IFN-γ to be released from lymphocytes and NK cells, which subsequently activated monocytes and resulted in the release of IL-6. As well as in epilepsy, these authors claimed (but did not show the data) similar effects in a series of hypogammaglobulinaemic patients.

The *in vivo* effects of IVIG on cytokine production were also studied by Aukrust *et al* (1994), who investigated the effects of a single dose of IVIG of 400 mg/kg on hypogammaglobulinaemic patients. Plasma levels of cytokines compared before and 1, 3, 20 and 44 hours post infusion showed a significant and rapid increase in plasma TNF-α as well as IL-6 and IL-8. There was an increase in soluble TNF-receptor, and a considerable increase (1000-fold molar excess) in IL-1 receptor antagonist levels.

TNF-α levels in the plasma of IVIG-treated antibody deficient patients were noted to be raised following adverse reactions to IVIG (Farber *et al*, 1994). The TNF-α level actually increased during the infusion itself, and was not abnormally elevated prior to treatment.

1.4.1.3.9 IVIG itself may contain cytokines

The cytokine content of commercial preparations of IVIG has been investigated. In van Schaik's study (1994), various cytokines from cell culture supernatants were used in an attempt to reverse IVIG-mediated suppression of proliferation, to no effect. Since there are similarities in effects between IVIG and transforming growth factor beta (TGF-β), it was conjectured that IVIG may contain TGF-β, but TGF-β neutralising antibodies had no effect on the anti-proliferative effect. Kekow *et al* (1998) examined the cytokine content of several batches of three commercial IVIG preparations and found no evidence of IL-6, IL-10 or TNF-α in any preparation. However, and in contrast to van Schaik's work, significant quantities of TGF-β1 and TGF-β2 (≥10 ng/ml) (but not TGF-β3) were found in all specimens. When these preparations of IVIG were given to

patients, their plasma TGF-β levels rose, along with TNF-α levels as previously demonstrated. The TGF-β was not bound to IgG in the IVIG preparations studied.

1.4.1.3.10 Anti-cytokine antibodies in IVIG

Anti-cytokine properties of IVIG preparations have also been studied (Abe *et al*, 1994). Since IVIG is derived from human donors, the presence of antibodies to cytokines in normal sera may also be present within IVIG preparations. Antibodies to IL-1α and TNF have been demonstrated in the sera of healthy individuals (Abe, 1988). Anti-IL-2 antibodies have been reported in autoimmune NZB, but not BALB/c mice (Tabata *et al*, 1986). Anti-IL-8 antibodies have been reported in connective tissue disorders and in normal volunteers (Reitamo *et al*, 1993). Anti-IFN-γ antibodies in IVIG have been shown to inhibit IFN-γ production *in vitro* (Toungouz *et al*, 1995) and such anti-IFN-γ antibodies were present at higher concentrations in IVIG preparations high in specific antibodies to hepatitis B and cytomegalovirus (Denys *et al*, 1997).

1.4.1.4 Replacement dose IVIG

Early attempts at using IVIG were abandoned because of the severe side effects caused by aggregates and fragments of IgG in the early IVIG preparations, and the first antibody deficiency patients were treated with intramuscular immunoglobulin. In the early 1980s newer preparatory techniques permitted the administration of IVIG to patients with primary antibody deficiencies without a high incidence of side effects, but with higher serum levels of IgG being achieved, and a reduction in the frequency and severity of infections compared with intramuscular administration. There is still no satisfactory evidence upon which to base a definitive schedule of dose or administration frequency for all patients (Yap, 1992), but in primary antibody deficiencies a replacement IVIG dose of 200-400 mg/kg, given approximately every three weeks is usual. This regimen is used in the large clinic of Dr David Webster at the Royal Free Hospital, from which samples used for this thesis were obtained.

1.4.1.5 High-dose IVIG

High-dose IVIG therapy at 1-2 g/kg is now used as an immunomodulating agent in an increasing number of disorders. Initial use of hdIVIG was for idiopathic thrombocytopenic purpura (ITP) in children (Imbach *et al*, 1981). Other disorders managed with hdIVIG include: autoimmune neutropenia (Pollack *et al*, 1982; Bussel *et al*, 1983;Ariizumi *et al*, 1983), various epilepsies (Pechadre *et al*, 1977; Ariizumi *et al*, 1983; Sandstedt *et al*, 1984), Kawasaki disease (Furusho *et al*, 1984), various neuropathies, and systemic vasculitis amongst others.

1.4.2 Ciprofloxacin

In addition to IVIG, the effects on intracellular cytokines of a second immunomodulatory drug, ciprofloxacin, was investigated. Ciprofloxacin is a fluoroquinolone antibiotic introduced in the 1980s; it has a broad spectrum of activity and is indicated for the treatment of bone, joint and systemic infections, and infections of the genital, gastrointestinal and respiratory tracts (Hooper & Wolfson, 1991). It is active against gram-negative bacilli, less active against gram-positive cocci and inactive against anaerobes (Hooper, 1998). It is of potential interest in an immunological setting, since there is some evidence that it has immunomodulatory properties as well as antimicrobial actions, where it functions as a bacterial DNA gyrase inhibitor. Shalit (1991) reviewed the immunological actions of ciprofloxacin as determined by *in vitro* studies. Whilst there was no direct effect on lymphocyte proliferation, phagocytic ability, immunoglobulin production or IFN-γ production, ciprofloxacin enhanced lymphocyte IL-2 secretion by PHA-stimulated lymphocytes. At high concentrations (>50 μg/ml) there was inhibition of IL-1 and TNF-α production by lymphocytes. However, *in vivo* studies have demonstrated an increase, due to ciprofloxacin, in monocyte TNF-α, IL-1 and IL-6 production following *ex vivo* stimulation with LPS (Bailly *et al*, 1991).

Ciprofloxacin has been found to increase IL-2 (and to a lesser extent IFN-γ) production by PHA-stimulated lymphocytes in culture supernatants (Riesbeck & Forsgren, 1990), and to increase IL-2 mRNA in ciprofloxacin-treated lymphocytes (Riesbeck *et al*, 1994a). The cytokine enhancing effect of ciprofloxacin was not inhibited by cyclosporin A (Riesbeck *et al*, 1994b), and similar effects were seen with other fluoroquinolones

(Riesbeck, 1994) including experimental cytotoxic fluoroquinolone (Riesbeck & Forsgren, 1995).

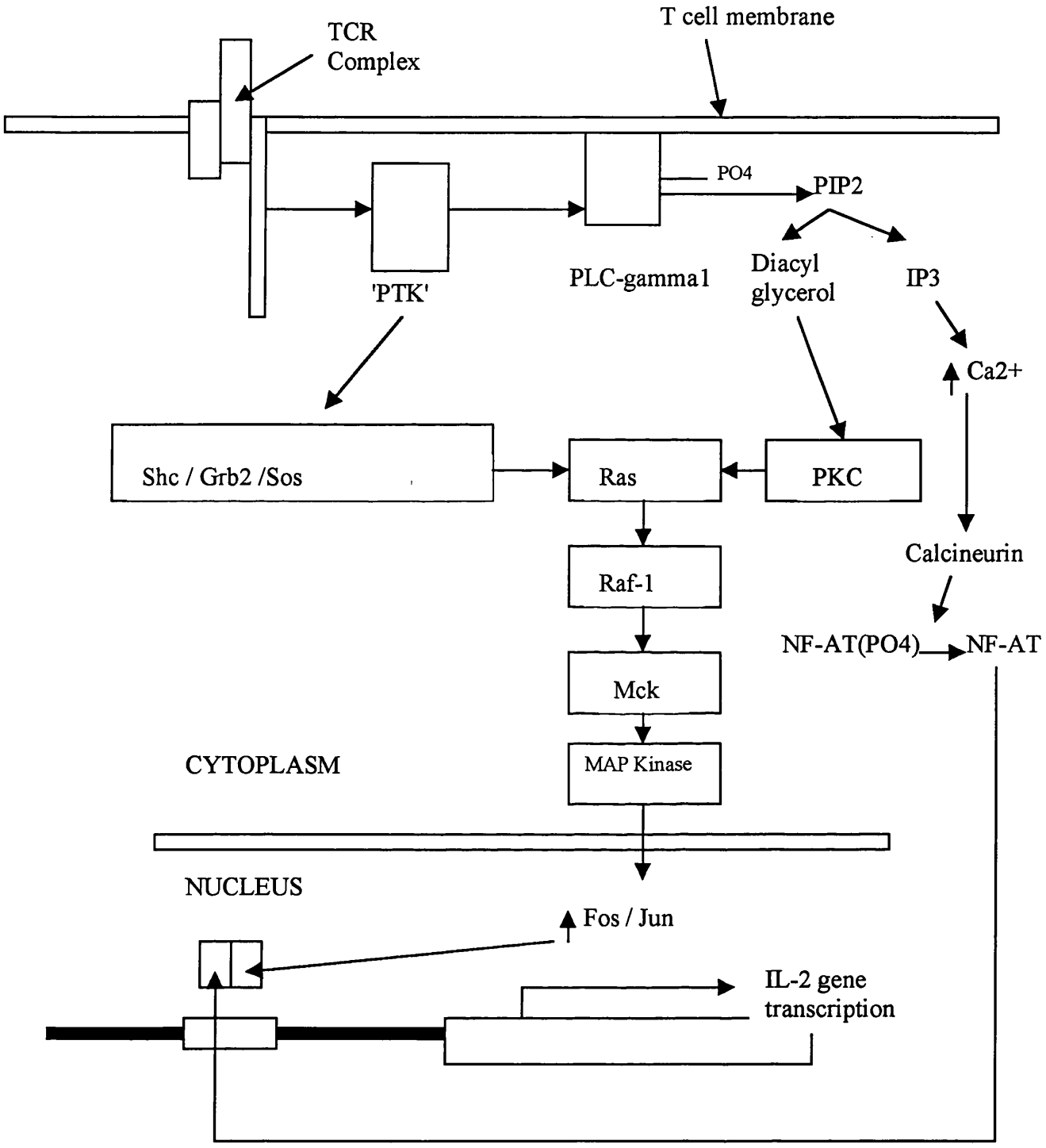
1.5 Lymphocyte activation

1.5.1 Important cell surface molecules in the activation process

Activation of a lymphocyte is the result of a large series of tightly integrated mechanisms (Crabtree, 1989). The activation of resting T cells enables that cell to exert its regulatory or effector function. A complex sequence of events drives the resting cell from G_0 to undergo a complex series of differentiation and proliferation processes. These processes have been extensively reviewed (e.g. Weiss, 1999) and are summarised in Figure 1.1.

In essence, a large number of cell surface molecules on the T cell engage with ligands on the antigen-presenting cell. Some of these receptor-ligand interactions are antigen-specific, e.g. between T cell receptor and antigenic peptide in the cleft of the major histocompatibility complex (MHC) molecule. The avidity of this interaction is low, so binding is stabilised by non-antigen specific interactions, e.g. between CD4/MHC Class II, CD2/lymphocyte functional antigen-3 (LFA-3), etc. These other receptor/ligand interactions provide a critical costimulatory signal, without which the naïve T cell is rendered anergic. The mechanism of signal transduction is highly complex, and involves a series of molecules within the T cell receptor complex undergoing tyrosine phosphorylation. This initiates a series of protein tyrosine kinases (Fyn, Lck, Syk, ZAP-70, Csk and Itk) which in turn activates phospholipase Cγ1 (PLCγ1), resulting in its translocation from the cytoplasm to the inner plasma membrane. (Absence of Btk, in XLA, results in failure of PLCγ1 activation). PLCγ1 activation results in generation of second messengers of the phosphoinositol pathway – inositol triphosphate (IP3) and diacylglycerol. IP3 activates an increase in intracellular calcium ion levels which in turn results in dephosphorylation of the transcription factor nuclear factor-activated T cells (NF-AT) (mediated by calcineurin) and translocation of the NF-AT to the nucleus. Diacyl glycerol (and phorbol esters) activate protein kinase C (PKC) which in turn activates the protooncogene Ras and leads to the subsequent activation of a sequence of downstream effector molecules. This pathway induces the intermediate-early genes Fos and Jun which are also transcription factors, resulting in the transcription of the IL-2

Figure 1.1 T cell Activation pathways.



Abbreviations: NF-AT (nuclear factor of activated T cells), PO4 (phosphate group), PLC (phospholipase C), PKC (protein kinase C), 'PTK' group of protein tyrosine kinases, PIP2 (phosphoinositol diphosphate), IP3 (inositol triphosphate), TCR (T cell receptor), MAP kinase (MEK activating protein kinase),MEK (tyrosine-serine threonine kinase), Raf-1 (serine-threonine kinase). Shc , Sos, Grb2, Fos, Jun are protein names, not abbreviations.
See text for details.

gene and subsequent cellular proliferation. Other cell surface molecules become upregulated and expressed on the cell surface including CD25, CD71, MHC Class II, CD69 etc.; each with varying kinetics.

An important counterpart to cell activation is that of apoptosis, or programmed cell death. The hallmark of apoptosis is controlled autodigestion of the dying cell. The activation of endogenous proteases, reviewed in Thompson (1999), results in disruption of the cytoskeleton, and expression of phosphatidyl serine on the outer leaflet of the plasma membrane. Endonucleases degrade nuclear DNA, causing the nucleus to shrink. A key feature of apoptosis is that cell membrane remains intact. The process of apoptosis must be actively suppressed in viable cells, e.g. by the supply of cytokines, particularly IL-2, and other environmental signals. Apoptosis may be initiated by ligation of a variety of cell surface receptors, particularly the tumour necrosis factor receptor (TNF-R) family, including CD95 (Fas). The processes involved in apoptosis are illustrated in Figure 1.2. The processes of cell activation and apoptosis are tightly inter-linked, since the balance of "death decision" factors within the lymphocyte is modulated by the activation status of the cell (outlined in Figure 1.3).

Some indication of the state of activation of a lymphocyte may be gained by examination of various surface molecules on the lymphocyte; makers examined in this study are described below.

Figure 1.2 Process outline of apoptosis

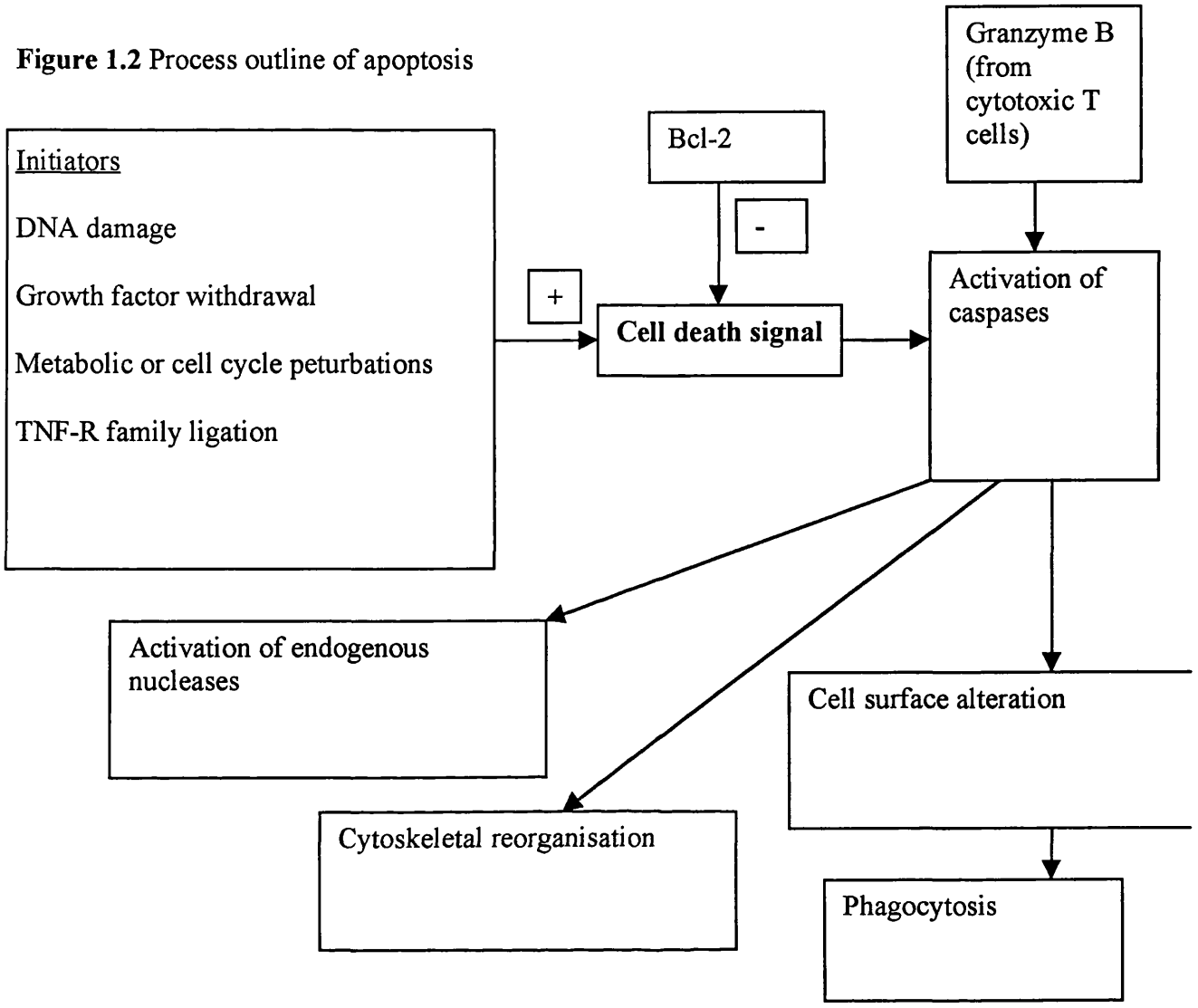
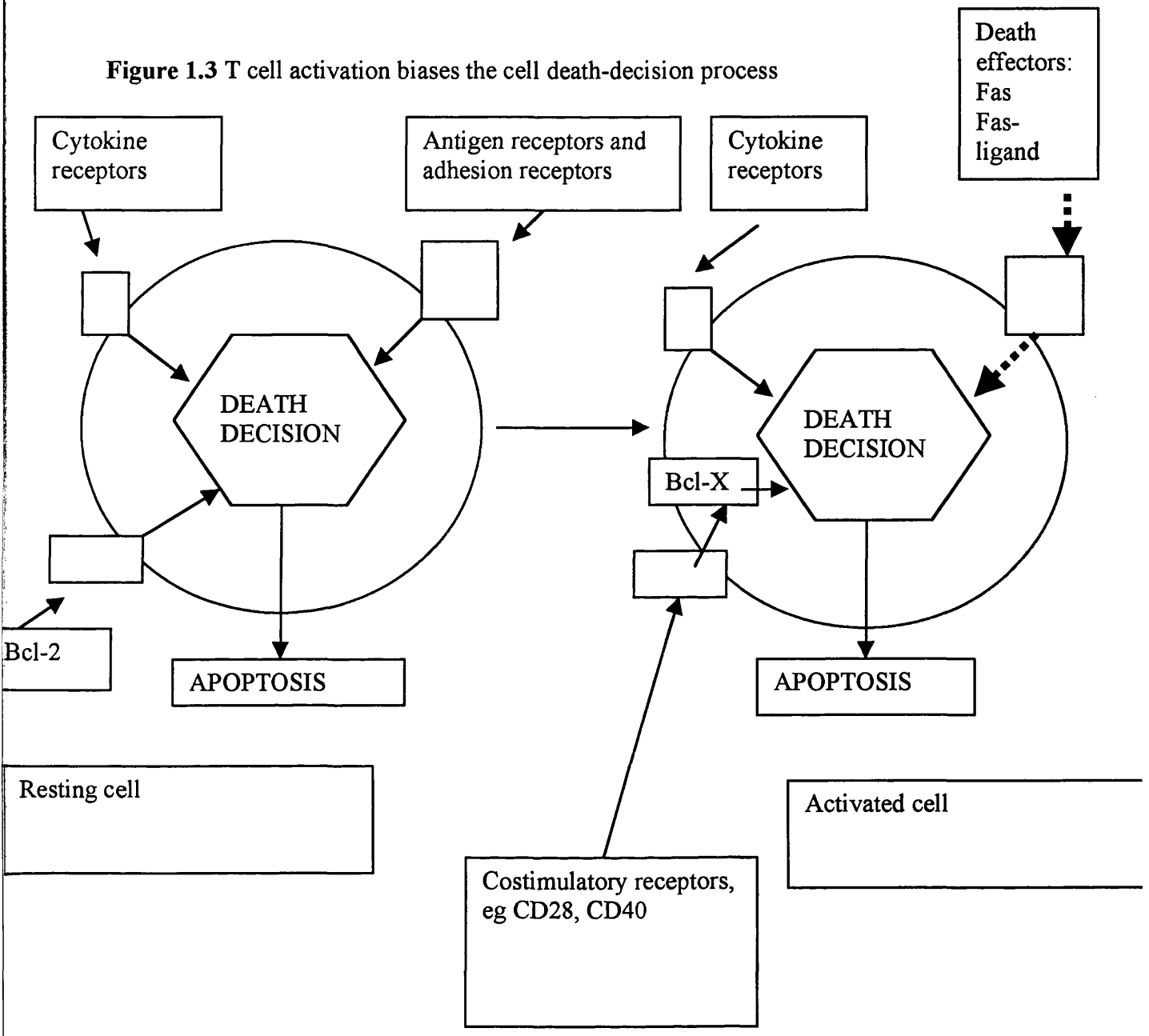


Figure 1.3 T cell activation biases the cell death-decision process



1.5.1.1 CD28

The major T cell co-stimulatory signal is mediated by a cell surface molecule termed CD28 (Lenschow *et al*, 1996), which is a ligand for the B7 family of cell surface molecules on antigen-presenting cells. CD28 is a disulphide linked homodimeric glycoprotein, composed of two 33-37 kDa chains (June *et al*, 1994). and is constitutively expressed on the majority of $CD4^+$ and about 50% of CD8+ cells (Linsley *et al*, 1993, June *et al*, 1990). CD28 expression increases on activated T cells, triggering a series of events inside the cell. Following CD28 ligation, a similar molecule CTLA-4 is upregulated, peaking about 48 h after activation and returning to background levels by 96 h (see CTLA-4 section below). During this time, CD28 becomes down-regulated. CD28- cells cannot upregulate CTLA-4, even after stimulation with PMA and ionomycin (Lindsten *et al*, 1993). CD28-/- mice have substantially impaired T cell proliferative responses (Green *et al*, 1994). The CD28 signal leads to upregulation of Bcl-XL, which protects the cell from apoptosis (Boise *et al*, 1995).

Once CD28 is engaged, the production of various cytokines (IL-1, IL-2, IL-4, IL-5, TNF-alpha and IFN-γ is enhanced (Linsley *et al*, 1993, McArthur *et al*, 1993, Thompson *et al*, 1989). Various studies have shown that CD28 is important in the development of the Th1/Th2 phenotype. In the absence of CD28 signals, naive cells become Th1-biased, shown by the failure of IL-4 production in cultures where CD28/B7 interaction was blocked by CTLA4Ig (Seder *et al*, 1994). In addition, pure CD3 signals in the absence of CD28 signalling, leads to IL-2 and IFN-γ production, whereas if anti-CD28 is added to the system IL-4 and IL-5 can be produced (King *et al*, 1995). The importance of CD28 in producing Th2 biased responses has also been demonstrated in CD28-/- mice, which can respond normally to LCMV, yet cannot make a Th2-biased antibody response to VZV (Shahinian *et al*, 1993). An interesting counterpart to this is in mice transgeneic for CTLA4Ig. These mice with a block in the CD28/B7 pathway, cannot make IL-4 and make excess IFN-γ, and are cannot make a primary antibody response (Lane *et al*, 1994, Ronchese *et al*, 1994). Mice treated with injections of CTLA4Ig to block CD28 signalling cannot make IL-4 responses to parasites. When infected with *Heligmosomoides polygyrus* these mice make fewer IL-4 secreting cells, less IgE and fewer eosinophils (Lu *et al*, 1994).

Blockade of the CD28/B7 pathway using CTLA4Ig therapy can profoundly impair humoral immunity, inhibiting the development of antibodies to neoantigens (Linsley *et al*, 1992), and suppressing secondary and tertiary reponses. Anti-B7 antibody therapy not only prevents the development of antibodies to immunisation, but also inhibits somatic hypermutation and impairs B cell memory responses (Han *et al*, 1995). CD28-/- mice have serum antibody levels only 20% that of normal mice, with lower levels of IgG1 and IgG2b (but increased levels of IgG2a) (Lenschow *et al*, 1996).

1.5.1.2 CTLA-4 (CD152)

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), now known as CD152, is an important regulator of T cell activation. CD152 is a 45 KDa glycoprotein (Linsley *et al*, 1995), expressed as a disulphide-linked homodimer or monomer (Lindsten *et al*, 1993). It is a counter-receptor for CD80 (B7.1) and CD86 (B7.2). This member of the immunoglobulin supergene family, with a single Ig V-like region, was initially thought to be specifically expressed on cytotoxic T cells (Brunet *et al*, 1987). Initial impressions were that both CD28 and CD152 were costimulatory receptors for T cell activation (Linsley *et al*, 1992; Freeman *et al*, 1992). It is now appreciated that CD152 ligation is inhibitory (Linsley *et al*, 1996), down-regulating CD28-induced T cell activation (Morton *et al*, 1996). CD152 is also involved in the induction of apoptosis (Gribben *et al*, 1995); murine T cells from CD152 knockout mice spontaneously proliferate (Waterhouse *et al*, 1995).

CD152 is not constitutively expressed on human T cells, although is often present in the cytoplasm. Following *in vitro* T cell activation, it becomes expressed on the cell surface peaking at day 3, and falling to undetectable levels by day 4 (Linsley *et al*, 1996). Even at the day-3 maximum, the level of expression is not high.

1.5.1.3 HLA-DR

HLA-DR is a Class II major histocompatibility antigen which although expressed primarily on antigen presenting cells, is also expressed on activated T cells following a variety of mitogenic stimuli (Ko *et al*, 1979; Robbins *et al*, 1988). Expression of HLA-DR occurs during the later phase of T cell activation, several days after the initial activating signal (Oshima & Eckels, 1990). Although HLA-DR expression follows activation by anti-CD3 and IL-2, PMA can result in a decrease in DR expression, implying that class II may be selectively activated by transduction of specific signals (Oshima & Eckels, 1990).

1.5.1.4 CD25

CD25 forms the α-chain of the IL-2 receptor (IL-2Rα) (properties reviewed in Greene & Leonard, 1986). CD25 alone is a low-affinity IL-2R, which together with CD122 (IL-2Rβ) and common gamma chain (IL-2Rγ) forms the high-affinity IL-2R. CD25 is a highly glycosylated, 55 kDa glycoprotein, encoded by a single gene on chromosome 10. It is expressed on activated T cells, B cells and monocytes. Only a low percentage (1-5%) of peripheral blood T cells express CD25 intrinsically (Jackson *et al*, 1990) although this result is influenced by the technique used to measure CD25 expression (reviewed in Amlot *et al*, 1996).

1.5.1.5 CD69

CD69, previously known as activation inducer molecule (AIM), is a disulphide-linked heterodimer consisting of a 27 and a 33 kDa polypeptide. It is the earliest membrane activation marker to appear following stimulation, is expressed on very few peripheral blood T cells, yet undergoes is rapidly expressed on peripheral blood lymphocytes following activation with phorbol esters, phyto-haemagglutinin, or anti-CD3 (reviewed in Maino *et al*, 1995). Its exact role has not been determined, but signalling via CD69 induces IL-2 synthesis, IL-2R expression and proliferation in T cells (Cebrián *et al*, 1988).

1.5.2 Lymphocyte activation in CVID

Numerous studies have identified abnormalities within various lymphocyte subpopulations in common variable immunodeficiency. Given that CVID is primarily an antibody deficiency, early reports concentrated on B cells, identifying low B cell numbers in some patients (Farrant *et al*, 1994), also confirmed in a larger series (Spickett *et al*, 1992). Patients with low B cell numbers were particularly prominent in the severe Group A subset of patients. There are of course concerns that some patients (including females) with very low B cell numbers may have autosomal recessive forms of X-linked agammaglobulinaemia, rather than true CVID. Later research shifted the focus to T cells

as the regulators of B cells, and many studies established that a substantial subgroup of CVID patients have reduced CD4 T cell numbers, and increased CD8 T cells, disturbing the CD4/8 ratio (Wright *et al*, 1990; Farrant *et al*, 1992). In those patients with elevated CD8 numbers, the CD8 T cells have major abnormalities of mitogen-induced proliferation, cytokine production and express increased amounts of CD57 and HLA-DR (Jaffe *et al*, 1993).

A study of 40 patients failed to find evidence of abnormal expression of the activation markers CD25 or HLA-DR (Spickett *et al*, 1991), confirming the findings of Wright *et al* (1990). However, these studies used 2 colour flow cytometry, and therefore were unable to simultaneously distinguish activation marker expression and CD4 or CD8 and lymphocyte subset marker (eg CD45RA or RO) simultaneously. Other molecules are expressed abnormally on CVID T cells, for example there are reduced numbers of CD45RO+ cells bearing L-selectin (Zhang *et al*, 1996), and a higher percentage of CD38+ T cells (Nordøy *et al*, 1998).

Subdividing CD4 and CD8 populations on the basis of CD45 isoform expression has shown that CD45RA+ cells are reduced in CVID, particularly within the population with low CD4 numbers (Farrant *et al*, 1994). Wright's group (1990) also confirmed that the low CD45RA expression was confined to the patients with low CD4 numbers. CD45RO expression was low in patients with the more severe CVID phenotype (Groups A and B) (Farrant *et al*, 1994), although the reciprocal expression of CD45RA and RO (Salmon *et al*, 1994) is such that a subgroup of patients with CVID co-express CD45RA and RO on CD8 cells (Richards *et al*, 1992).

Using CD28 expression to subdivide the CD8+ T cell population into CD28+ (cytotoxic) and CD28- (suppressor) cells (Lum *et al*, 1982; Koide & Englemann, 1990; Freedman *et al*, 1991) can be used to demonstrate that not only is the CD8+28- subset expanded in CVID, but that the CD28+ subset over-expressed IFN-γ on activation (North *et al*, 1998). Numerous studies have delineated similar CD8/28 subset abnormalities in diseases with chronic inflammation (eg. Dalod *et al*, 1999), and parallels can be drawn with CVID.

2 General materials & methods

2.1 Introduction

This section details materials and methods common to the subsequent chapters. Methods specific to an individual chapter are described in detail as they occur in the text.

2.2 Donors

Blood donations were provided after informed consent and were used with the approval of the ethics committee of the Royal Free Hospital. Samples were collected in Lithium-heparin containing tubes (Sarstedt Monovette) unless otherwise stated.

2.2.1 Normal subjects

Peripheral blood was taken from healthy volunteers (laboratory workers) of both sexes, aged 20-65 years.

2.2.2 Hypogammaglobulinemic patients

Peripheral blood was taken from patients with common variable immunodeficiency and X-linked agammaglobulinaemia attending the hospital for routine intravenous immunoglobulin infusions. Except where stated, samples were taken immediately prior to the infusion.

2.2.3 Other subjects

Peripheral blood was taken from patients with other conditions (sarcoidosis, hyper IgE syndrome, and atopic eczema) as described in the relevant chapter.

2.3 Cell Cultures

2.3.1 Culture media

2.3.1.1 RPMI 1640 medium

RPMI 1640 (ICN-Flow Laboratories, UK) was supplemented with penicillin/streptomycin (100 IU/ml each, ICN-Flow Lab) and 2 mmol/l L-glutamine except where stated. Where indicated, heat-inactivated fetal calf serum (10%) was added.

2.3.2 Cell preparations

Cells preparations were carried out in a Class II microbiological safety cabinet under sterile conditions.

2.3.2.1 Peripheral blood mononuclear cell preparations

Peripheral blood mononuclear cells (PBMCs) were prepared by layering heparinised blood (10ml) onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) in a sterile universal container. Samples were centrifuged at 400*g* for 25 minutes at room temperature, and PBMCs were removed from the appropriate interface using a Pasteur pipette. The PBMCs were washed twice using RMPI 1640 (approx. 10 ml), before being counted using a modified Neubauer haemocytometer and the concentration was adjusted by the addition of culture medium to a concentration of 2×10^6 PBMC/ml.

2.3.3 Stimulants & other reagents

2.3.3.1 Phorbol myristate acetate

Phorbol 12-myristate, 13-acetate (PMA; Calbiochem-Novabiochem Corporation, La Jolla, CA) was dissolved in dimethyl sulphoxide (Sigma) at a concentration of 1 mg/ml, and stored in 10 µl aliqots at -80°C until required.

2.3.3.2 Ionomycin

Ionomycin (1mg, Calbiochem) was dissolved in dimethylsulphoxide (352.5 µl) and stored in 7.5 µl aliquots at -80°C until required.

2.3.3.3 Monensin

Monensin (Calbiochem) was dissolved in absolute ethanol (41.57 mg in 5 ml) and stored in 7.5 µl aliquots at -20°C until required.

2.3.3.4 Fixation and permeabilisation reagents

Leukoperm A (Serotec, Kidlington, Oxford, UK) was used as a fixative for the intracellular cytokine assays, and Leukoperm B (Serotec) was used as a permeabilising agent. Both reagents were used at 250 µl per 50 µl blood.

2.3.3.5 Lysing agents

Optilyse C Lysing Solution (Immunotech, Marseilles, France) was used as a lysing solution, at volumes of 250 µl per 50 µl blood.

2.3.3.6 Intravenous immunoglobulin preparations

For the *in vitro* studies, various commercial immunoglobulin preparations for therapeutic use were used, including Alphaglobin (Grupo Grifols, kind gift of Carl Wheeler), Sandoglobulin (Novartis) and Vigam S (BPL)(kind gifts of Sister Cilla Freud, Royal Free Hospital, UK). For the *in vivo* studies at replacement dose, a range of IVIG preparations were used since the patients received their routine IVIG product, including Alphaglobin, Sandoglobulin and Vigam S. For the high-dose *in vivo* IVIG study, Alphaglobulin was used.

2.4 Flow cytometry

The flow cytometer used in the majority of experiments was an Epics XL with multi-channel loader (MCL) (Beckman Coulter Ltd, High Wycombe, UK), equipped with 15 mW argon laser operating at 488 nm, and four photo-multiplier tubes with a spectral range of 200-800 nm. Appropriate filters were used to provide fluorescence detection for four fluorescence channels: FL1 as fluoroscein isothiocyanate (FITC), FL2 as phycoerythrin (PE), FL3 as Energy Coupled Dye (ECD, a Texas Red/PE tandem dye) and FL4 as a PE/cyanin-5 tandem dye (PC5). Some of the earlier experiments were performed on a Becton Dickinson 'Facscan' equipped with a 488 nm argon laser, and photodetector/filter combinations suitable for FL1: FITC, FL2: PE and FL3: peridinin chlorophyll protein conjugate (PerCP)

2.4.1 Calibration and standardisation

The flow cytometers were calibrated and operated in accordance with the manufacturer's instructions.

2.4.1.1 Flow cytometer laser alignment

To ensure that the optical alignment of the laser and sensors in the flow cytometer was correct, FlowCheck beads (Coulter) were used each time the Epics XL was operated, and half-peak coefficients of variation (HPCV) of <2 from single parameter fluorescence peaks were accepted as evidence of correct alignment.

2.4.1.2 Flow cytometer voltage settings

To ensure that photomultiplier voltages and gains were providing identical signal amplification each time the flow cytometer was used, FlowSet beads (Coulter) were used. These were used in conjunction with a subroutine on the Epics cytometer that automatically adjusted all photomultiplier voltages until identical readings were obtained on each occasion the machine was operated. This ensured that the same compensation settings were applicable on every run when on-line machine compensation was used, since the performance characteristics of each photomultiplier tube was corrected for using the standard FlowSet beads.

2.4.2 Absolute counting procedures

All events were acquired with the exception of events below a threshold set on the forward light scatter to exclude cell debris and other small particules. Use of a 'live gate' to acquire (for example) only lymphocytes defined by light scatter characteristics was not done, one reason being that this would exclude the acquisition of the FlowCount beads. However, within the 'all-event' acquisition, a minimum of 10 000 events within a lymphocyte light scatter gate was always acquired for offline analysis. Where absolute cell counts were required, FlowCount beads (Coulter) were used, according to the method of Schlenke *et al* (1998). These beads are of standard concentration, and have light scatter and fluorescent characteristics that clearly distinguishes them from haematopoeitic cells. They were added to the flow cytometry samples within 2 h before reading on the

cytometer, using exactly the same pipette and volume setting as was used to dispense the cells. The volume of beads added was the same as the volume of the cell sample.

In this way, the number of events registering as FlowCount beads (of known concentration) could be related to the number of events of the cell population of interest (of unknown concentration) using the following formula:

$$C = B \times \frac{n_C}{n_B},$$

Where C is the required concentration of the cell population of interest (cells/μl), B is the known concentration of the batch of beads added to the sample (typically ≈1000/μl), n_B is the number of beads in the bead-containing region on a plot of forward scatter *vs.* FL1, and n_C is the number of cells in the appropriate region of interest.

2.4.3 Colour compensation

For each experimental series, compensation for spectral overlap between photodetectors ('colour compensation') was performed in one of three ways:

2.4.3.1 Electronic compensation offline

Data from the Epics flow cytometer was usually collected without colour compensation being applied at the time of collection. Instead, single- and dual-stained standards were used, usually stained for either CD3 alone, or CD4 and CD8. The Compensation Toolbox within the WinList 4.0 (Verity, Topsham, USA) flow cytometry analysis package was then used to apply colour compensation retrospectively based on the overlap detected with the standards, according to the principles outlined by Bagwell & Adams (1993).

2.4.3.2 Epics XL compensation

For the study of high-dose intravenous immunoglobulin in atopic eczema, a compensation package built-in to the Epics XL was used. This relies on commercial lymphocyte preparations (CytoTrol, Coulter) being dual-stained for two mutually exclusive markers (CD4 and CD8) using CytoComp (Coulter) reagents. A subroutine on the Epics system then electronically adjusted the compensation settings between those fluorescence channels with significant spectral overlap (FL1 and FL2, FL2 and FL3, FL2 and FL3, FL3 and FL4). In comparative preliminary experiments this technique gave identical results to the Compensation Toolbox technique outlined above (data not shown), with the advantages of external standards being used. A disadvantage was that the listmode data produced using this method had fluorescence-negative cells assigned to very low channel numbers, which resulted in dots being plotted against the axis on two-parameter dot plots. To visualise the data more clearly, a 'log-bias' of 0.5 decade was applied to the lowest channel number events. This had the effect of producing a 'cloud' of cells, around which regions could be clearly drawn.

Preliminary experiments established no difference between these colour compensation techniques for the data from the Epics XL (data not shown).

2.4.3.3 Facscan compensation

Colour compensation was applied in real-time to the Facscan listmode data during acquisition, according to the manufacturer's instructions.

2.4.4 Data transmission

Listmode data from each flow cytometer was stored on removable medium (Zip disk for Epics XL, Magneto-optical drive for Facscan) and transferred to a personal computer (Dan Pentium) for off-line analysis.

2.4.5 Data analysis

Off-line analysis was performed using WinList 4.0 (Verity). A variety of analytical protocols were used, described in each chapter. Results were either manually entered into Excel spreadsheets (Microsoft) or for large experiments, were directly transferred from WinList to Excel using a Dynamic Data Exchange (DDE) link. Individually written software subroutines were used to establish the link and to assign data to individual cells. Great care was taken, with multiple checks by several people, to ensure that the correct data had been entered into the correct spreadsheet cell.

2.5 Assessment of intracellular cytokines within PBMCs

Assessment of intracellular cytokines in PBMCs was done using a modified method of North *et al* (1996). PBMCs were obtained at a concentration of 2×10^6/ml in RPMI1640 + 10% fetal calf serum. Aliquots (500 µl) were cultured in 24-well multi-well plates (Falcon, Marathon Supplies, London) with monensin (3 µmol/l) alone ('unstimulated' cultures), or monensin (3 µmol/l) with PMA (5 ng/ml) and ionomycin (1 µg/ml) ('stimulated' cultures). All concentrations are expressed as final concentrations in a culture volume of 1 ml. Cells were incubated at 37°C in a humid 5% CO_2-in air atmosphere for 2 h.

Following culture, cells were harvested by washing each well twice with phosphate buffered saline (PBS; GibcoBRL) containing bovine serum albumin (0.2%; Sigma, St Louis, MO) and sodium azide (0.02%; Sigma) (PBS/A/A). The cell pellet was then fixed using Leucoperm Reagent A (Serotec; 50 µl per 0.5×10^6 cells) for 15 min at room temperature. The cells were then washed once in PBS/A/A before permeabilising with Leucoperm Reagent B (Serotec; 50 µl per 0.5×10^6 cells). Aliquots (50 µl) of the permeabilised cells were immediately added to cocktails of monoclonal antibodies at previously-determined saturating concentrations. A typical antibody cocktail might be: (FL1) anti-IFN-γ/FITC, (FL2) anti-CD28/PE, (FL3) anti-CD4/ECD, (FL4) anti-CD8/PC5. Cells were stained for 30 min at room temperature in the dark, before being washed once in PBS/A/A and then stored in 0.5% paraformaldehyde in PBS (400 µl) at +4°C until flow cytometric assessment within 18 h.

For the PBMC, following 'all event' acquisition on the flow cytometer, listmode data from 10 000 events in a 'lymphocyte gate' was analysed using WinList 4.0 (Verity). From a plot of forward- against side-scatter, a tight region (R1) was drawn around the lymphocyte population. On an R1-gated plot of CD4-fluorescence against CD8-fluorescence, rectangular regions were defined for $CD4^+$ (R2) and $CD8^{bright}$ (R3) cells. Histograms of intracellular cytokine-fluorescence were then produced, gated on R1 AND R2 (for $CD4^+$ cells) and R1 AND R3 (for $CD8^+$ cells). Regions were set using the 'unstimulated' cultures (cultured with monensin only) to define cytokine-negative cells. Data were then expressed as the percentage of cytokine-positive $CD4^+$ (or $CD8^+$) cells. Additionally, if absolute counts of cytokine-positive cells were required, FlowCount beads were used as described above.

2.6 Statistical analysis

Descriptive statistics (mean, standard deviation, etc.) were determined using an Excel 97 (Microsoft) spreadsheet. Parametric tests, mainly Student's t test (paired and unpaired), were also performed using Excel. Non-parametric comparisons for paired data (sign test for paired data) and for unpaired data (Mann-Whitney U test) were carried out using Minitab 7.1 (Minitab Inc, Pasadena). For all statistical tests, statistical significance was set at the p=0.05 level.

3 Developing a whole-blood intracellular cytokine assay

3.1 Introduction

Disruption of the mechanisms involved in the regulation of cytokine production by lymphocytes plays a major role in the pathogenesis of immunological diseases (Scott & Kaufmann, 1991). Suitable tools to investigate this process are an essential requirement for a better understanding of the aetiology of immunological disorders. Recent developments in cell permeabilisation reagents (Halldén *et al*, 1989; Sander *et al*, 1995) and the production of anti-cytokine monoclonal antibodies (Prussin & Metcalfe, 1995) has resulted in new assays for analysing the cytokine production of individual cells within mononuclear cell preparations (reviewed in Chapter 1) using flow cytometry of lymphocytes stimulated in culture.

A major drawback with these techniques is that they have required large volumes of blood (10-20 ml), that limits the use of the assays for example in small children, or in individuals being tested frequently. A second disadvantage is the use of cell separation techniques using density gradient centrifugation, since there is evidence that this can activate lymphocytes, so disturbing their cytokine profile (Maino *et al*, 1995; 1996). Finally, several intracellular cytokine assays based on isolated cells involve culturing the cells for long periods (up to 72 h, e.g. Elson *et al*, 1995) during which many changes in cell phenotype can occur, particularly the loss of CD4 antigen, which makes positive-identification of $CD4^+$ cells difficult or impossible (Petersen *et al*, 1992).

The initial aim of the present study was therefore to develop an assay for intracellular cytokines that was rapid, that did not require cell separation processes using density gradient centrifugation, and that used very small volumes of blood.

3.2 Whole blood assay

The development of a whole blood assay for intracellular cytokines involved first the selection of concentrations of stimulants to express the cytokines maximally, and then

the appropriate time course for culture. Normal subjects (5 male, 5 female, mean age 39.7 years) were used to establish the assay. Blood (1-5 ml) was collected in lithium-heparin-containing Monovette tubes (Sarstedt) and immediately processed; preliminary experiments showed that if analysis was not started within approximately 4 h of sampling irreproducible changes in cytokine production occurred (data not shown). It is important to note that EDTA cannot be used as the anti-coagulant, as it chelates the calcium required for the ionomycin-based stimulus; presumably citrated samples would also be unsuitable for this reason. To detect as much as possible of the cytokine expressed within each cell, monensin (3 μmol/l) was added to all cultures to block the export of newly synthesised cytokine from the Golgi apparatus (Jung *et al*, 1993). The optimum monensin concentrations had previously been determined (North *et al*, 1996 and Ivory, 1997).

Blood (250 μl) was added to culture medium (RPMI1640 without supplementation; 500 μl) containing monensin, with PMA and ionomycin ('stimulated' cultures) or without PMA and ionomycin ('unstimulated' cultures). The ratio of blood:medium of 1:2 was established after preliminary experiments demonstrated that other ratios were suboptimal for various reasons including the formation of gels or precipitates during subsequent procedures (data not shown). Cultures were carried out in ventilation-capped 5 ml polystyrene round-bottomed plastic tubes (Greiner Labortechnik, Gloucestershire).

Both stimulated and unstimulated cultures were incubated at 37°C for various times in a humid 5% CO_2 atmosphere. After incubation, red cells were lysed using a lysing solution, Optilyse C (2 ml for 15 min, Coulter) following the manufacturer's instructions for use with Coulter Epics-XL flow cytometers. Other flow cytometers require different lysing solutions, for example Optilyse B (Coulter) for Ortho Diagnostics or Becton Dickinson machines. After the red cells were lysed, the remaining cells were washed with phosphate buffered saline (2 ml) containing sodium azide (0.02%, Sigma) and bovine serum albumin (0.2%, Sigma) (PBS/A/A) at 300 *g* for 5 min and the cell pellet was resuspended.

The cells were then fixed by the addition of Leucoperm Reagent A (250 μl, Serotec) for 15 min, following which the cells were again washed in PBS/A/A and then permeabilised by the addition of Leucoperm Reagent B (250 μl, Serotec). The exact composition of these commercial solutions is not publicly available, but they are quality

controlled and according to the manufacturers, Reagent A is formaldehyde based and Reagent B is detergent based. Leucoperm was previously known as Cytoperm.

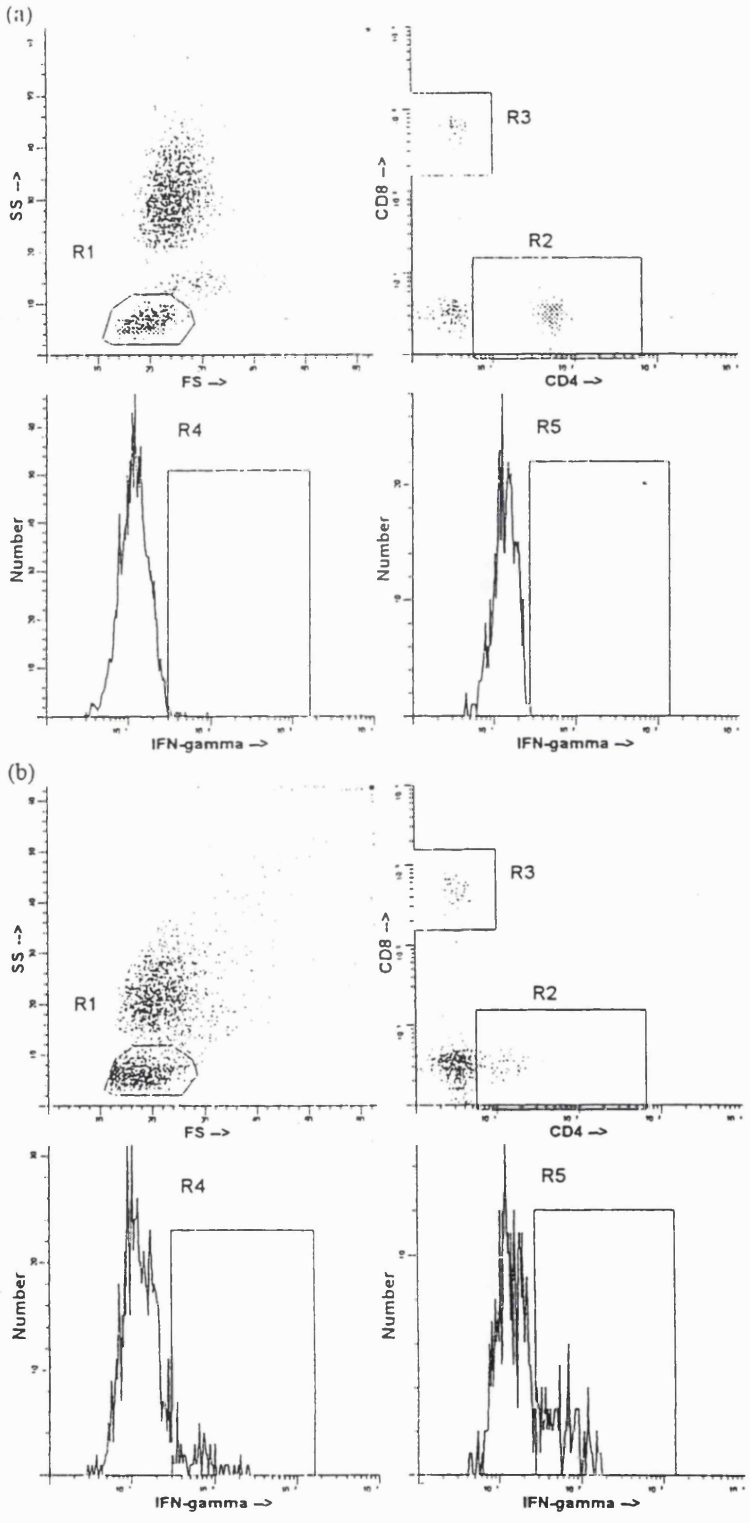
Aliquots (50 μl) of cells in Reagent B were then added to tubes containing directly-conjugated monoclonal antibodies against various combinations of cytokines and cell surface markers. Saturating concentrations of monoclonal antibodies were used, previously determined in other experiments (data not shown). All samples were stained in the dark at room temperature for 30 min. Samples were then washed once in PBS/A/A and stored in 0.5% paraformaldehyde in PBS (400 μl) at 4°C until acquisition within 18 h.

The following monoclonal antibodies were used: CD4/PE, CD8/ECD, CD3/PC5 (Coulter), CD69/FITC (Becton Dickinson), anti-human TNF-α/FITC (clone B-D9), anti-human IFN-γ/FITC (clone B-B1) and anti-human IL-2/FITC (clone (B-G5) (all anti-cytokine antibodies were the kind gift of Dr Andrew Lane, Serotec).

Following acquisition on an Epics XL flow cytometer, listmode data from 10 000 'lymphocyte gate' events was analysed in a similar manner to that detailed in Chapter 2 for intracellular analysis of cytokines in PBMCs (See Figure 3.1). In this experiment, data were expressed as the percentage of $CD4^+$ or $CD8^{bright}$ cells (termed '$CD8^+$' from now on) within a lymphocyte gate that were positive for cytokine. The early activation surface antigen CD69 was also measured on these fixed and permeabilised cells to determine the activation status of the lymphocytes. An isotype control antibody was used to define the region of CD69-positivity.

Figure 3.1 (On next page)

Gating procedure for flow cytometric analysis of intracellular cytokines. IFN-γ production by $CD4^+$ and $CD8^+$ cells shown as an example. List mode data were collected with forward scatter threshold set to exclude the majority of residual red cells and debris. Lymphocytes (R1) were gated to a plot of CD4 (R2) and CD8 (R3) fluorescence. Histograms of cytokine fluorescence were then constructed gated on R1 and R2 for $CD4^+$ IFN-γ^+ (cells in R4 = 8.9% in this example) and R1 and R3 for $CD8^+$ IFN-γ^+ (cells in R5 = 26.8% in this example).


(a)
SS -->
FS -->
R1
CD8 -->
CD4 -->
R3
R2
Number
IFN-gamma -->
R4
R5
(b)
SS -->
FS -->
R1
CD8 -->
CD4 -->
R3
R2
Number
IFN-gamma -->
R4
R5

3.2.1 Optimisation of stimulant dose

3.2.1.1 PMA

Cultures were established as above, with ionomycin at a final concentration of 2 μmol/l, monensin 3 μmol/l and various concentrations of PMA (5, 10 and 15 ng/ml). Intracellular TNF-α and IFN-γ as well as surface CD69 expression were assessed in $CD4^+$ and $CD8^+$ lymphocytes. Cultures were incubated for 2 h.

The results from this experiment are shown in Figure 3.2. The optimal PMA concentration, i.e. the concentration producing the maximal cytokine expression, was found to be 10 ng/ml, when the ionomycin concentration was held constant at 2 μmol/l.

3.2.1.2 Ionomycin

The experiment was repeated holding the PMA concentration constant at 10 ng/ml (16.2 nmol/l) with various concentrations of ionomycin (1, 2, 5 and 10 μmol/l). The result is shown in Figure 3.3.

When this concentration of PMA was used with various concentrations of ionomycin, a 2 μmol/l concentration of ionomycin produced the maximal cytokine expression. In subsequent experiments, PMA at 10 ng/ml and ionomycin at 2 μmol/l were used for the whole-blood intracellular cytokine assays, together with monensin at a concentration of 3 μmol/l.

Although different concentrations of stimulants gave slightly different expression levels of individual cytokines, the concentrations given were chosen as the best compromise for TNF-α, IFN-γ and CD69 in the same culture system, combined with the minimum down-regulation of surface CD4 antigen.

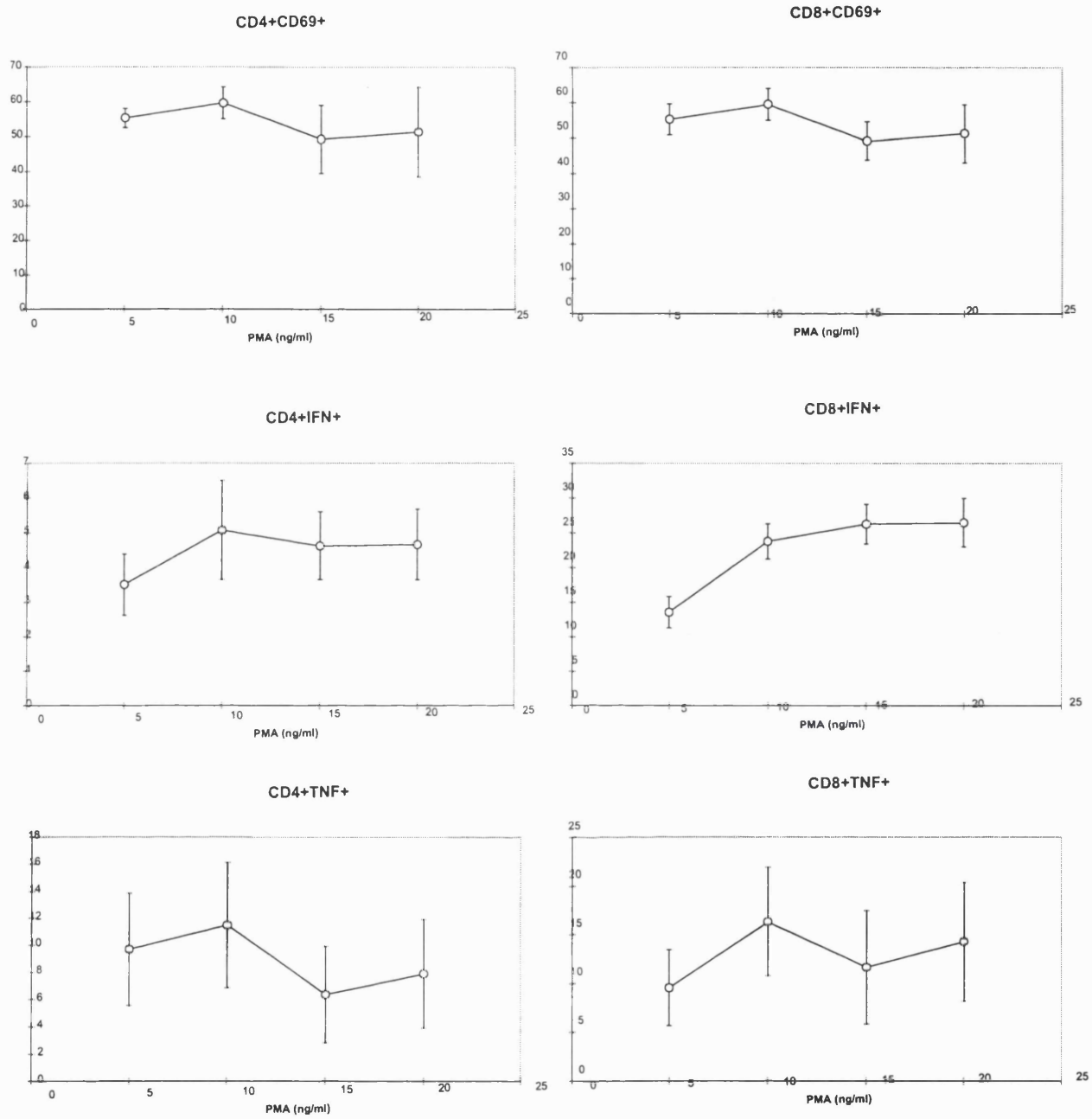


Figure 3.2 PMA dose-response curve. Whole blood samples diluted 1:3 with RMPI1640 were cultured (2 h) with monensin (3 µmol/l) and ionomycin (2 µmol/l) and various concentrations of PMA. Data plotted is the mean percentage of cells expressing the stated marker ± SEM shown for n=5 donors. The y-axis shows the percentage of $CD4^+$ or $CD8^+$ cells expressing the marker shown in the title to each graph.

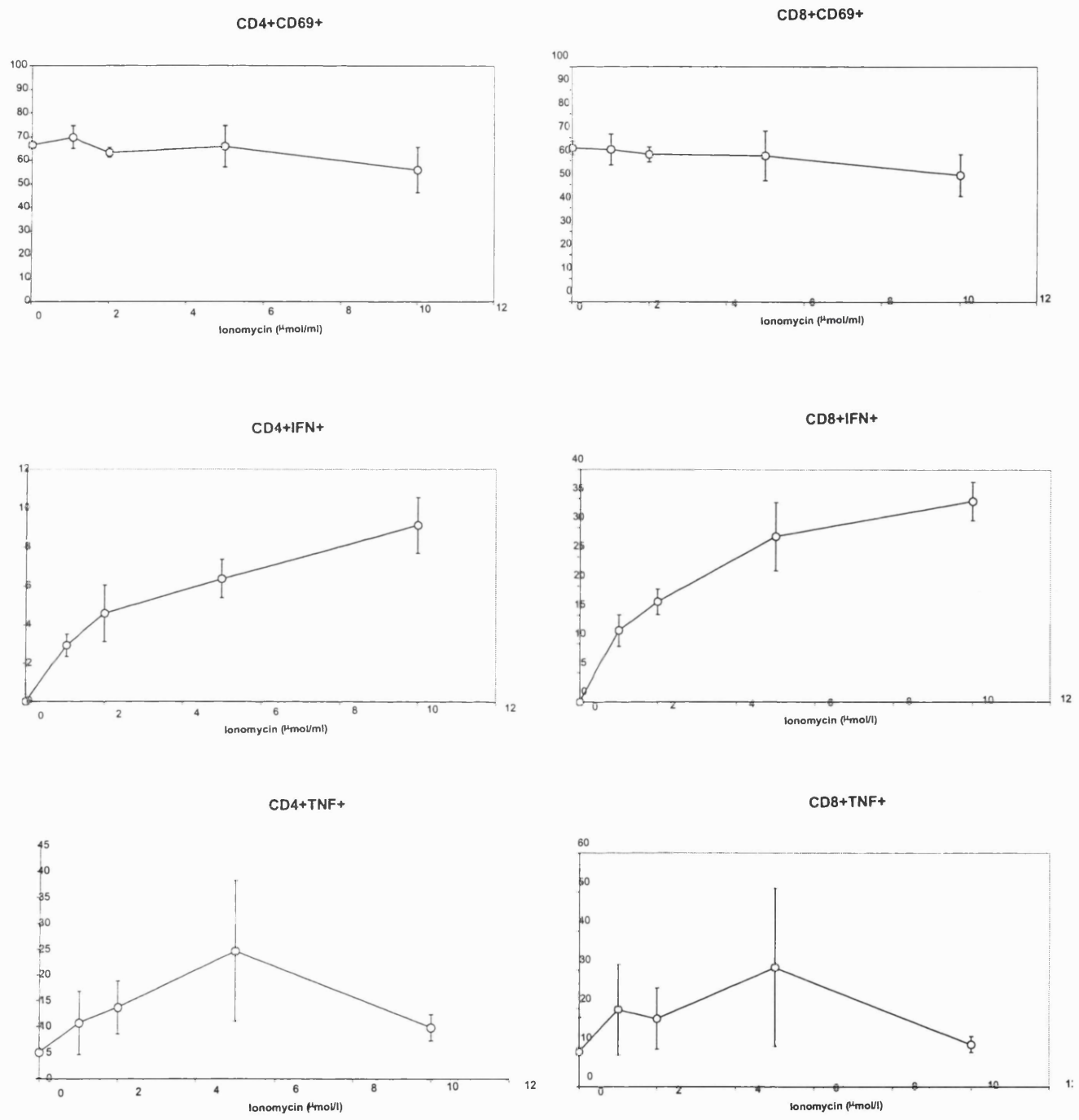


Figure 3.3 Ionomycin dose-response curve. Whole blood samples diluted 1:3 with RMPI1640 were cultured (2 h) with monensin (3 µmol/l) and PMA (10 ng/l) and various concentrations of ionomycin. Data plotted is the mean ± SEM shown for n=5 donors. The y-axis shows the percentage of $CD4^+$ or $CD8^+$ cells expressing the marker shown in the title to each graph.

3.2.2 Time course

Blood from 3 normal volunteers was cultured with stimulant concentrations as above, for various time intervals from 0 to 6 hours. TNF-α, IFN-γ and IL-2 production as well as CD69 expression were again determined in $CD4^+$ and $CD8^+$ cells. These short time intervals were chosen to make the assay suitable for possible eventual use in a routine clinical laboratory, where a same-day service might be required.

The results are shown in Figure 3.4. For both $CD4^+$ and $CD8^+$ cells, a 2 h stimulus yielded maximal CD69, IFN-γ and TNF-α expression, whereas a 4 h stimulus yielded peak IL-2 production.

In the time course experiment, surface CD4 became down regulated on prolonged stimulation with PMA, making positive identification of $CD4^+$ cells difficult. To determine the degree of CD4 down-regulation, four-colour flow cytometry was used, measuring CD3, CD4 and CD8 with three separate colours and cytokine fluorescence in the fourth. The mean CD4 channel fluorescence was measured in $CD3^+8^-$ cells cultured for 0, 2, 4 and 6 h with PMA and ionomycin. For comparison, the mean CD4 fluorescence signal of CD8 cells (i.e. totally CD4-negative cells) was also measured.

The down-regulation of surface CD4 expression with increasing culture time is illustrated in Figure 3.5. At time 0, CD4 cells had a clearly higher CD4 fluorescence signal than CD8 cells, and hence were easily distinguished from CD4-negative cells. By 2 h, CD4 brightness had decreased as the number of surface CD4 molecules became down regulated, but it was still possible to clearly distinguish CD4-positive and CD4-negative cells. There was further CD4 down-regulation at 4 h but it was still just possible to distinguish $CD4^+$ from $CD4^-$ cells. At 6 h of stimulation, however, the CD4 fluorescence of $CD3^+8^-$ cells was similar to that of $CD8^+$ cells, making it impossible to distinguish between $CD4^+$ and $CD4^-$ populations.

3.2.3 Assay performance characteristics

3.2.3.1 Intra-assay variation

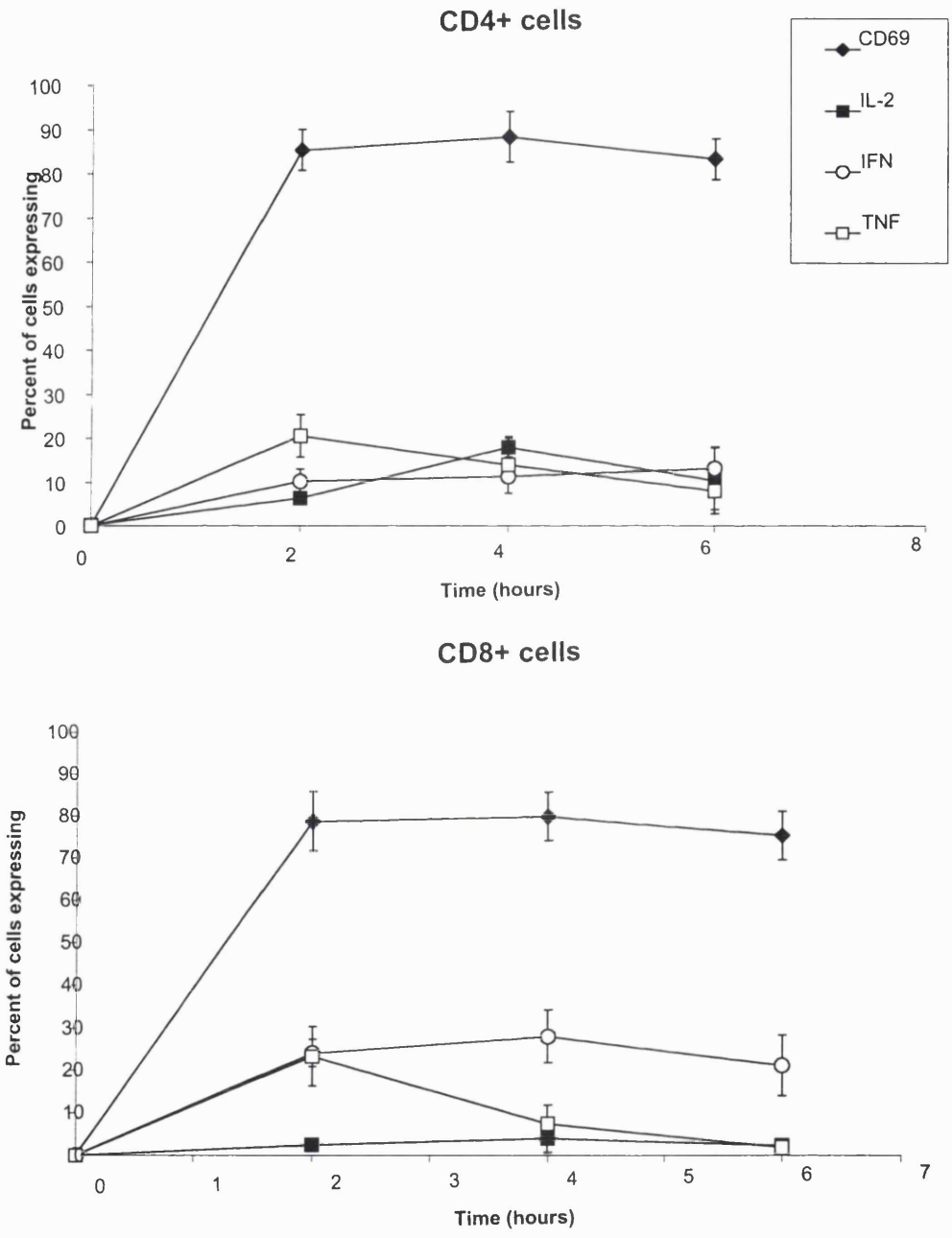


Figure 3.4

Kinetics of cytokine production under optimal conditions. Whole blood was cultured with PMA (10 ng/ml), ionomycin (2 μmol/l) and monensin (3 μmol/l) for various times. Stimulation for 2 h gave maximum IFN-γ and TNF-α production and activation marker (CD69) expression, 4 h gave maximum IL-2 production. Error bars show the SEM.

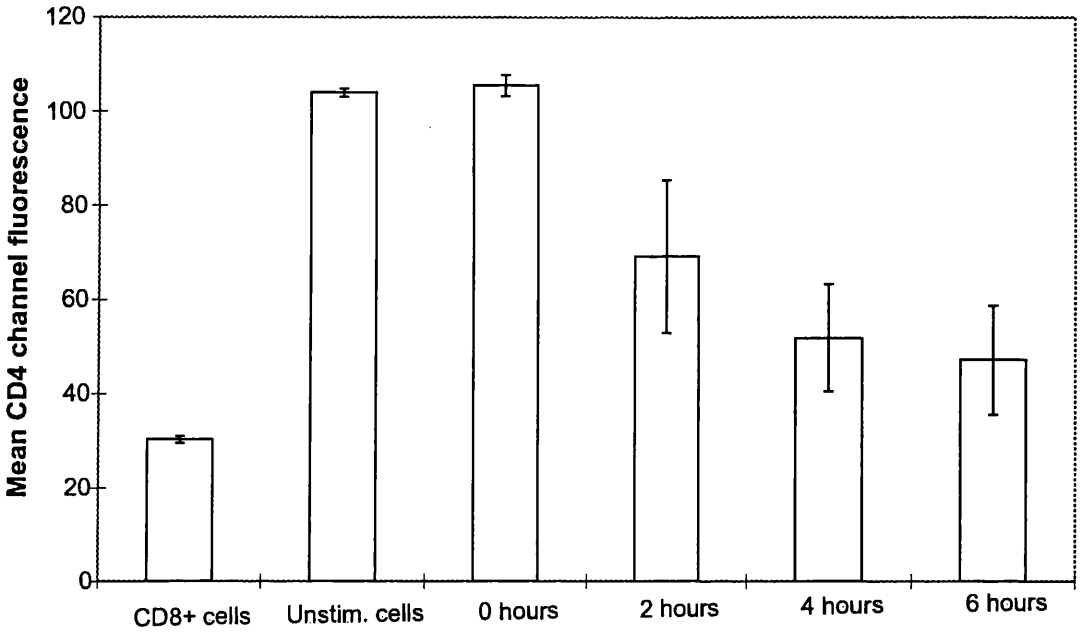


Figure 3.5

Down-regulation of surface CD4 expression with increasing culture time. The mean ± SEM mean CD4 channel fluorescence of $CD3^{+}8^{-}$ cells for 3 normal donors is shown with CD4 fluorescence in $CD8^{+}$ cells (i.e. $CD4^{-}$) for comparison.

In order to establish the intra-assay variation of this intracellular cytokine assay, the assay was repeated five times on the same occasion, for a single subject, and CD69, IFN-γ, TNF-α and IL-2 expression were measured in $CD4^+$ and $CD8^+$ cells.

The results are shown in Table 3.1.

Tube	CD69		IFN-γ		TNF-α		IL-2	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
1	66.2	56.0	7.3	21.0	8.6	6.9	11.9	2.2
2	69.6	54.9	12.8	21.6	14.3	6.1	18.9	8.0
3	71.3	65.9	14.5	26.3	19.2	9.7	13.5	4.0
4	78.2	62.0	11.7	28.3	20.6	11.5	13.7	4.2
5	77.2	65.1	11.1	28.5	17.8	8.9	19.4	11.1
Mean	72.5	60.8	11.5	25.1	16.1	8.6	15.5	5.9
SD	5.1	5.1	2.7	3.6	4.8	2.2	3.4	3.6
CoV	7.0%	8.4%	23.4%	14.5%	29.9%	25.5%	22.1%	61.4%

Table 3.1 Intra-assay coefficient of variation (CoV) for the whole-blood intracellular cytokine assay. Samples are from one subject, assayed simultaneously. Results shown are the percentages of $CD4^+$ or $CD8^+$ cells expressing the stated marker. SD, standard deviation.

The coefficient of variation (CoV = 100 × standard deviation/mean, expressed as a percentage) is a measure of the reproducibility of the assay. As expected, the CoV was reasonably low (<10%) for expression of a surface marker (CD69) after activation. For intracellular cytokine expression apart from IL-2, the CoVs were larger, but not unreasonable. The large CoV for IL-2 expression in $CD8^+$ cells, calls into question the suitability of that assay, although the range of variability is low.

3.2.3.2 Inter-assay variation

Given the problem of the assay lacking a standard or reference preparation against which to compare subsequent runs of the assay, it is difficult to determine the true inter-assay variation. In the section '3.1.4.3 Standard Donor' below the inter-assay variation for a single donor over the course of one year is discussed.

3.2.3.3 Direct immunofluorescence verification of staining pattern

Early intracellular cytokine techniques used direct immunofluorescence techniques to directly visualise cytokine staining within individual cells using a fluorescence microscope (Sander *et al*, 1991). These methods were laborious, and were not able to identify the subset of lymphocyte producing the cytokine, but were able to confirm that the cytokine was being retained within the Golgi apparatus of the cell. To establish that the whole blood assay described here was detecting cytokine within the Golgi apparatus, as opposed to non-specific cytoplasmic staining, direct fluorescence microscopy was carried out on one occasion to illustrate the process:

The whole-blood technique was carried out as above up to the point of cell permeabilisation using Leucoperm Reagent B, and stained with the directly conjugated anti-IFN-γ/FITC monoclonal antibody (Serotec, 10 µl) for 30 min, in the dark. Glass microscope slides were mounted inside the centrifuge holder, containing an application funnel and positioning filter paper, and the slides were wetted by applying 100 µl of PBS, and centrifuging at 80*g* for 4 minutes in a cytocentrifuge ('cytospin', Shandon Southern Products Ltd, Runcorn, UK). Aliquots of stained cells (250 µl) were placed in the cytospin funnel , and the slides were centrifuged at 80*g* for 4 min at room temperature. The slide was air dried for approximately 15 minutes, and a coverslip was applied over a drop of mounting medium (Citifluor). The slides were then immediately examined using a fluorescence microscope (Zeiss). As illustrated in Figure 3.6, unstimulated cells (cultured with monensin only) did not stain for IFN-γ, but cells stimulated with monensin, PMA and ionomycin stained brightly within the golgi apparatus only, confirming that newly synthesised IFN-γ was being detected in the appropriate location within the lymphocyte.

3.2.4 Standardisation attempts

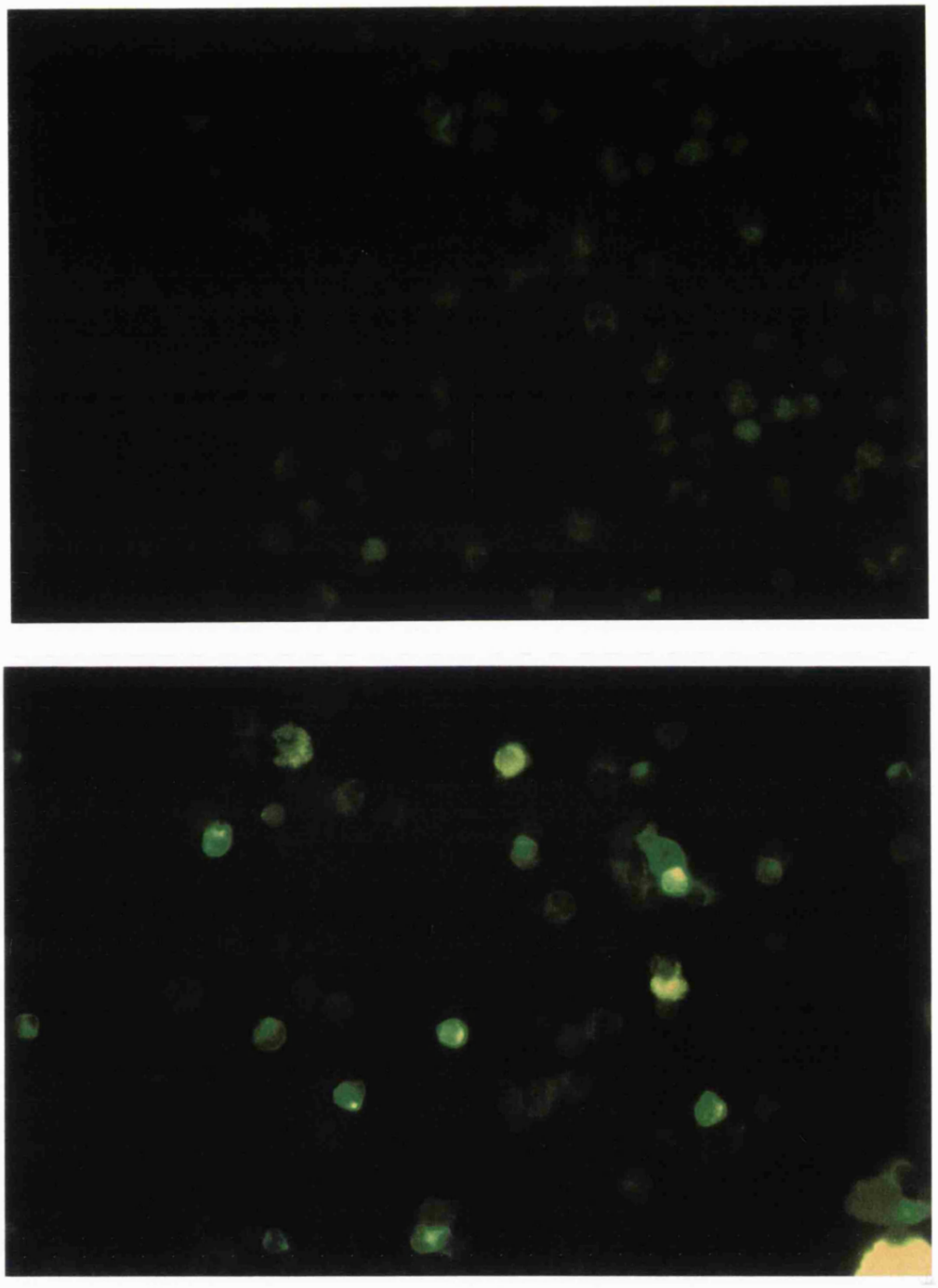


Figure 3.6(Top) Cells cultured in the whole blood system with monensin only, and stained for IFN-γ. (bottom) Cells cultured in the whole blood system in the presence of monensin, PMA and ionomycin, and stained for IFN-γ, showing localisation of staining to the Golgi apparatus.

A limitation of all published intracellular cytokine assays, as well as the whole blood technique described above, is the lack of a standard or reference preparation against which to compare assay performance on repeated measurements. In an attempt to address this, several approaches were taken.

3.2.4.1 Cell lines and frozen lymphocytes

The first approach to produce a reference preparation, which could be cultured and stained as a control in every intracellular cytokine assay, was to use a T cell line. Jurkat cells (the kind gift of Dr Kamal Ivory) were rapidly defrosted from liquid nitrogen in a 37°C water bath, and cultured at an initial concentration of 10^6/ml in 260 ml ventilation capped tissue culture grade flasks (Nunclon) in RPMI1640, supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml), L-glutamine (2 mmol/l) and 10% fetal calf serum. Aliquots of cells (250 µl) were removed after various times in culture and intracellular cytokines were assessed using the PBMC technique described in the general materials and methods section. Unfortunately, the cells used did not express any detectable cytokine. This may have been because the cell line was not a cytokine-producing line, or that the activation requirements were different to normal human cells. This approach was therefore abandoned.

One previous study of intracellular cytokines referred to a frozen lymphocyte standard which was defrosted and cultured with patient samples in every assay, but did not describe the method used (Meyaard *et al*, 1996). It transpires that this approach gave >20% variability and is no longer used (Frank Miedema, personal communication). Preliminary experiments to establish a standard in this study for the intracellular cytokine assay using frozen lymphocytes was unsuccessful as although cell viability was high (>80%) after defrosting, the cells invariably underwent spontaneous lysis following fixation and permeabilisation (data not shown).

3.2.4.2 Standard donor

The reproducibility of the whole blood intracellular cytokine technique was established by repeated samples from a single donor over a period of >1 year. The same batch of monoclonal antibodies, and aliquots of the same frozen (-80°C) stimulants were used throughout the year. Venous blood samples were withdrawn from the same male donor

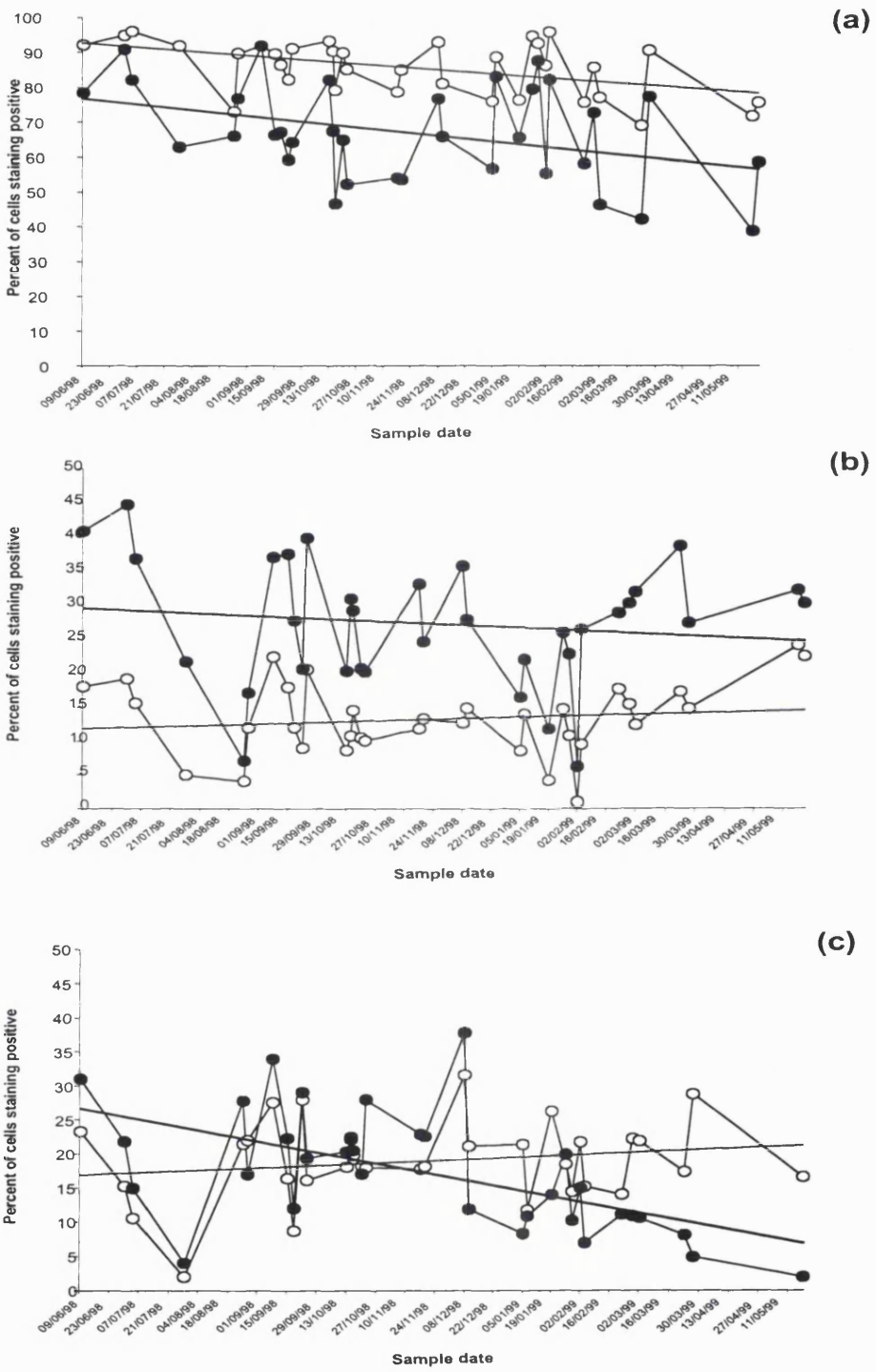


Figure 3.7: Expression of (a) CD69 expression, (b) IFN-γ, (c) TNF-α over a one year period in (o) CD4 and (•) CD8 cells from one donor. Trend lines were calculated by least-squares best fit (Microsoft Excel).

(myself) during a >1 year period at various intervals ranging from 1 day to 1 month, and intracellular IFN-γ, TNF-α and surface CD69 expression were determined in $CD4^+$ and $CD8^+$ cells. The results are plotted in Figure 3.7, and the coefficients of variation are shown in Table 3.2.

	CD69		IFN-γ		TNF-α	
	CD4	CD8	CD4	CD8	CD4	CD8
N	33	33	33	33	32	32
Mean	85.6	67.3	13.5	27.8	19.1	17.5
SD	7.9	14.1	5.1	8.7	6.1	9.0
CoV %	9.2	20.9	37.5	31.2	31.7	51.4

Table 3.2: Coefficients of variation for various parameters measured using the whole blood intracellular cytokine assay on one normal subject during the course of a year, giving an estimate of inter-assay variability. Data are expressed as percentage of $CD4^+$ or $CD8^+$ cells expressing the marker shown. SD, standard deviation.

As with the assessment of within-assay variability, the CoVs for the CD69 expression was the smallest. The CoV for the cytokines (except for CD8 TNF) remained around 35%, which is reasonably acceptable for a biological assay of this kind. The trend lines do not show a consistent increase or decease in cytokine expression with time. In addition, where there is low expression of cytokine (e.g. one occasion of low IFN-γ expression in February 1999) this does not coincide with low CD69 expression. This suggests that the variation is due in some way to the state of immunoregulation within the donor, rather than the stimulants decaying with storage, for example.

Because of the difficulties with developing a reference preparation for subsequent use of the assay, two important steps were taken throughout this work to control for inter-assay variability: (1) for comparisons between subject groups, subjects from each comparison group would be analysed in the same run together whenever possible. (2) Where comparisons were to be made over time, for example in the high-dose intravenous immunoglobulin experiment (Chapter 5), the same normal donor would be bled at each time point as the subject.

3.2.5 Further developments of the assay

Once the whole blood intracellular cytokine assay had been established, a number of other developments were attempted, as described below:

3.2.5.1 IL-4 producing cells

Using the whole blood technique with the same concentrations of PMA and ionomycin as for IFN-γ resulted in very low percentages of $CD4^+$ (and even fewer $CD8^+$) cells being positive for IL-4 (mean of 0.5% and 0.1%, respectively, in the measurements taken over one year in one normal subject). In addition, the CoV for the IL-4 assay was extremely large (90.5% for CD4 cells and 187.7% for CD8 cells). Varying the concentrations of PMA and ionomycin did not increase the IL-4 production (data not shown), even if extremely atopic subjects were used. Following suggestions that PMA may specifically inhibit IL-4 production in normal T cells (as opposed to T cell lines) (van der Pouw-Kraan *et al*, 1993), attempts were made to stimulate cells with various concentrations of phytohaemagglutinin (PHA) or concanavalin A (conA), but these were similarly unsuccessful (data not shown). Other workers have used double staining techniques to identify $IL\text{-}4^+$ cells, but only in mononuclear cell preparations, not with whole blood (Ivory, 1997). This technique is time consuming, and as it also requires PBMCs instead of whole blood, was not developed in this study.

3.2.5.2 Other cytokines

The ability to examine cells for expression of additional cytokines would be useful, and attempts were made to adapt the technique using other antibodies to other cytokines. These included: IL-10 (Serotec), TGF-β (Serotec), and IFN-α (Serotec). Unfortunately these antibodies did not give consistently positive staining of cells. This may have been because the stimulant types, concentrations or culture times were not appropriate for these cytokines, or that the monoclonal antibodies were not suitable for intracellular staining. Since the whole blood intracellular cytokine assay was successful for the major Th1 cytokines (IFN-γ, TNF-α) as well as IL-2, and IL-4 (with the proviso given above) it was decided to confine future work to these cytokines.

3.2.5.3 NK cells

The assay was also extended to examine cytokines in $CD16^+$ and $CD56^+$ NK cells in cultured whole blood. Preliminary work established that the red cell lysis medium (Optilyse C) and the fixation and permeabilisation reagents (Leucoperm A and B) interfered with the staining for CD16 and CD56 antigens. An alternative protocol was therefore established, in which blood was cultured in exactly the same manner as for the

IFN-γ assay up to the point of red cell lysis. At this stage, before permeabilisation, the cells were stained with saturating concentrations of anti-CD16 and anti-CD56 monoclonal antibodies (15 min). Red cells were then lysed, and the remaining cells were fixed, permeabilised and stained as for the IFN-γ technique. As with the CD4 antigen on T cells, CD16 became down-regulated during cell culture with PMA and ionomycin (see Figure 3.8), therefore NK cells were defined as $CD3^{-}CD56^{+}$ in this study.

3.2.5.4 Monocytes

Because of the importance of IL-12 from monocytes as an inducing factor for the expression of IFN-γ in T cells and NK cells, staining for intracellular monocyte IL-12 in whole blood samples was also done following the procedure developed jointly with Dr Rosario Cambronero (Cambronero, Sewell, *et al*, manuscript submitted). Aliquots (250 µl) of heparinised blood were diluted 1:2 in round bottomed polystyrene tubes with RMPI1640 (Life Technologies) and cultured with monensin (3 µmol/l) with or without lipopolysaccharide (0.1 µg/ml, Sigma) for 5 h at 37°C in a humid 5% CO_2 atmosphere. After culture, the cells were washed once in RPMI1640 and the cell pellet was resuspended in 250 µl medium. Aliquots (50 µl) were added to conjugated monoclonal antibodies directed against surface markers (CD3, CD14 and HLA/DR) and incubated at room temperature for 15 min. Red cells were then lysed by the addition of Optilyse C (500 µl, 15 min) and the remaining cells were washed again prior to fixation, permeabilisation and staining as for the IFN-γ method, using anti-IL-12/PE (Pharmingen). In the flow cytometric analysis, monocytes were defined as $CD3^{-}$ $CD14^{+}DR^{+}$. Cytokine-negative cells were again defined using regions set with the monensin-only treated cells, to measure the increase in IL-12 expression on activation, since there was a very low level (<1%) of IL-12 expression in the monensin-only treated cells.

3.2.5.5 V-beta family

Since the precursor frequency of antigen-specific T cells is very low in normal individuals, and particularly in CVID studied here (Kondratenko *et al*, 1997) this intracellular cytokine technique was not attempted for detecting antigen specific responses. Attempts were however made to use the assay to distinguish antigen-

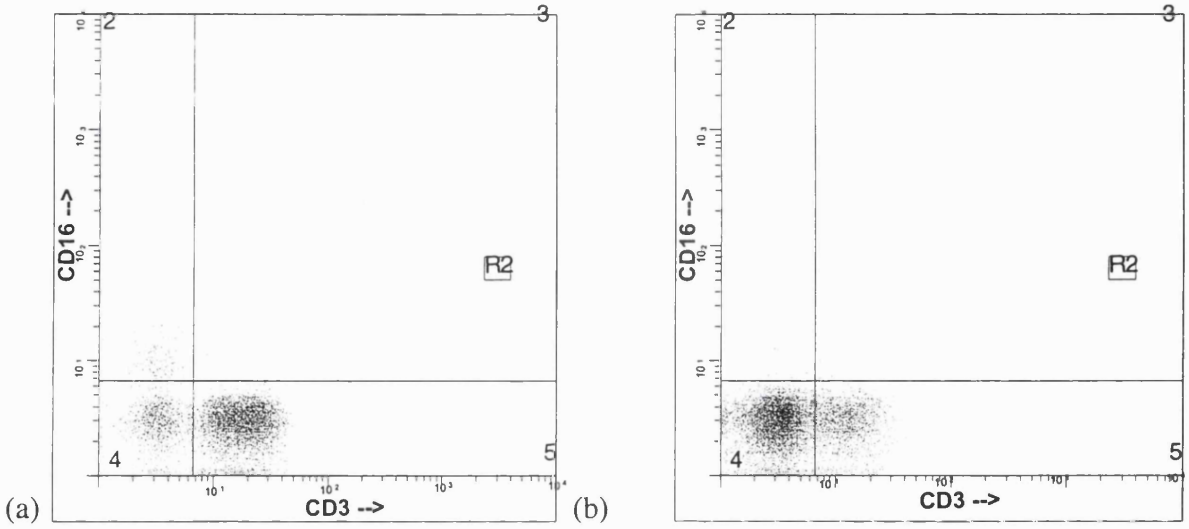


Figure 3.8 Down-regulation of CD16 expression on NK cells following stimulation with PMA and ionomycin. (a) Cells cultured with monensin only, (b) cells cultured with PMA, ionomycin and monensin.

responsive cells on the basis of their T cell receptor (TCR) Vβ usage. In patients with melanomas, there is often an oligoclonal T cell response against the tumour, for example, Sensi *et al* (1995) demonstrated that the HLA-A2-restricted cytotoxic T cell response against melan-1/MART-1 is restricted to TCR Vβ14 or TCR Vβ7. For this reason, in an attempt to use the assay as an approach to monitoring intracellular cytokines during immunotherapy of melanoma patients, cells were also stained with anti-Vβ14/PE monoclonal antibodies (Immunotech). The aim was to be able to analyse the IFN-γ content of $V\beta14^+$ cells separately, following stimulation with PMA and ionomycin as before. Vβ14-bearing T cells constitute a minor population in humans, of approximately 2.2-5.6% of $CD3^+$ T cells (Immunotech Vβ14 monoclonal antibody data sheet.). It was hoped that IFN-γ produced by this subpopulation would reflect an anti-melanoma response more specifically than studying all $CD8^+$ cells. Unfortunately, $V\beta14^+$ cells could not be clearly distinguished using this technique (see Figure 3.9), and therefore the subset-specific cytokine production could not be adequately determined. This failure to distinguish Vβ14 adequately was due to the dim fluorescence of the Vβ14 antibody on Vβ14-positive cells. This may reflect the fluorochrome conjugation of the monoclonal antibody used, or the low numbers of $V\beta14^+$ cells in the donors used. Further work in this area could be done using different Vβ14 antibodies, or perhaps a two-layer staining technique to amplify the Vβ14 signal.

3.2.5.6 Subset specific responses

The ability to identify lymphocyte subsets by expression of up to three surface markers in addition to a cytokine-fluorescence signal using four-colour flow cytometry has great potential to determine the mechanisms of cytokine regulation in disease. This is particularly applicable to common variable immunodeficiency, since cytokine expression within (say) just the $CD8^+$ population may not reveal an abnormality when compared with $CD8^+$ cells from normal controls, yet closer examination of lymphocyte subsets, such as subdivision of $CD8^+$ cells into $CD8^+28^-$ and $CD8^+28^+$ populations is helpful (North *et al*, 1998). Several sub-setting strategies were therefore used in this study including: (1) Division of $CD4^+$ cells into $CD4^+7^-$ and $CD4^+7^+$ cells, since the $CD7^-$ population may represent a separate memory compartment (Reinhold & Abken, 1997)

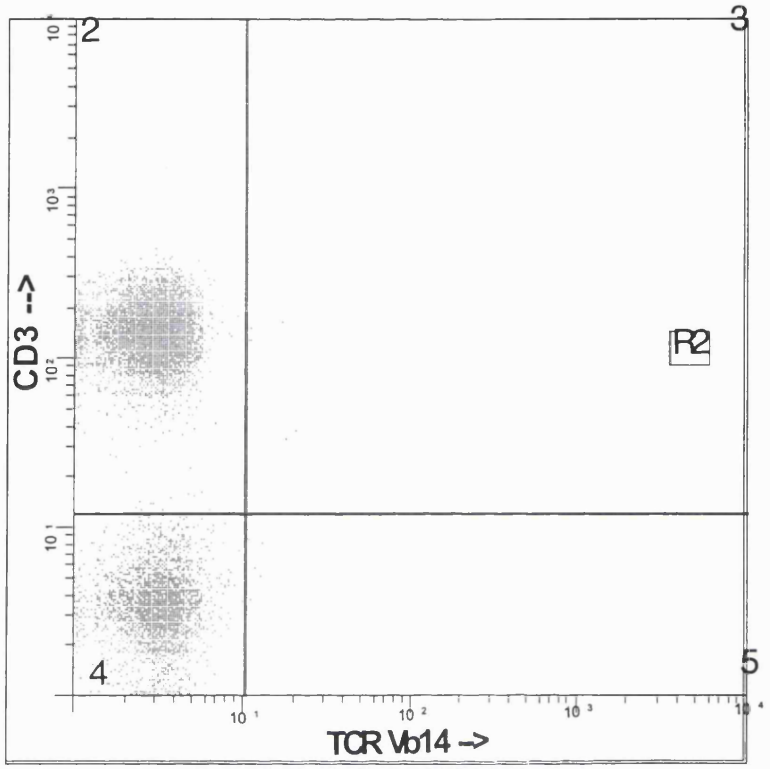


Figure 3.9 Vβ14 staining of human T cells. Note that despite high concentrations of antibody, positive cells are not bright enough for a clear Vβ14 region to be defined around them.

and may be Th2-biased (Autran *et al*, 1995). (2) Division of $CD8^+$ cells into $CD8^+28^-$ and $CD8^+28^+$ illustrated in Figure 3.10. The results of these are given in Chapter 4.

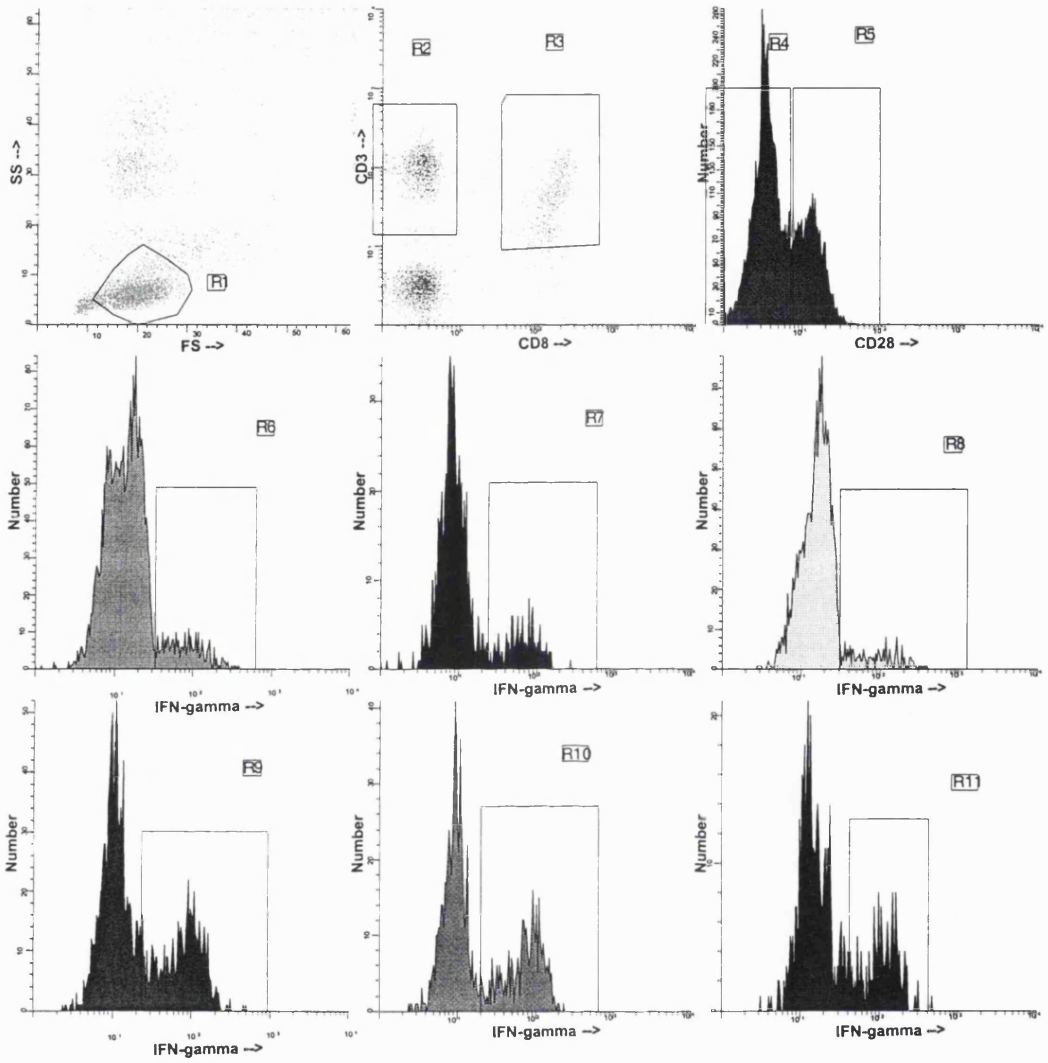


Figure 3.10 Cytokine expression in lymphocyte subsets. In this example, IFN-γ expression in $CD4^+$ ($CD3^+8^-$) and $CD8^+$ cells and their $CD28^-$ and $CD28^+$ subsets are shown. Lymphocytes are defined by region R1 in the first panel, so all panels after the first are gated on R1. Regions R2 and R3 define CD4 and CD8 cells respectively. Region R4 defines CD28- lymphocytes, R5 defines CD28+ lymphocytes. The middle row shows IFN-γ expression in CD4+ lymphocytes, as CD4+ (ie gated on R1 AND R2), then IFN-γ in CD4+CD28- lymphocytes (ie R1 AND R2 AND R4), finally IFN-γ in CD4+CD28+ cells (ie R1 AND R2 AND R5). The bottom row is similar, showing IFN-γ expression in CD8 cells, CD8+28- cells and CD8+CD28+ cells respectively.

3.3 Discussion

In this part of the project, a rapid, small sample volume, whole-blood technique was developed for the determination of intracellular cytokines. Commercially available reagents were used, concentrations of stimulants were optimised and the technique appears to be suitable for adaptation for use in routine clinical immunology laboratories. The advantages of PMA and ionomycin as stimulants, independent of accessory cells, were demonstrated in earlier work (Sander *et al*, 1991) and these reagents were found to be superior to phytohaemagglutinin (PHA) (North *et al*, 1996). The stimulus chosen is deliberately supra-physiological in order to activate as many cells as possible capable of producing cytokine under the conditions that the cell is in at the time. Antigen-specific stimuli were not used, as the antigen-specific T cell frequency in normal subjects, but particularly in common variable immunodeficiency is very low (Kondratenko *et al*, 1997). Furthermore, it is not yet clear whether antigen stimulation through the T cell receptor, in combination with co-stimuli, is capable *in vitro* of inducing adequate cytokine expression measurable by current flow-cytometric techniques, although there are some encouraging preliminary results emerging that this approach is feasible (Margaret North, personal communication).

The kinetic data demonstrates that short culture times (e.g. 2 h) for whole blood samples are sufficient for IFN-γ and TNF-α expression; longer culture times resulted in lower levels of these two cytokines. The optimal conditions used are a compromise between maximising cytokine expression and minimising down-regulation of surface CD4 which prevents efficient discrimination of $CD4^+$ cells. However, for IL-2, 2 h of stimulation was insufficient, so a 4 h period was chosen, accepting the increased difficulties in discriminating $CD4^+$ cells. One obvious way round the difficulty of the down regulation of the CD4 antigen is to use Boolean algebra to gate the CD4 cells as being [within the lymphocyte region] AND [CD3 positive], AND [CD8 negative].

There are noticeable differences in the kinetics of cytokine production in this whole blood system when compared with other studies using PBMC, illustrating the physiological importance of the whole-blood system used here. For example, cytokine

production in whole-blood systems has been reported to be greater than in PBMC systems (de Groote *et al*, 1992). Although this has been contested (Jason and Larned, 1997) the assay systems used in each system were not comparable.

The difficulties in standardising the measurement of intracellular cytokines have been confirmed by other workers (e.g. Jason & Larned, 1997), with the consensus view being that standardisation of protocols, and simultaneous comparison of test and control subjects is essential. The use of cell lines or frozen standards was not successful in the present work, although standards for intracellular cytokines have recently been marketed as HicK2 cells (Pharmingen). The performance characteristics of these were not available from the manufacturer, and samples were not available to assess during the period of this study.

Difficulties are also widely recognised in the reliable determination of IL-4^+ cells. Most reports suggest a very low proportion of these cells (a maximum of approximately 1% of $CD4^+$ cells). Although there have been reports of high percentages of IL-4^+ cells being detected (e.g. Pharmingen Research Products Catalog, 1988, page 359), these have used complex approaches, with multiple stimulants (including IL-4 itself) and are not readily adaptable to routine clinical practice. Other techniques have used PBMC as opposed to whole blood samples, with all the disadvantages that this entails (Jolles *et al*, 1999). Also, the choice of stimulant used in the present project (PMA & ionomycin) has been suggested to be inappropriate for IL-4 production by fresh T cells as opposed to T cell lines (van der Pouw-Kraan *et al*, 1993), yet alternative stimulants (PHA, ConA) gave no improvement in the IL-4 signal in this study. For these reasons, IL-4 was determined using the same protocol as for IFN-γ, with cautious interpretation of the results given the problems discussed.

In conclusion, the optimal conditions worked out for the whole blood intracellular cytokine assay (for use in lymphocytes) and used in the following sections were therefore:

Dilute blood 1:2 with RPMI1640 in round-bottomed polystyrene tubes containing the following stimulants:

For assessment of IFN-γ, TNF-α, IL-4 and CD69 expression:

PMA (10 ng/ml), ionomycin (2 μmol/l), monensin (3 μmol/l) cultured for 2 h

For assessment of IL-2 expression:

PMA (10 ng/ml), ionomycin (2 μmol/l), monensin (3 μmol/l) cultured for 4 h

4 Intracellular cytokine measurements in immunological disorders

4.1 Introduction

Having developed the whole blood method for the determination of intracellular cytokines described in Chapter 3, studies were undertaken of its usefulness in a variety of immunological disorders. This enabled an assessment of diseases with presumed Th1/Th2 'imbalances' and also a comparison of whole blood versus PBMC methods. Furthermore, the subsetting approach using four colour flow cytometry, permitted new insights into cytokine regulation at the individual lymphocyte subset level which have not been previously determined, for example within γ/δ^+ T cells, within $CD4^+7^{-/+}$ subsets and within $CD8^+$ cells where a 'Th1/Th2-like' system is now being recognised.

The principal disease studied was the primary immunodeficiency common variable immunodeficiency (CVID) using XLA patients as a control. In addition, patients with sarcoidosis, hyper IgE syndrome and atopic eczema were studied.

4.2 Results

4.2.1 Normal subjects

Ten normal volunteers: 5 males, 5 females, mean age 39.7 years, were used as a control group for comparison with the patient data. Peripheral blood was drawn into heparinised tubes and intracellular cytokines were measured using the whole blood technique developed and described in Chapter 3. Intracellular cytokine analysis was undertaken within 1 hour of sampling. IFN-γ and TNF-α expression within $CD4^+$ and $CD8^+$ cells was examined, as well as surface CD69 expression as a measure of cell activation.

The results are illustrated in Figure 4.1. Despite variability in cytokine expression between donors, several patterns emerge, namely: that IFN-γ expression following cell activation is higher in $CD8^+$ (mean 41.8% ± SD 20.1) than $CD4^+$ cells (Mean 12.0% ± SD 7.4) (p=0.0002, Student's paired t-test), and that TNF-α expression is the same in

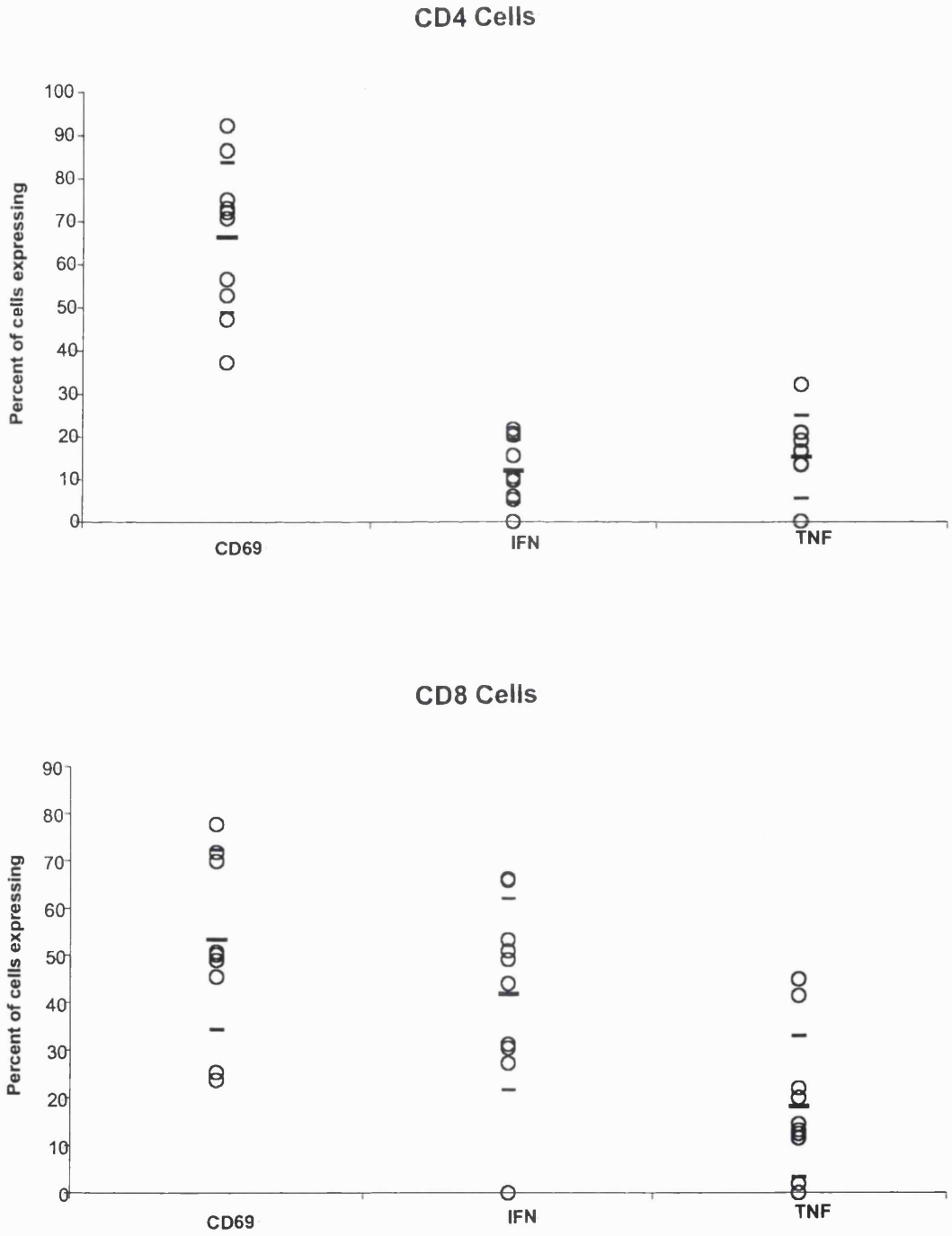


Figure 4.1 Percentage of $CD4^+$ and $CD8^+$ cells expressing CD69, IFN-γ and TNF-α in normal subjects following stimulation with PMA and ionomycin in whole-blood culture. Each symbol represents one donor. Mean ± SD shown by horizontal lines.

$CD4^+$ (mean 15.3% ±9.7) and $CD8^+$ (mean 18.1% ±14.9) cells (p not significant). $CD4^+$ cells also show a greater percentage of $CD69^+$ cells (66.5% ± 17.6) following stimulation than do $CD8^+$ cells (53.3% ±19.0) (p=0.0016, by student's paired t-test).

The difference in CD4 and CD8 IFN-γ expression is interesting, given that the $CD4^+$ cells still show greater activation in terms of CD69 expression. No clear explanation exists for this finding, which has also been noted in PBMC cultures (North *et al*, 1996), although it may be that the cytotoxic role of CD8 cells necessitates a greater proportion of cells to generate cytotoxic cytokines. It certainly shows that although the 'Th1/Th2' paradigm was established in $CD4^+$ cells, $CD8^+$ cells are also (more) potent producers of some cytokines and must be considered in models of cytokine immunoregulation to explain immunological disorders.

4.2.2 Common variable immunodeficiency

4.1.1.1 Cytokines in $CD4^+$ and $CD8^+$ cells

Initial studies were performed with a cohort of 12 CVID patients receiving regular intravenous immunoglobulin replacement therapy. Samples were taken immediately prior to infusion of the IVIG therapy, and intracellular cytokines were assayed within one hour of sampling.

Comparison of the expression of CD69, IFN-γ and TNF-α in $CD4^+$ and $CD8^+$ cells between CVID and normal donors is shown in Figure 4.2.

In the whole blood preparation, there was no significant difference between normal subjects and patients with CVID in terms of CD69 expression for either $CD4^+$ cells (66.5% ±17.6 in normals versus 71.1% ±22.3 in CVID, mean ± SD) or $CD8^+$ cells (53.3±19.0 versus 57.0±23.7). Similarly, there was no significant difference between CVID and normals for TNF-α expression in $CD4^+$ cells (15.3±9.7 versus 14.8±9.7) or $CD8^+$ cells (18.4±14.9 versus 10.3±10.0). However, IFN-γ expression was significantly elevated in CVID compared with normal subjects, in both $CD4^+$ cells (26.0±11.0 versus 12.0±7.35, p=0.014) and $CD8^+$ cells (49.0±22.4 versus 41.8±20.1, p<0.0001).

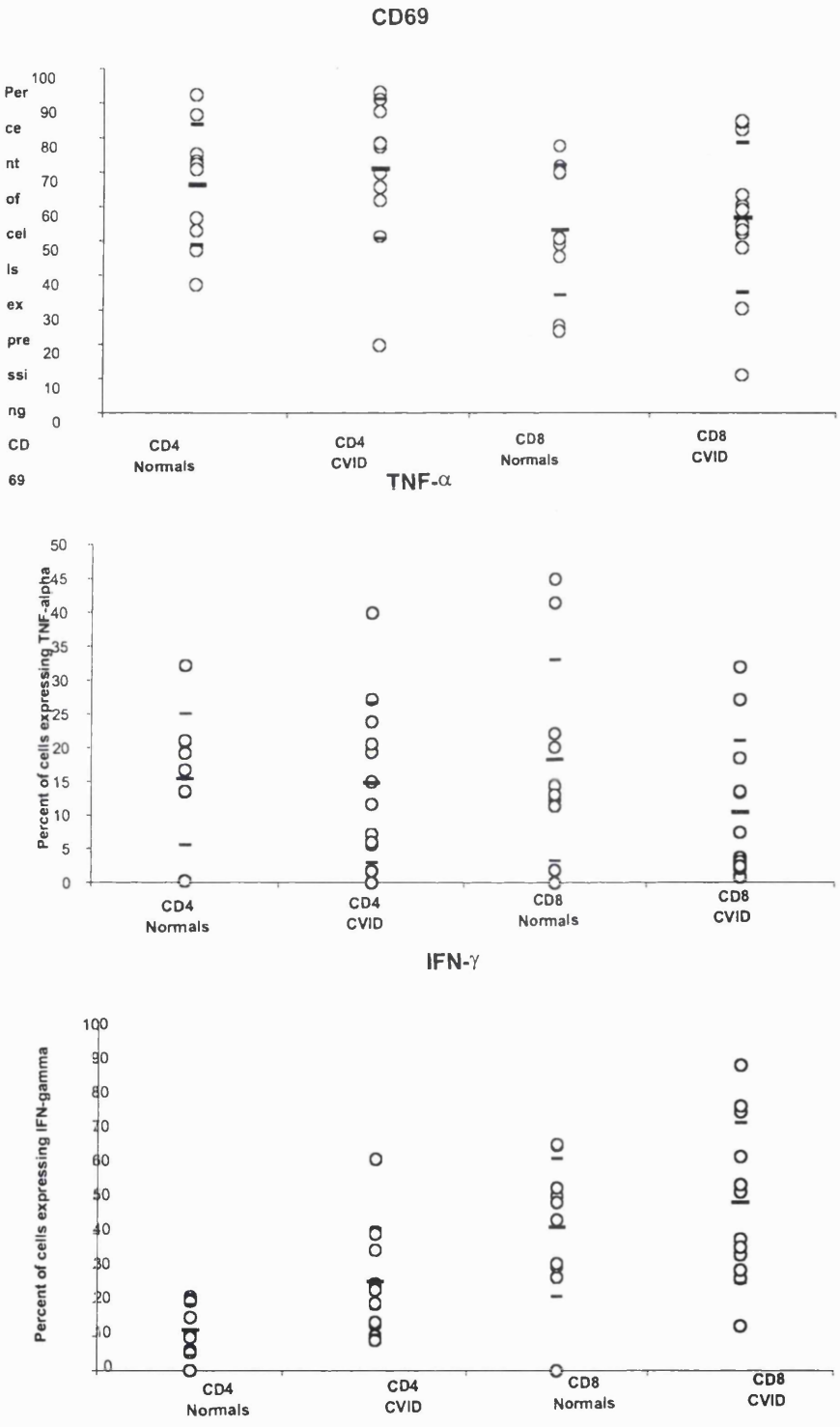


Figure 4.2 Comparison of CD69 (top), intracellular IFN-γ (middle), and TNF-α (bottom) expression in $CD4^+$ and $CD8^+$ cells following 2 h culture with PMA and ionomycin in normal subjects and patients with CVID. Horizontal lines show mean and standard deviation.

The pattern of cytokine and activation marker expression between $CD4^+$ and $CD8^+$ cells was similar to that in normal subjects, with greater CD69 expression in $CD4^+$ cells than $CD8^+$ cells (71.7±22.3 versus 57.0±23.7, p=0.0004), similar TNF-α expression in $CD4^+$ and $CD8^+$ cells (14.8±9.7 versus 10.4±10.0, p not significant), and higher IFN-γ in $CD8^+$ cells than $CD4^+$ cells (49.0±22.4 versus 26.0±11.0, p=0.003).

4.1.1.2 Cytokines in CD28 subsets in normals and CVID patients

Using four-colour flow cytometry, division of CD4 and CD8 subsets into their $CD28^-$ and $CD28^+$ subpopulations was possible, with cytokine determination within each subpopulation. A small cohort of CVID patients was available for this study (n=9) with a smaller cohort of normal controls (n=5). The results of samples stimulated with PMA and ionomycin are shown in Figure 4.3.

In this experiment, CD69 expression following activation with PMA and ionomycin was uniformly lower in CVID than in normal controls, particularly in $CD8^+$ cells (median expression 43.1% versus 16.5%, Mann-Whitney U=58, p=0.008) and $CD8^+28^-$ cells (a population which is expanded in CVID (North *et al*, 1998)) where the median expression was 14.3% versus 31.7% in normals (U=53, p=0.046). Although there was also a significantly reduced expression of CD69 on $CD4^+28^-$ cells, these cells form a numerically very small population in both normals and CVID (see Chapter 7). Interestingly, the expression of CD69 was considerably greater on $CD8^+28^-$ cells than $CD8^+28^+$ cells in normal subjects, yet this was not the case in CVID, where CD69 expression on 28^- and 28^+ cells was the same. The reason for this may reflect differences in activation status of circulating cells between normal subjects and CVID patients. There is evidence that CD28- cells represent memory cells with different activation requirements to CD28+ cells (Mugnaini *et al*, 1999). The lower proportion of CD28-69+ cells in CVID may reflect some abnormality in activating memory cells in CVID.

TNF-α expression was generally increased in CVID in all these subpopulations, although this increase was only significant in the $CD4^+28^+$ population (median of 1.8% in normal subjects versus 8.3% in CVID, U=22, p=0.046).

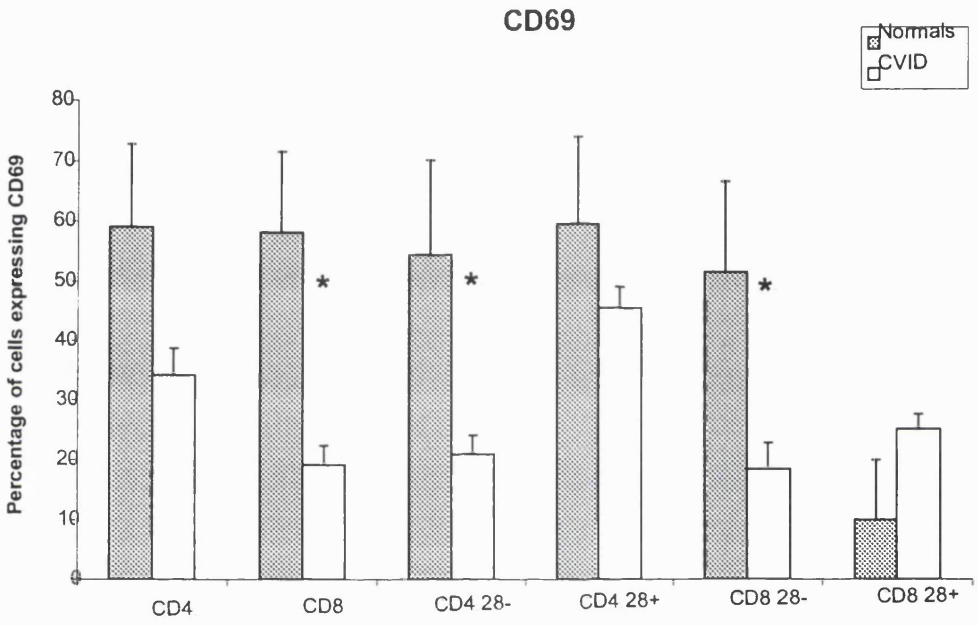
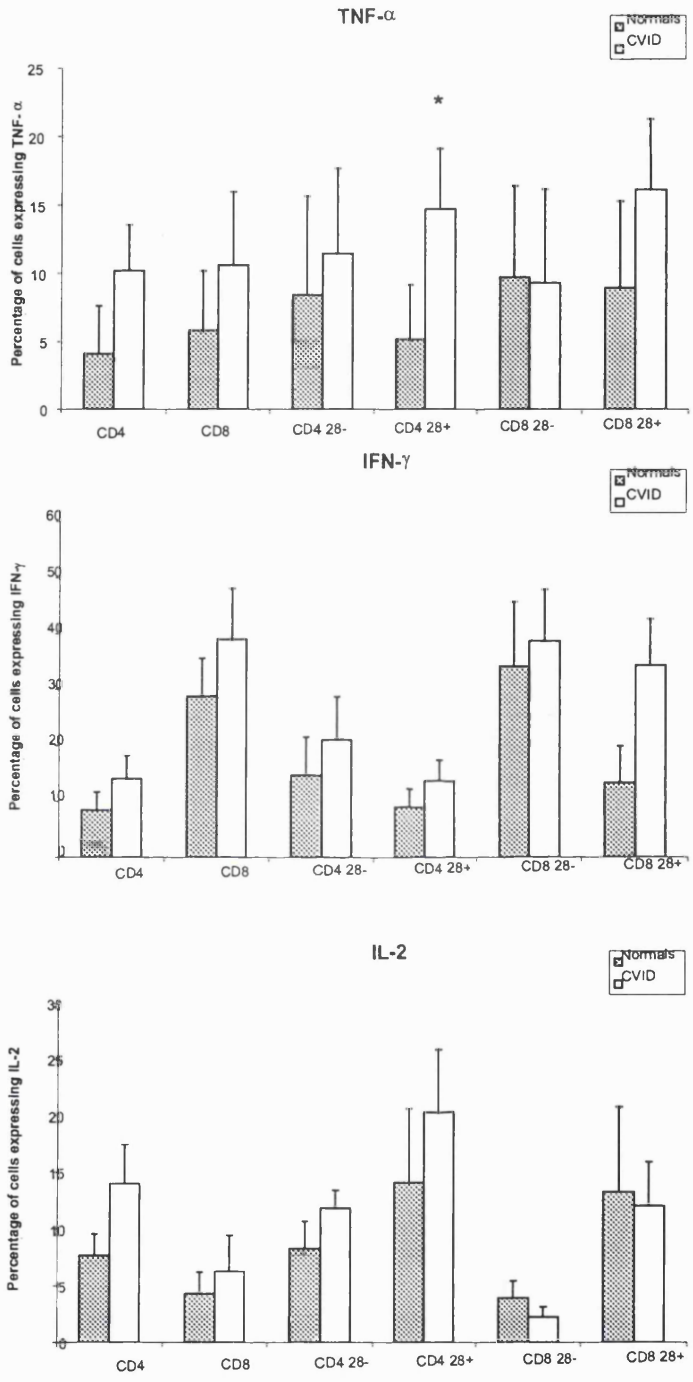


Figure 4.3 Expression of CD69 in subsets of CD4 and CD8 cells subdivided by CD28 expression. Bars show the mean and SEM. Subject groups were small and not normally distributed, so statistical comparisons between normals and CVID patients were made with the Mann-Whitney U test. * $p<0.05$



Figures 4.3 continued/...

Expression of TNF-α, IFN-γ and IL-2 in subsets of CD4 and CD8 cells subdivided by CD28 expression. Bars show the mean and SEM. Note that the subject groups were small and not normally distributed, so statistical comparisons between CVID patients and normal subjects were made with the Mann-Whitney U test. * $p<0.05$

Although when $CD8^+$ cells as a whole were examined there was a difference between IFN-γ expression between normal subjects and patients with CVID, IFN-γ expression in this experiment which subdivided $CD8^+$ cells on the basis of CD28 expression did not show a significant difference between normal controls and CVID patients. Since there was a significant difference in the proportion of cells which became activated and expressed CD69, it was anticipated that there would be a significant difference in IFN-γ expression in $CD8^+28^+$ cells, yet this was not found, although there was a trend to a greater expression in CVID. This contrasts with the finding of North *et al* (1998). The finding in the present study may have failed to reach statistical significance due to the small numbers in the normal control group, since there was a sizeable but non-significant difference between IFN-γ expression in $CD8^+28^-$ cells between normals and CVID patients.

IL-2 expression was similar in both CVID and normal controls. More $CD28^+$ cells (in both $CD4^+$ and $CD8^+$ subsets) expressed IL-2 than their $CD28^-$ counter-parts. This may reflect a genuine difference in cytokine regulation between these populations of cells, since it does not correlate with the extent of cell activation in terms of CD69 expression in Figure 4.3(a).

This experiment demonstrated that it was possible to measure cytokines in subdivisions of cell populations in whole blood beyond simple CD4/CD8 and that differences between normal and patient groups could be revealed. TNF-α was raised in CVID, especially in CD4+28+ cells, but expression of TFN-γ and IL-2 was not significantly different.

4.1.1.3 Cytokines in CD7 subsets in normals and CVID patients

CD7 is expressed on the majority of T cells, and recent work has suggested that $CD7^-$ helper T cells may represent a different memory compartment to $CD7^+$ cells (Autran *et al*, 1995). Additional work has suggested that $CD7^-$ T cells may be biased towards Th2-cytokines. There are no indications of differences in the function of $CD8^+7^-$ compared with $CD8^+7^+$ cells. A small cohort of normals (n=5) and patients with CVID (n=9) was therefore used to determine cytokine and activation marker expression following culture

with PMA and ionomycin using the whole-blood technique. The $CD7^-$ population is extremely small, so results are shown as the percentage of cells expressing cytokine (or activation marker) within the named subset. Comparisons for such a small cohort of donors were made using the Mann-Whitney U test. CD4 or CD8 populations were considerably less activated by PMA & ionomycin in CVID patients than in normal controls (median of 11% versus 49% for CD4, U=59, p=0.005; median of 5.9% versus 33.3% for CD8, Mann Whitney U (U)=60, p=0.003). Despite these differences in activation status, there were no statistically significant differences between normal subjects and CVID patients in terms of the percentages of cells expressing IFN-γ, TNF-α or IL-2 in CD4 or CD8 subsets of $CD7^+$ or $CD7^-$ cells (Figure 4.4)

Interestingly, there were also no statistically significant differences in cytokine expression between CD4 $CD7^+$ or $CD7^-$ cells, nor between CD8 $CD7^+$ or $CD7^-$ cells for either normal controls or CVID patients. This suggests that there is no bias away from a Th1-like cytokine profile in the $CD7^-$ helper cells, and neither does such a bias exist in the CD8 $CD7^-$ subset. This finding contrasts with Autran's study in HIV patients (1995), where $CD7^-$ cells had a Th2-like bias, and may reflect differences in methodology: intracellular cytokines were not measured in that study, culture periods were different and only cell lines were assessed.

4.1.2 X-linked agammaglobulinaemia

Patients with X-linked agammaglobulinaemia (XLA) are an important control group when examining the mechanisms behind CVID, since they are also hypogammaglobulinaemic, they receive the same dose of IVIG, and they are subject to a similar range of infections. XLA is less common than CVID, and the majority of patients at the Royal Free immunodeficiency clinic are young men receiving IVIG home therapy. For these reasons, there were relatively few occasions where an XLA patient was available for donating blood..

4.1.2.1 Cytokines in $CD4^+$ and $CD8^+$ cells in XLA

XLA patients (n=3) were bled immediately prior to IVIG therapy and the intracellular cytokine analysis was begun within 1 hour. Comparisons were made with 5 normal

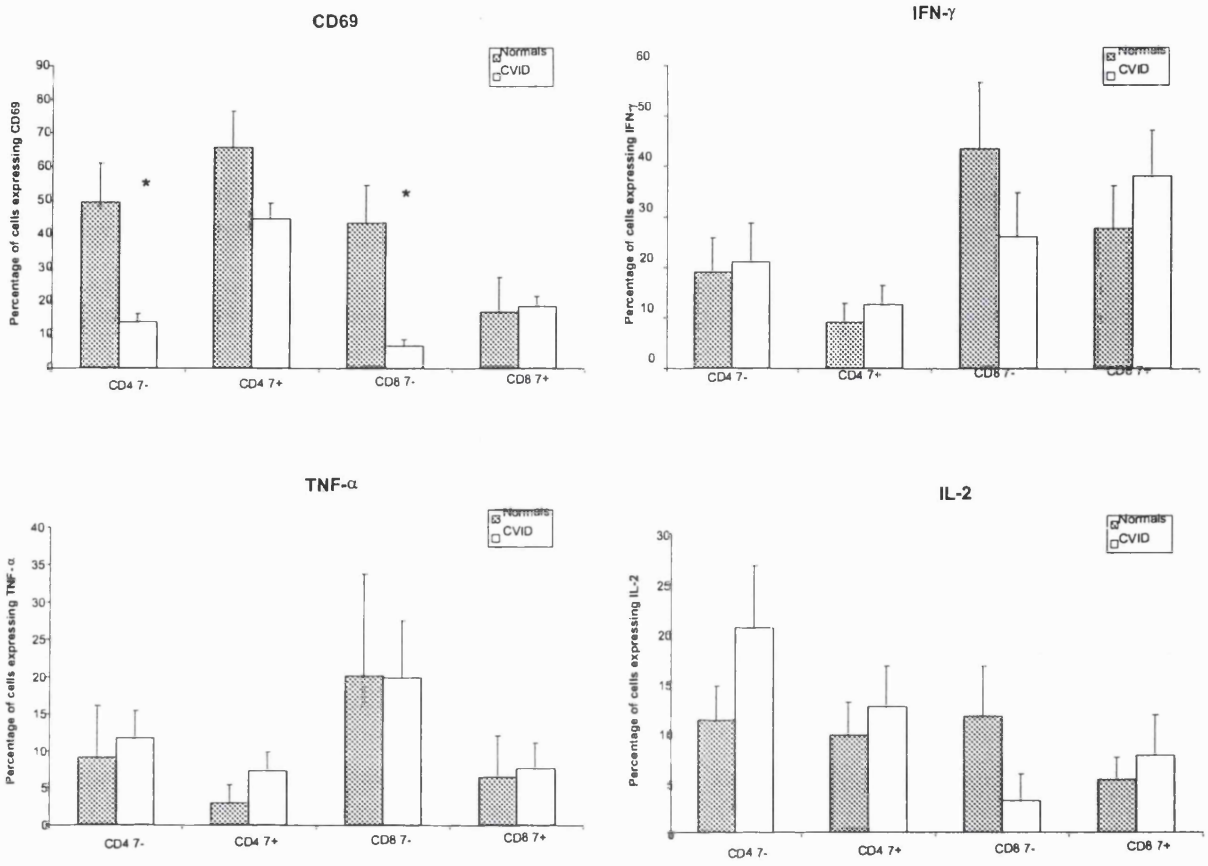


Figure 4.4 Cytokine expression within CD7 subsets of $CD4^+$ and $CD8^+$ cells. Although results are illustrated as the mean ±SEM, because of the small number of donors, statistical comparisons were made using the non-parametric Mann-Whitney U test. * $p<0.05$

control subjects. The results are shown as individual data points in Figure 4.5. Because of the small number of data points, formal statistical comparisons were not made.

As can be seen in Figure 4.5, the distribution of percentage of cytokine or CD69 expression following stimulation with PMA & ionomycin is similar in the 3 XLA subjects to that in normal controls, and was not like that of CVID patients who had a trend towards greater cytokine expression (discussed above).

4.1.2.2 Cytokines in CD28 subsets in XLA

Cytokine expression in CD28 subsets of CD4 and CD8 XLA cells was analysed in the same experiment as section 4.4.1, and is also illustrated in Figure 4.5.

CD69 expression in stimulated $CD8^+28^+$ cells was zero in 4/5 normals in this experiment, yet was 55-85% (range) in XLA. This failure of $CD8^+28^+$ activation by PMA & ionomycin in XLA was not reflected in cytokine expression, which was similar to that in the total $CD8^+$ cells. The CD8 cytokine and CD69 expression values were from cells in the same tube as the CD8/28 subset data so were not due to a failure of detection, for example by not adding anti-CD69 staining antibody. Unlike CVID, IFN-γ expression in $CD8^+28^+$ cells from the XLA patients was not elevated above that of the $CD8^+28^-$ cells (see Figure 4.3).

4.1.3 Sarcoidosis

The aim of this experiment was to apply the whole-blood intracellular cytokine assay to a patient group with a presumed Th1-like cytokine bias, namely patients with sarcoidosis. Patients with sarcoidosis were from Dr Huw Beynon's sarcoidosis clinic at the Royal Free Hospital. Samples were collected into lithium-heparin tubes and analysed within 4 hours of sampling. All patients had a clinical diagnosis of sarcoidosis made using clinical criteria, serum calcium and angiotensin-converting enzyme levels, and chest radiograph findings. Ten patients were used, 4 males, 6 females, mean age 50.9 years. Some patients were taking oral prednisolone, and one was also on methotrexate. Comparisons were made with a group of 12 normal controls.

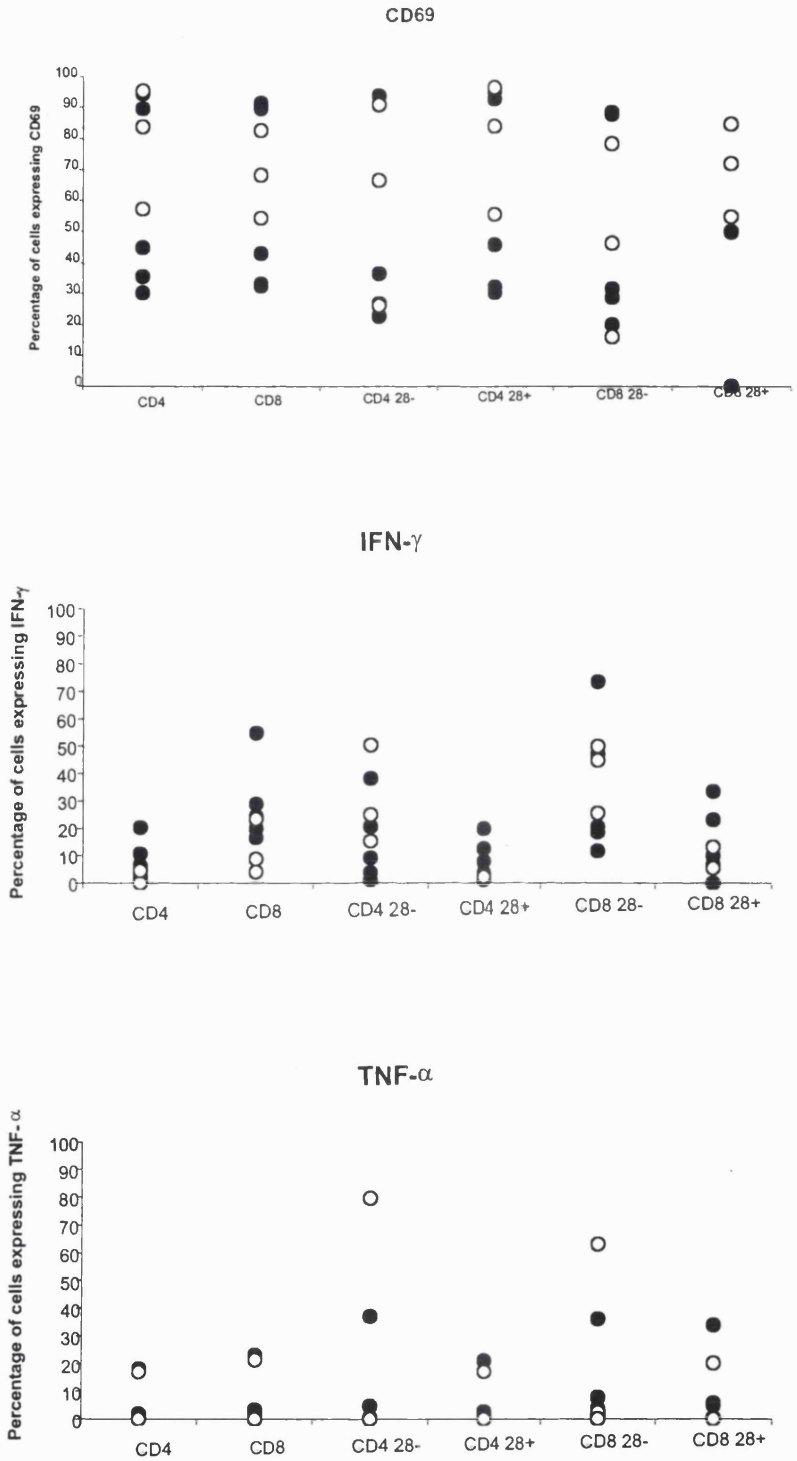


Figure 4.5

Expression of CD69, IFN-γ and TNF-α in $CD4^+$ and $CD8^+$ cells, and their CD28 subsets in normal subjects (black symbols) and XLA patients (white symbols). (Note that CD69 expression in $CD8^+28^+$ cells is 0 for 4 normal subjects, the data points lie on top of each other).

4.1.3.1 Cytokines in $CD4^+$ and $CD8^+$ cells in sarcoidosis

The distribution of CD69 expression and intracellular IFN-γ and TNF-α within $CD4^+$ and $CD8^+$ cells is shown in Figure 4.6.

There was no statistically significant difference in CD69 or TNF-α expression in $CD4^+$ or $CD8^+$ cells between normal subjects and sarcoid patients. However, there was significantly lower IFN-γ expression in $CD4^+$ cells from sarcoidosis patients compared with normal control subjects (mean ± SD of 7.3 ± 5.7% versus 26.0 ± 15.6%, p=0.0017 by Student's t-test). IFN-γ expression was also lower in $CD8^+$ cells (30.6 ± 19.1% versus 49.0 ± 23.4%) but this did not reach statistical significance (p=0.056).

This data was unlike the anticipated 'Th1-like' pattern expected. It may be that the Th1 phenotype cells involved in the sarcoid lesions are in granulomas within lung, lymph nodes, etc. and that whilst those cells are Th-1 biased, the cells sampled from the peripheral blood may not be. There is evidence in sarcoidosis for a difference in behaviour of peripheral blood cells compared with cells in broncheoalveolar lavage (BAL) fluid. Müller-Quernheim (1996) found high levels of spontaneous TNF-α production from BAL cells, especially in active sarcoidosis, yet normal TNF-α production by peripheral blood mononuclear cells. Following LPS stimulation, BAL cells from the sarcoidosis patients again produced more TNF-α than normal controls and the patient's peripheral blood cells produced equivalent amounts to normal subjects.

4.1.3.2 Cytokines in TCR γ/δ^+ T cells from patients with sarcoidosis

The aim of this experiment was to validate the use of the whole blood intracellular cytokine method in determining cytokines within minority populations of lymphocytes. An example is within TCR γ/δ^+ T cells. Since sarcoidosis is characterised by granuloma formation (Müller-Quernheim, 1996), and since granulomas (at least in animal models) contain many γ/δ T cells (Bluestone *et al*, 1989), the intracellular cytokine technique was adapted to examine IFN-γ expression within γ/δ T cells from patients with sarcoidosis, using the following four-colour staining combination: IFN-γ/FITC, TCR γ/δ/PE, CD4/ECD, CD8/PC5. Cells within a lymphoid forward/side scatter gate which

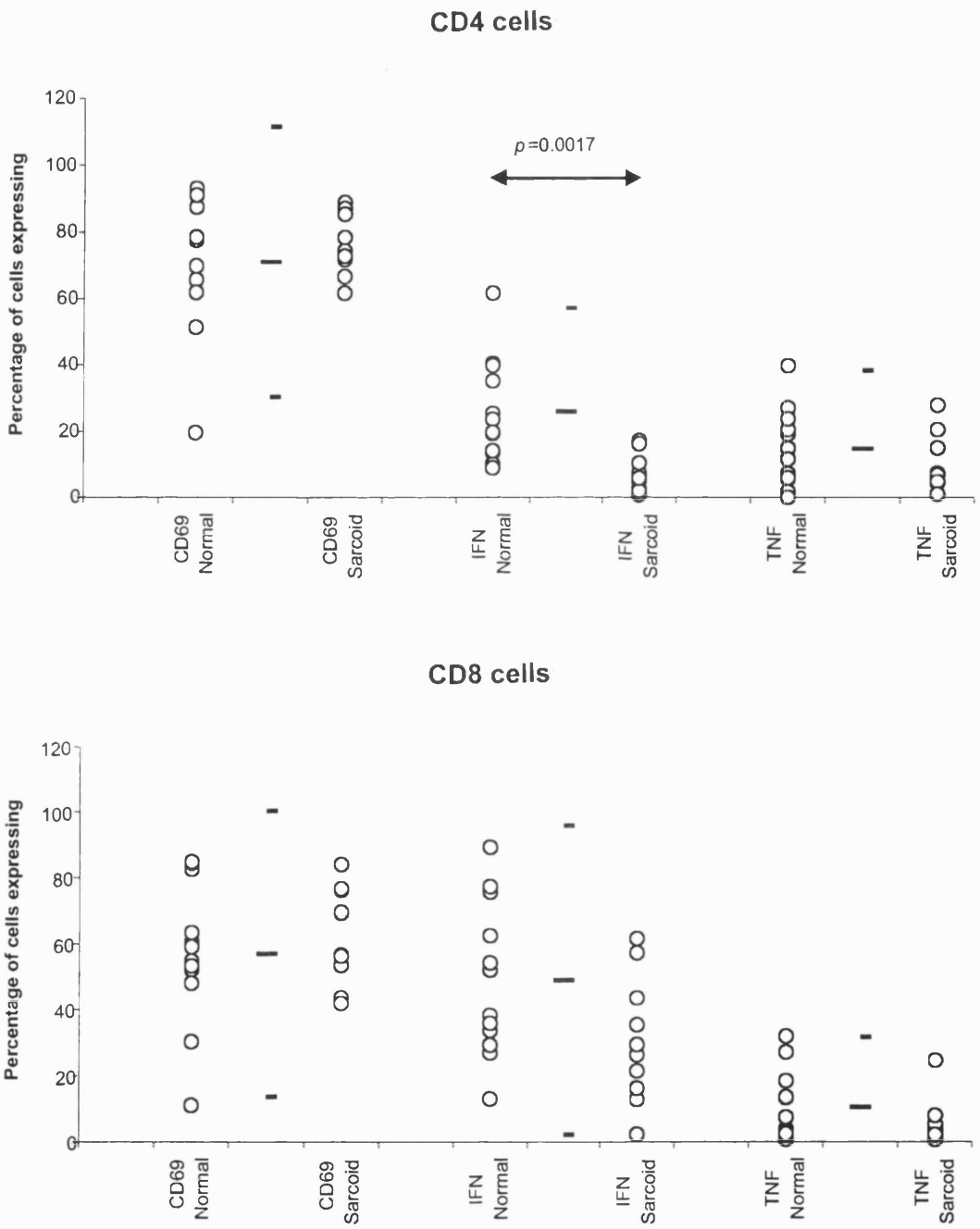


Figure 4.6 Expression of CD69, IFN-γ and TNF-α in $CD4^+$ and $CD8^+$ cells in normal subjects (n=12) and patients with sarcoidosis (n=10). Horizontal lines denote mean and 95% confidence interval based on normal subjects.

expressed TCR γ/δ but neither CD4 nor CD8 were designated as γ/δ$^+$ T cells. Cytokine-negative cells were again defined using monensin-only treated cultures, with other stimulus conditions the same as for the determination of IFN-γ in CD4/CD8 cells. The expression of IFN-γ in γ/δ T cells is shown in Figure 4.7. The percentages of CD4$^+$, CD8$^+$ and γ/δ T cells within the lymphocyte gate are shown in Table 4.1.

Patient	% CD4$^+$	% CD8$^+$	% γ/δ$^+$
1	16.1	15.5	14.8
2	31.8	10.0	6.8
3	36.6	22.7	1.6
4	33.9	38.8	3.1
5	34.1	12.2	5.8
6	18.6	23.2	0.6
7	36.5	8.5	0.6
8	41.1	10.4	0.8
9	41.9	7.4	1.1
10	49.1	18.1	0.7
Mean	34.0	16.7	3.6
(SD)	(10.1)	(9.6)	(4.5)

Table 4.1

Percentages of lymphocyte populations in 10 sarcoidosis patients. Note that CD8$^+$ was exclusively CD8^{bright} cells only. SD = standard deviation.

This experiment demonstrated that, in agreement with other studies, patients with sarcoidosis do not have a substantial percentage of circulating peripheral blood γ/δ T cells. Wilsher *et al* (1995), for example found that the majority of sarcoidosis patients have similar percentages of circulating γ/δ T cells as found in normal controls (<5%) but with occasional patients with higher levels (~15%, similar to patient 1 in table 4.1). γ/δ T cells from sarcoidosis patients are quite capable of synthesising TNF-α and IFN-γ when stimulated, and of expressing CD69 on activation. Comparisons between the subsets using Student's paired t-test, shows that γ/δ T cells have comparable levels of CD69

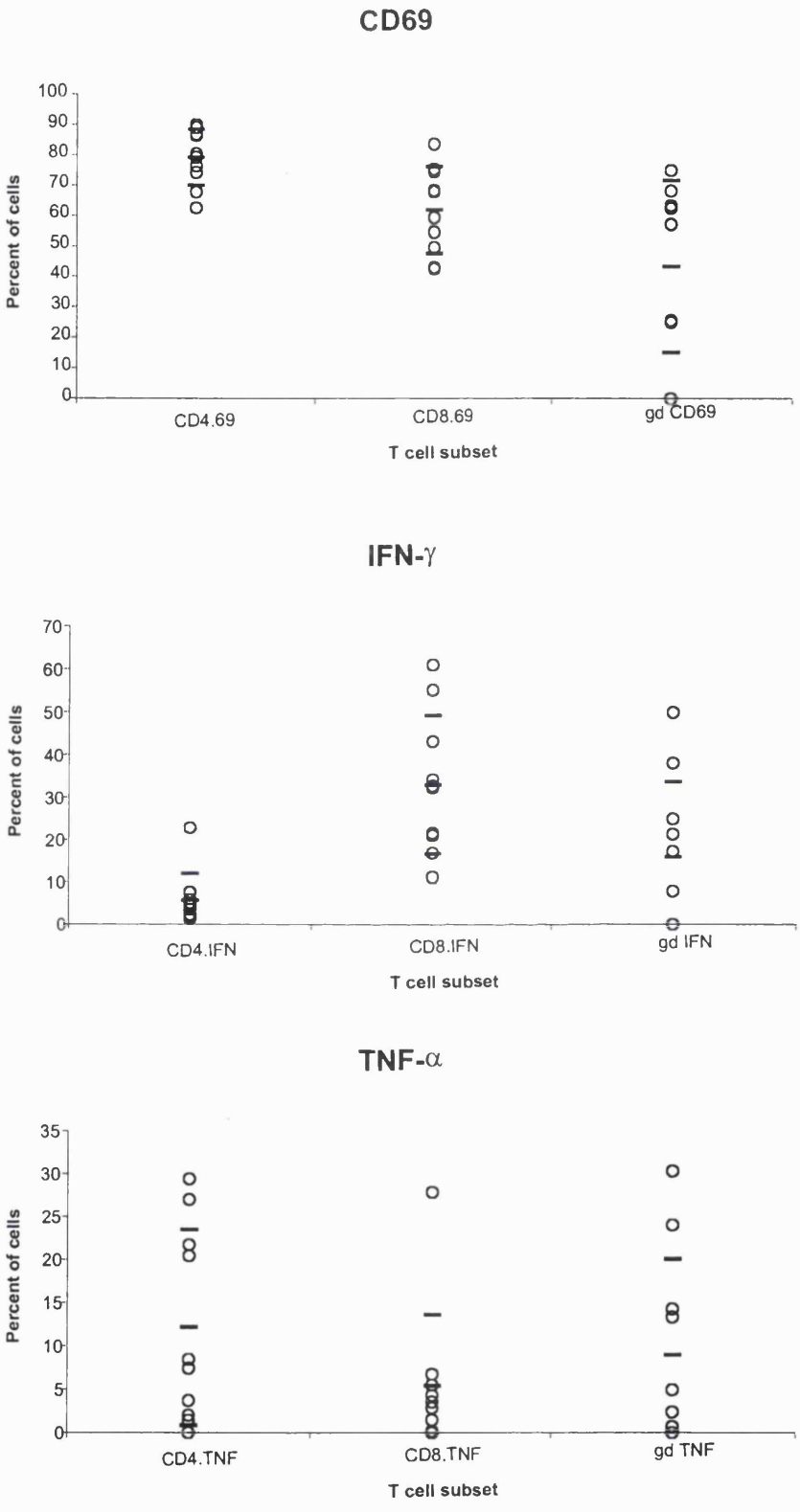


Figure 4.7
CD69, IFN-γ and TNF-α expression within γ/δ T cells in sarcoidosis. 'gd' = γ/δ T cells. Horizontal lines indicate mean and standard deviation.

expression (mean ± SD 43.4±28.3%) on activation to $CD8^+$ cells (62.0±14.3%), but significantly lower levels than $CD4^+$ cells (79.3±9.2%, p=0.0019). IFN-γ expression was higher in γ/δ T cells (16.0±17.8%) than $CD4^+$ cells (5.7±6.4%) but this difference was not statistically significant, mainly because of the substantial overlap of low values between these populations. However, $CD8^+$ cells expressed higher percentages of IFN-γ (32.8±16.3%) than γ/δ T cells versus (16.0±17.8%) which was statistically significant (p=0.042). TNF-α expression was similar in all three populations of cells: 12.2±11.3% ($CD4^+$), 5.4±8.2% ($CD8^+$) and 9.0±11.1% (γ/δ T cells).

In summary, sarcoidosis patients have different cytokine profiles to either CVID or XLA. There is lower IFN-γ expression than in normal controls, which argues against the concept of a Th1-like bias in peripheral blood cells from these patients. Also, there is no obvious γ/δ T cell expansion, and this minority population of cells does not over-express inflammatory cytokines. Further work in this area would be to examine the IFN-γ expression in γ/δ T cells stimulated with PMA & ionomycin in a larger group of patients with sarcoidosis, and compare this with γ/δ T cell INF-γ expression in a similarly sized group of normal controls.

4.1.4 Hyper-IgE syndrome

To determine the behaviour in the whole-blood intracellular cytokine assay of a disease which has a putative Th2-bias, it was fortunate to be able to obtain blood from a single patient with hyper IgE syndrome (HIGES). This patient was a boy aged 17 years, with typical high IgE levels, recurrent *Staphylococcal* skin infections, neutrophilia and other features of HIGES. Samples were obtained with the kind help of Dr Jackie Parkin at St Bartholomew's Hospital, and were processed within 4 hours of donation.

The whole-blood technique was used to determine intracellular IFN-γ, TNF-α, IL-2, IL-4 and CD69 expression in $CD4^+$ and $CD8^+$ T cells. Comparisons were made with a single normal control subject (the same subject previously used to study longitudinal variation in the intracellular cytokine assay). The results are shown in Figure 4.8.

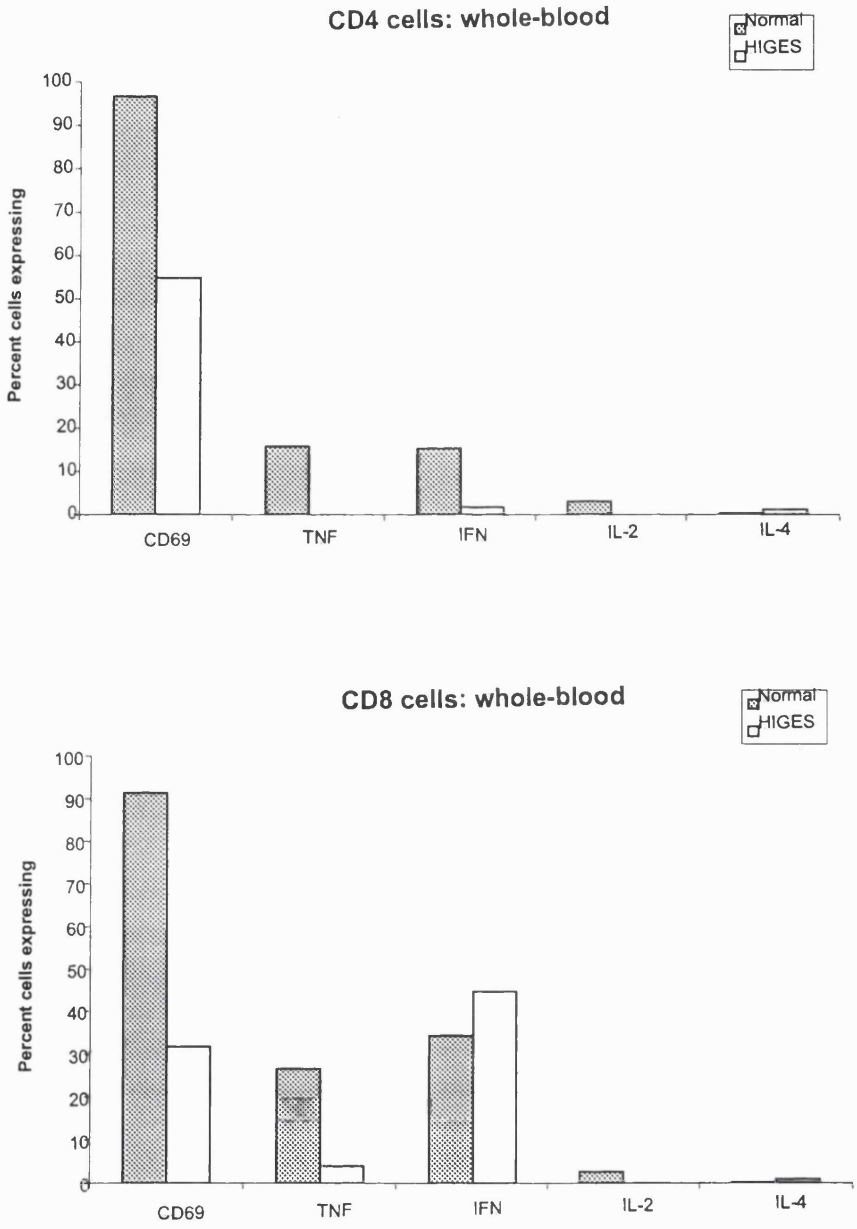


Figure 4.8
Cytokines in a patient with hyper-IgE syndrome (HIGES) compared with a normal control subject. Expression of CD69, TNF-α, IFN-γ, IL-2 and IL-4 in $CD4^+$ and $CD8^+$ cells using the whole-blood intracellular cytokine technique.

The differences between the HIGES patient and the normal control are striking: there is substantially less TNF-α, IFN-γ (in $CD4^+$ cells) and IL-2 along with increased IL-4 in the patient compared with the normal control. This is consistent with the hypothesis that patients with HIGES have an inherent Th2-like bias (discussed in Buckley, 1996 a & b).

It is unexpected that $CD8^+$ IFN-γ production is higher in the patient than in the normal control. The reason for this is not clear, but may reflect the inflammatory nature of the skin lesions in HIGES, since the cytokines in the CD8 population are regulated differently to the CD4 population (North *et al*, 1996). Del Prete, *et al* (1989) reported that IFN-γ production was reduced in HIGES, but used a different assay system, namely measuring IFN-γ in culture supernatants, using cell lines derived from patients with HIGES. In contrast, the technique used in this experiment examined cells in the more physiological environment of whole blood. Simultaneous analysis of IL-4 and IFN-γ in the same cell was not done in this experiment.

4.1.5 Atopic eczema

4.1.5.1 Cytokines in $CD4^+$ and $CD8^+$ cells in atopic eczema

Another disease with a putative 'Th2-like' bias is atopic eczema (discussed in Chapter 1). Four patients with extremely severe atopic eczema enrolled in a trial of experimental high-dose intravenous immunoglobulin therapy had blood samples taken before the start of therapy. The samples were processed within one hour. For comparison, the same normal donor was used in each assay (see Figure 3.7). Samples from the different patients entering the trial were analysed on separate occasions during a one-year period. All patients were male, mean age 36 years, and had a history of severe atopic eczema with a clinical score of ≥8 on the scale of Rajka & Langeland (1989). Patients had Eczema Area and Severity Indexes ('EASI scores') as detailed in Table 4.2. EASI Scores are a highly reproducible, observer independent measure of eczema severity, with a maximum score of 180 points. Treatments which reduce the EASI score to <100 points are usually deemed successful. Patients who responded to the high-dose IVIG therapy, i.e. reduced their EASI scores to <100 points are shown in Table 4.2, and are termed 'responders' in this experiment.

The results are shown in Figure 4.9.

	Patient 01	Patient 03	Patient 04	Patient 05
Pre-treatment	150	115.5	135.3	141
After therapy	71.52	113.0	161.7	30.8

Table 4.2

EASI scores of atopic eczema patients at entry to trial and following treatment. Patients reducing their EASI score to <100 points are termed 'responders' (Patient 01 and Patient 05).

All eczema patients had slightly higher levels of CD69 expression following cell culture than the normal control. This may reflect a lower level of CD69 expression in this particular normal control, however, this subject has cytokine and activation levels in the mid-range when compared against other normal controls. Alternatively, there may be an intrinsically higher level of cell activation in eczema, given the inflammatory nature of the skin lesions. This would need further experiments to confirm, and has not been addressed in other studies of intracellular cytokines in atopic eczema (e.g. Ferry *et al*, 1997).

The cytokine expression showed lower IFN-γ and TNF-α (i.e. Th1-like cytokine) expression than the normal control in all but one patient (Patient 01 for IFN-γ in both $CD4^+$ and $CD8^+$ cells). It is not clear why this subject has higher IFN-γ levels, and yet lower TNF-α expression. There was no clear-cut difference in baseline cytokine (or CD69) expression between responders and non-responders. IL-4 levels are always difficult to detect, and given the large CoV shown earlier for IL-4 assays (Chapter 3), little weight should be put on the IL-4 results. However, it is interesting that one atopic subject should have higher IL-4 levels than the normal control, particularly as this was the same subject with high IFN-γ levels.

In summary, the whole blood intracellular cytokine assay demonstrated a cytokine profile biased away from a 'Th1-like' pattern in severe atopic eczema. However, the IL-4 assay is too insensitive to confidently say that the patients are positively 'Th2-biased'.

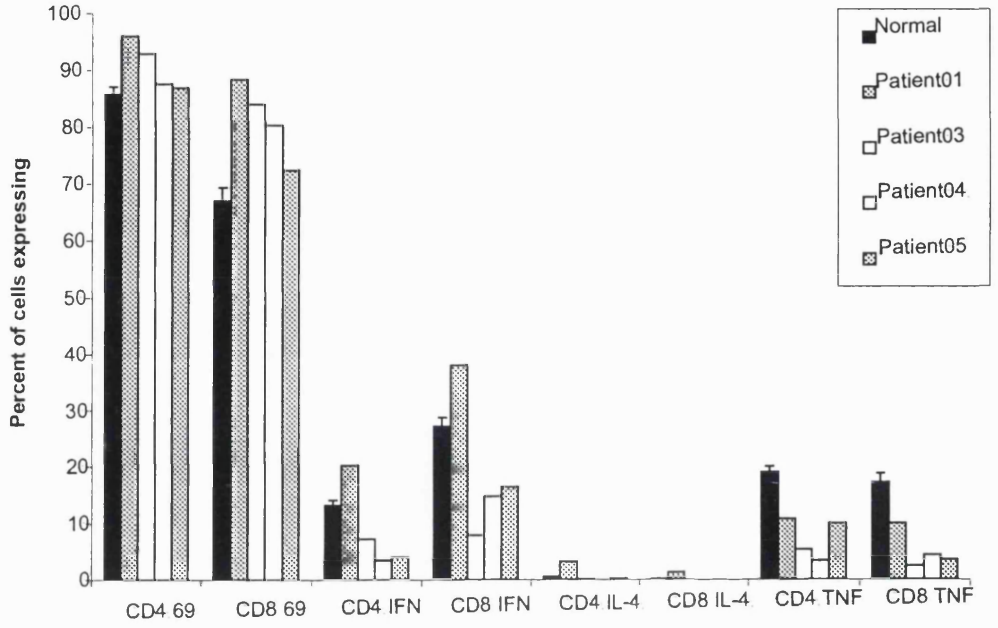


Figure 4.9

Intracellular cytokines in four patients with severe atopic eczema. In each assay the same normal control was used (mean over one year ± SEM plotted). Each patient is shown as a separate bar. Responders are shown in grey, non-responders in white.

4.2 Discussion

The whole blood assay for intracellular cytokines was straightforward to perform and reasonably reproducible. Attempts were made to make a reference preparation with which to standardise the assay, but these attempts were not successful. Other groups have tried to produce controls for intracellular cytokine assays, with limited success (e.g. Mayaard *et al*, 1996). A standard cytokine-expressing cell line (HiCK2; Pharmingen) has been produced, but was not used in this study. Although the coefficient of variation for the intracellular cytokine assays was relatively large, it compares favourably with another group's attempts to make a reference preparation using frozen lymphocytes (Mayaard *et al*, 1996). It was decided, therefore, to always run patient materials with normal subjects in the same batch. If this was not possible, blood from the same volunteer was used wherever possible to reduce variability.

The whole-blood assay for intracellular cytokines was applied to a variety of immunological disorders, and demonstrated some interesting findings. The ability of the whole blood method to detect abnormalities of cytokine expression previously found using PBMC was confirmed. Similarities between whole blood and isolated cell techniques have been noted by Jason & Larned (1997), but they compared cytokine production and did not include the activation status of the cells separated by density gradient centrifugation (see Maino *et al*, 1995). The ability to use small volumes of blood in this new technique was extremely advantageous in terms of venesection burden to the patient, avoidance of density gradient centrifugation and also reduced the overall assay time considerably.

The new technique confirmed previously reported cytokine imbalances in two sets of conditions. In terms of the ability to detect a Th1-bias, a preponderance of IFN-γ secreting T cells had previously been noted in CVID (North *et al*, 1996) and this was confirmed using the whole blood approach. However, the suggestion that sarcoidosis is a Th1-biased disease (Moller, 1999) was not confirmed using this technique. This may reflect the sampling bias of using peripheral blood samples, when the pathological

changes are actually occurring in tissues. It also underlines an important difference between studies which examine cytokines in plasma (reporting cytokine actually produced and secreted) and intracellular techniques (which examine the potential of lymphocytes to produce a cytokine when activated). Given the problems with reproducibility of plasma cytokine levels, and the doubtful physiological significance of such measurements, a recent conference consensus was that intracellular cytokine techniques may have more validity in examining cytokines in the clinical setting (reviewed in Klein, 1999). The technique developed in this study has several advantages over other intracellular cytokine assays, which need larger blood samples and take longer.

Validation of the whole blood technique to identify Th2-bias was also confirmed using two conditions thought to be examples of 'Th2-like' diseases, hyper IgE syndrome and atopic eczema, where a reduction of Th1-cytokines compared with that found in T cells from normal controls was found. The IL-4 assay was used in the study of the patient with HIGES to confirm that the shift away from Th1 cytokine production was accompanied by an increase in Th2 cytokine production. This confirms Paganelli *et al*'s findings (1991) of reduced IFN-γ production in culture supernatants in HIGES patients, yet used smaller volume blood samples and a more rapid technique. It is notable that the present data shows that not all patients with atopic eczema have a reduced proportion of IFN-γ-producing cells, particularly in the $CD8^+$ cells. This may reflect the extremely inflamed skin seen in this cohort of patients, who by their inclusion in the hdIVIG in atopic eczema study had such severe eczema that it was viewed as 'intractable'. It would have been of great interest to monitor intracellular cytokines in King's study (1989) in which HIGES patients were treated with IFN-γ, in order to investigate whether exogenous IFN-γ biases the proportions of Th1 cells in the peripheral blood. The whole blood technique could also be used to investigate the effects of high-dose intravenous immunoglobulin (hdIVIG) therapy in HIGES. The effects of hdIVIG in atopic eczema were monitored using this technique and are discussed in Chapter 5.

Additional adaptations of the assay demonstrated that cytokine production by minor lymphocyte subsets could also be measured. The first subsetting validation for the whole blood assay was confirmed using lymphocyte subsets defined by CD28 expression. Within the immunodeficiency disease CVID, significant differences were found between CD28- and CD28+ cells in terms of IFN-γ production, confirming earlier findings using

PBMC-based techniques (North *et al*, 1998). Smaller subsets were also examined. For example, the $CD4^+7^-$ subset has been suggested to be Th0/Th2 weighted (Autran *et al*, 1995) by measuring cytokines in the culture supernatant of $CD4^+7^-$ and $CD4^+7^+$ separated using immunomagnetic beads. Using the whole blood technique, $CD7^+$ and $CD7^-$ cells had similar cytokine expression profiles for IFN-γ, which was the same finding as in the Autran paper. However, her study suggested that CD7- cells make only 50% of the IL-2 produced by CD7+ cells. In our experiment this was not so, and probably reflects the different methods used to measure cytokine production since Autran's group only examined culture supernatants.

An additional subsetting validation of the whole blood intracellular cytokine method was confirmed using γ/δ T cells as another example of a minor T cell population. These cells may be important in sarcoidosis, since γ/δ T cells are found in large quantities within granulomas (Wynn *et al*, 1993), and may contribute to granuloma formation by the local production of pro-inflammatory cytokines (Agostini *et al*, 1997). However, the present whole blood study did not find IFN-γ expression by peripheral blood γ/δ T cells to be different to that in peripheral blood $CD4^+$ and $CD8^+$ T cells. Sampling bias may account for this, since the immunologically active cells in sarcoidosis may have localised to the inflamed tissues (lung, etc.) and would be missed by sampling peripheral blood.

This new method compares favourably with other, recently published whole blood intracellular cytokine techniques. The technique of Ferry *et al* (1997) used a similar whole-blood technique, but with longer (10 h) stimulation by lower concentrations of PMA and ionomycin, at a greater dilution of blood. Although the results are similar, the longer culture times would be a drawback in routine clinical practice. In addition, her technique was not assessed using four-colour flow cytometry, although it could readily be adapted to this. The method of Jason & Larned (1997) used a higher concentration of PMA (50 ng/ml) with brefeldin A instead of monensin for a longer culture period (4-5.5h) which makes discrimination of $CD4^+$ cells impossible. Again, only three colour flow cytometry was used in the Jason & Larned study.

In conclusion, this new technique for measuring intracellular cytokine production using small-volume whole-blood samples was rapid, straightforward to perform and reasonably robust, and was sufficiently sensitive to distinguish patients from normal controls in a

number of situations where differences in cytokine profile could be expected. The use of four-colour flow cytometry for analysis of the stained lymphocytes enabled the cytokine production of minor lymphocyte populations to be determined, without the complications of prolonged cell separation techniques.

5 Therapeutic modulation of cytokine synthesis

5.1 Introduction

Since many studies have clearly established that cytokine regulation has a substantial influence on disease pathogenesis (Romagnani, 1994), it follows that an appreciation of the effect of therapeutic agents on cytokine regulation is important in understanding how to improve treatment of disease. In this chapter, the effects of two immunomodulatory agents (chosen as discussed in Chapter 1) were investigated using the whole-blood intracellular cytokine technique described in Chapter 3: intravenous immunoglobulin (IVIG) used both at replacement dose (200-400 mg/kg body weight) and high-dose (hdIVIG, 1-2 g/kg), and the 4-fluoroquinolone antimicrobial agent ciprofloxacin.

5.2 Immunomodulation by IVIG

5.2.1 Effects of IVIG on lymphocytes *in vitro*

The effects of any drug may differ when examined using *in vivo* compared with *in vitro* systems. In this first experiment, the whole-blood intracellular cytokine assay was used to determine the effects of IVIG *in vitro* over a wide range of IgG concentrations, in both normal subjects and patients with CVID. The range of IgG concentrations was chosen to reflect three situations, namely IgG levels typical of: immunodeficient patients receiving inadequate doses of IVIG therapy, normal subjects, and patients receiving hdIVIG therapy. Blood was taken from 11 normal subjects and four patients with CVID. The experimental protocol involved culturing whole blood (250 µl) with dilutions of a commercial IVIG preparation (Alphaglobin, Grupo Grifols, Barcelona; kind gift of Carl Wheeler) (250 µl, in RPMI1640) for 18 h at 37°C in a humid CO_2 atmosphere. Samples were cultured in polystyrene 5 ml round-bottomed ventilation-capped tubes (Greiner Labortechnik). This overnight incubation period was followed by addition of RPMI1640 (250 µl) containing monensin (3 µmol/l, final concentration) ('unstimulated' cultures) or monensin (3 µmol/l), PMA (10 ng/ml) and ionomycin (2 µmol/l) ('stimulated' cultures) making a final blood dilution of 1:3. Samples were then incubated at 37°C for 2 h for

CD69, IFN-γ and TNF-α assays, and 4 h for IL-2. Red cells were lysed, and the remaining cells fixed, permeabilised and stained according to the method in Chapter 3.

The results of the effects of overnight incubation of whole-blood samples with *in vitro* IVIG on cytokine production followed by stimulation with PMA and ionomycin are shown in Figure 5.1.

As can be seen in Figure 5.1, IVIG had no effect on CD69 expression following activation with PMA & ionomycin in CD4 or CD8 cells, in either normal subjects or patients with CVID. However, the overnight incubation with IVIG did reduce the expression of CD69 and cytokines compared with that expressed in samples cultured immediately after sampling (see results in section 4.1.1.1 from samples analysed immediately after sampling).

This effect was particularly apparent for IFN-γ, where normal subjects had a mean expression in CD4 cells of 12±7% (section 4.1.1.1) when analysed immediately yet 3.1±1.2% when incubated overnight with IVIG. Interestingly, CVID patients were less effected by the overnight incubation, with CD4 IFN-γ expressions of 26.0±11.0% analysed immediately, and 15±2% analysed after an overnight culture, and 49±22% of CVID CD8 cells expressed IFN-γ when analysed immediately compared with 45±9% overnight

IVIG had no effect on IFN-γ expression in normal or CVID $CD8^+$ cells, nor normal $CD4^+$ cells. However, there was a small but significant reduction in IFN-γ expression in CVID $CD4^+$ cells with increasing concentrations of IVIG from 15±1.87% with no IgG down to 10.5±1.7% with 10 mg/ml IgG (comparison by Student's paired t-test, p=0.006) and 8.8±2.6% with 20 mg/ml IgG (p=0.04).

Under the experimental *in vitro* conditions, there was no consistent effect of IVIG on TNF-α or IL-2 expression in $CD4^+$ or $CD8^+$ cells in either normal subjects or CVID patients.

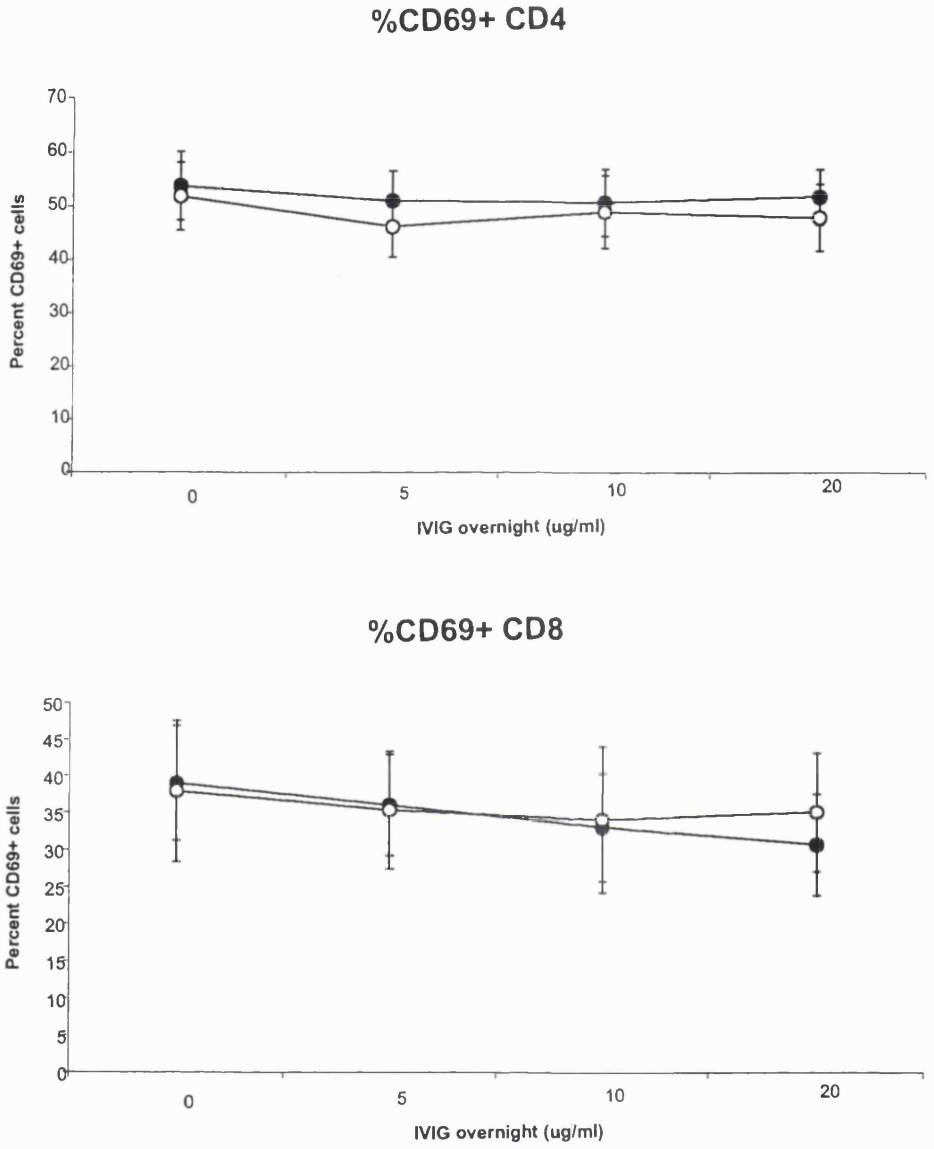


Figure 5.1 Effects of overnight exposure to *in vitro* IVIG on CD69 expression following activation with PMA & ionomycin, in $CD4^+$ and $CD8^+$ cells, in normal subjects (•) and patients with CVID (o).

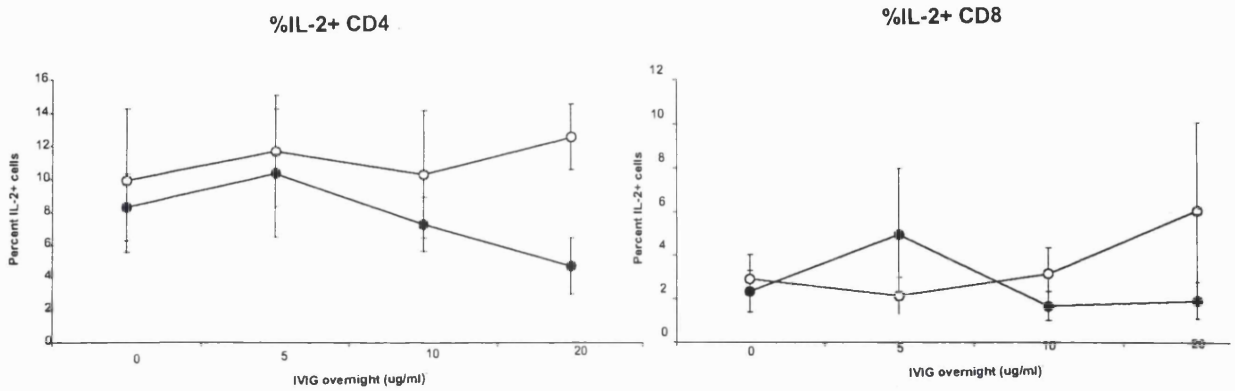


Figure 5.1 Contd. Effects of overnight exposure to *in vitro* IVIG on IFN-γ, TNF-α and IL-2 expression following activation with PMA & ionomycin, in $CD4^+$ and $CD8^+$ cells, in normal subjects (•) and patients with CVID (o).

In summary, the overnight incubation (even without the presence of IVIG) lead to reduced CD69 and IFN-γ expression in normal subjects, with less effect in CVID, particularly in CVID $CD8^+$ cells. IVIG itself had no effect on CD69 expression or intracellular TNF-α, IL-2 or IFN-γ expression. However, increasing concentrations of IgG caused a dose-dependent fall in $CD4^+$ IFN-γ expression in CVID patients but not in normal control subjects. $CD8^+$ cell IFN-γ expression was not affected by IVIG concentration.

5.1.1 Effects of replacement-dose IVIG in vivo

5.1.1.1 Effects of replacement-dose IVIG on lymphocytes in CVID & XLA

In order to examine the effects of IVIG on lymphocytes *in vivo*, CVID (n=9) and XLA (n=3) patients who normally receive IVIG therapy for treatment of their condition, had samples taken immediately prior to their routine IVIG infusion, and immediately following IVIG. Care was taken to flush out the line before taking the post-IVIG sample, to ensure that it was not diluted with residual IVIG. Both pre-IVIG ('pre') and post-IVIG ('post') samples were analysed together, immediately after the 'post' sample was taken. In case there were changes in cytokine expression *ex vivo*, blood samples were also taken from normal subjects (n=5), and left *ex vivo* for 3 h (the mean duration of IVIG infusion), equivalent to the 'pre' sample from the patients. A second sample, equivalent to the 'post' sample was then taken from each normal subject, and both 'pre' and 'post' were analysed together. It is important to clarify that this was a control for time *ex vivo* only. No normal subject received IVIG.

The results for individual donors are shown in Figures 5.2 (a-d). The median change of expression of cytokine in the 'pre' compared with the 'post' samples is given in Tables 5.1 (a-d), where the differences in cytokine (or CD69) expression between the before and after IVIG are compared using the sign test for paired data (Minitab). Negative values indicate a reduction in expression following IVIG, positive values indicate an increase in expression following IVIG. Comparison of 'pre' and 'post' samples in the normal subjects showed that the 3 h *ex vivo* delay had no significant effect on CD69 or cytokine expression (therefore this analysis is not shown in the tables). Also, since samples from only three XLA patients were examined, no statistical analysis was performed on their

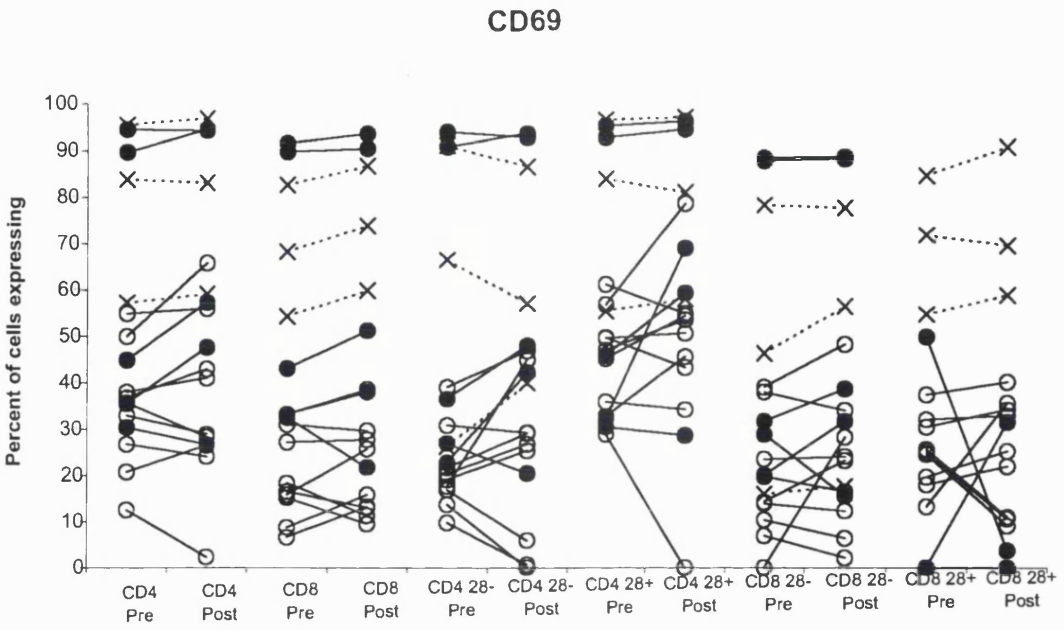


Figure 5.2 (a) Expression of CD69 following activation with PMA and ionomycin in CD4 and CD8 cells and their CD28 subsets. 'Pre' indicates samples taken prior to IVIG infusion, 'post' indicates samples taken following IVIG infusion in CVID patients (o) and XLA patients (×). Normal donors (•) are shown for comparison, with 'pre' samples taken 3 h before 'post' samples.

Cell population	Normals	CVID	CVID 'pre' compared with 'post'
CD4	4.85 (-0.25, 12.1)	1.1 (-4.05, 5.75)	NS
CD8	2.05 (0.8, 4.9)	0.55 (-3.45, 6.65)	NS
CD4 28-	2.8 (-1.11, 11.4)	6.3 (-9.2, 7.5)	NS
CD4 28+	1.7 (1, 13.5)	1 (-6.3, 9.2)	NS
CD8 28-	0.4 (0.2, 7)	-1.7 (-4.9)	NS
CD8 28+	0 (0, 0)	2.8 (-14, 4)	NS

Table 5.1(a) Effect of *in vivo* IVIG infusions on expression of CD69. For the CVID patients, the difference of (%$CD69^+$'after') - (%$CD69^+$'before' the 3 hour IVIG infusion) is shown, (median with inter-quartile range). For the normal subjects, the difference over a 3 hour *ex vivo* period prior to analysis is shown, (median with inter-quartile range). The differences before and after IVIG are compared using the sign test for paired data. NS = not significant. There were no significant differences over the 3 hour *ex vivo* period for normal subjects (not shown in the table).

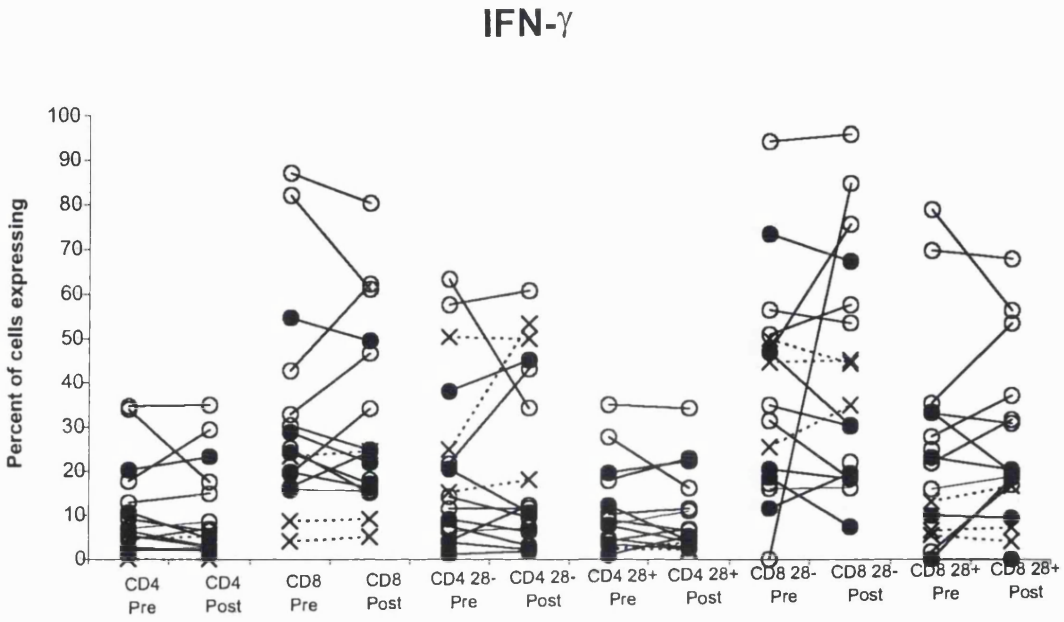


Figure 5.2 (b) Expression of IFN-γ following activation with PMA and ionomycin in CD4 and CD8 cells and their CD28 subsets. 'Pre' indicates samples taken prior to IVIG infusion, 'post' indicates samples taken following IVIG infusion in CVID patients (o) and XLA patients (×). Normal donors (•) are shown for comparison, with 'pre' samples taken 3 h before 'post' samples.

Cell population	Normals	CVID	CVID 'pre' compared with 'post'
CD4	-0.35 (-3.45, 0.05)	0.35 (-2.6, 2.1)	NS
CD8	-5.15 (-6.85, -3.5)	-0.25 (-6.0, 13.8)	NS
CD4 28-	-1.6 (-2.9, 0.7)	0.7 (-4.4, 3.2)	NS
CD4 28+	-1 (-3.7, 2.7)	0.4 (-2.1, 2.8)	NS
CD8 28-	-6.1 (-11.2, -2.2)	1.6 (-2.9, 6.6)	NS
CD8 28+	-0.5 (-2.8, 0)	2.8 (-2.3, 9.9)	NS

Table 5.1(b) Effect of *in vivo* IVIG infusions on expression of CD69. For the CVID patients, the difference of (%IFN-γ^+'after') - (%IFN-γ^+'before' the 3 hour IVIG infusion) is shown, (median with inter-quartile range). For the normal subjects, the difference over a 3 hour *ex vivo* period prior to analysis is shown, (median with inter-quartile range). The differences before and after IVIG are compared using the sign test for paired data. NS = not significant. There were no significant differences over the 3 hour *ex vivo* period for normal subjects (not shown in the table

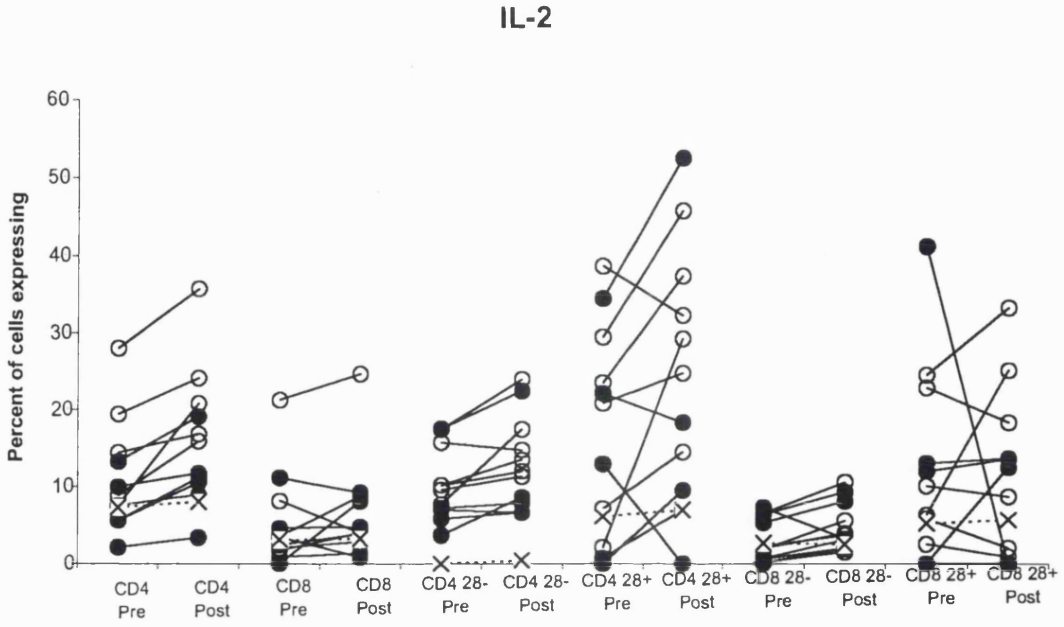


Figure 5.2 (c) Expression of IL-2 following activation with PMA and ionomycin in CD4 and CD8 cells and their CD28 subsets. 'Pre' indicates samples taken prior to IVIG infusion, 'post' indicates samples taken following IVIG infusion in CVID patients (o) and XLA patients (×). Normal donors (•) are shown for comparison, with 'pre' samples taken 3 h before 'post' samples.

Cell population	Normals	CVID	CVID 'pre' compared with 'post'
CD4	1.8	6.3	p=0.031
	(1.4, 4.9)	(4.9, 7.5)	
CD8	0.15	1.55	NS
	(-1.9, 1.4)	(0.59, 3.11)	
CD4 28-	0.8	2.55	NS
	(0.6, 4.9)	(1.73, 5.7)	
CD4 28+	6.3	10.6	NS
	(-3.8, 9.5)	(4.8, 15.7)	
CD8 28-	1.8	2.15	p=0.031
	(1.4, 2.8)	(1.53, 3.45)	
CD8 28+	0.6	-1.5	NS
	(0, 1.6)	(-3.25, 6.2)	

Table 5.1(c) Effect of *in vivo* IVIG infusions on expression of CD69. For the CVID patients, the difference of (%IL-2$^+$'after') - (%IL-2$^+$'before' the 3 hour IVIG infusion) is shown, (median with inter-quartile range). For the normal subjects, the difference over a 3 hour *ex vivo* period prior to analysis is shown, (median with inter-quartile range). The differences before and after IVIG are compared using the sign test for paired data. NS = not significant. There were no significant differences over the 3 hour *ex vivo* period for normal subjects (not

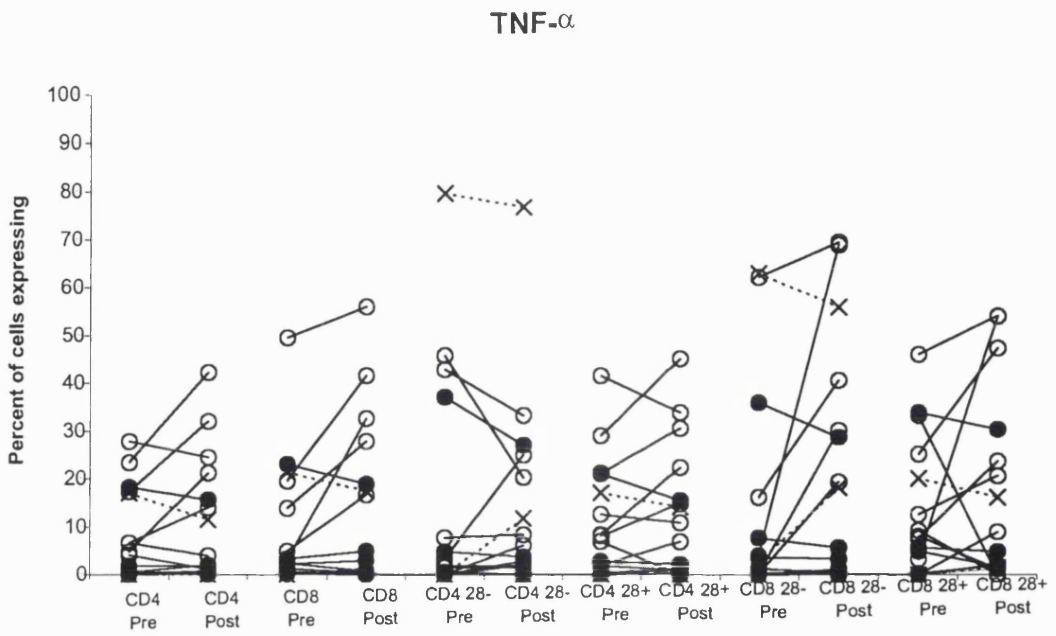


Figure 5.2 (d) Expression of TNF-α following activation with PMA and ionomycin in CD4 and CD8 cells and their CD28 subsets. 'Pre' indicates samples taken prior to IVIG infusion, 'post' indicates samples taken following IVIG infusion in CVID patients (o) and XLA patients (×). Normal donors (•) are shown for comparison, with 'pre' samples taken 3 h before 'post' samples.

Cell population	Normals	CVID	CVID 'pre' compared with 'post'
CD4	0 (-0.3, 0.05)	1.6 (-2.65, 14.7)	NS
CD8	-0.15 (-0.3, 0.7)	6.45 (-0.55, 14)	NS
CD4 28-	-0.5 (-1.0)	0 (-0.1, 2)	NS
CD4 28+	-0.4 (-0.8, 0.2)	4.9 (-1.7, 9.4)	NS
CD8 28-	-1.4 (-2, -0.2)	7.3 (0.9, 24.5)	p=0.039
CD8 28+	-0.9 (-3.2, 1.5)	8.1 (-6.8, 16.2)	NS

Table 5.1(d) Effect of *in vivo* IVIG infusions on expression of CD69. For the CVID patients, the difference of (%TNF-α$^+$'after') - (%TNF-α$^+$'before' the 3 hour IVIG infusion) is shown, (median with inter-quartile range). For the normal subjects, the difference over a 3 hour *ex vivo* period prior to analysis is shown, (median with inter-quartile range). The differences before and after IVIG are compared using the sign test for paired data. NS = not significant. There were no significant differences over the 3 hour *ex vivo* period for normal subjects (not shown in the table).

data, although the results are plotted in the figures for comparison with CVID and normal subjects.

IVIG had no effect on CD69 expression in either $CD4^+$ or $CD8^+$ cells, or their CD28+/- subsets in CVID or XLA patients. (Figure 5.2(a)). Greater numbers of $CD8^+$ cells than $CD4^+$ cells expressed IFN-γ in all of the donor groups studied (Figure 5.2(b)). The highest levels of IFN-γ expression were in CVID. The findings of North *et al* (1998) were confirmed, with the expanded $CD8^+28^-$ subset from CVID patients expressing high amounts of IFN-γ together with significant overproduction of IFN-γ from the $CD8^+28^+$ subset, compared with that seen in normal controls. There was no significant effect of IVIG on IFN-γ expression in either CVID or XLA.

IL-2 expression was greater in $CD4^+$ than $CD8^+$ cells in all subsets, and for all groups studied (Figure 5.2(c)). Most strikingly, there was a significantly greater proportion of $CD4^+$ cells expressing IL-2 following IVIG in CVID patients; but there was no significant increase in IL-2 expression in $CD8^+$ cells after IVIG (Table 5.1(c)). Moreover, subset analysis revealed that $CD8^+28^-$ cells from CVID patients significantly increased IL-2 expression following IVIG, although the increase was small. IL-2 expression was not affected by IVIG in the single XLA subject in whom this cytokine was measured.

Expression of TNF-α was similar in both $CD4^+$ and $CD8^+$ cells (Figure 5.2(d)) and there was no significant difference in levels of expression between subject groups. However, the proportion of $CD8^+$ cells positive for TNF-α increased significantly following IVIG in the CVID patients, but only in the $CD8^+28^-$ subset (Table 5.1(d)). IVIG had no effect on TNF-α expression in the three XLA patients.

In conclusion, this experiment demonstrated for the first time, the direct *in vivo* effects of replacement-dose IVIG on intracellular cytokines in CVID, namely that it can increase the potential of $CD8^+28^-$ cells to make TNF-α, and $CD4^+$ lymphocytes to make IL-2.

Effects of replacement-dose IVIG on monocyte IL-12 expression in CVID

Using a modification of the whole blood intracellular cytokine technique developed in Chapter 3, Dr Cambronero in our laboratory found an increase in the percentage of

monocytes expressing IL-12 following *in vitro* stimulation by LPS in CVID compared with that seen in stimulated monocytes from normal controls (Cambronero, Sewell, *et al*, 2000). This joint work does not form part of this thesis. It was therefore decided to apply this modified technique within the thesis to investigate the effects of IVIG therapy on IL-12 expression in monocytes.

Six CVID patients receiving IVIG were bled immediately before their routine IVIG infusion, and immediately following the infusion, in exactly the same manner as in the previous experiment. Monocyte IL-12 was determined (see above). Briefly, whole blood was cultured for 5 h with LPS and then lymphocyte and monocyte surface markers were stained prior to lysis of red cells, fixation and permeabilisation of cell membranes and finally staining for intracellular IL-12. Comparisons were again made with unstimulated cultures (without LPS, but with monensin) to define cytokine-positive cells. The results are shown in Figure 5.3

After replacement-dose IVIG there was no significant change in monocyte IL-12 production following LPS stimulation (Mean±SEM of 5.8±1.4 % of monocytes IL-12$^+$ before and 5.8±2.1% after IVIG, p =not significant by Student's paired t-test).

5.1.1.2 Effects of replacement-dose IVIG on NK cell IFN-γ expression in CVID

Although this study has concentrated on the effects of immunomodulatory agents on T cell cytokines, an adaptation of the technique was used to examine the effects of replacement-dose IVIG on NK cell IFN-γ expression, since NK cells as well as T cells are a potent source of IFN-γ production (discussed in de Maeyer & de Maeyer-Gruignard, 1998)

The culture technique was described in Chapter 3. Briefly, whole blood was cultured with PMA and ionomycin in the same way as for the T lymphocyte cytokine assays. Since preliminary experiments had shown that the combination of the red cell lysis, and the fixation and permeabilisation steps resulted in loss of NK markers (particularly CD56, data not shown), surface markers were stained first and then the cells were fixed and permeabilised prior to intracellular cytokine staining. Since preliminary work had also demonstrated that activation of NK cells with PMA & ionomycin resulted in the loss of

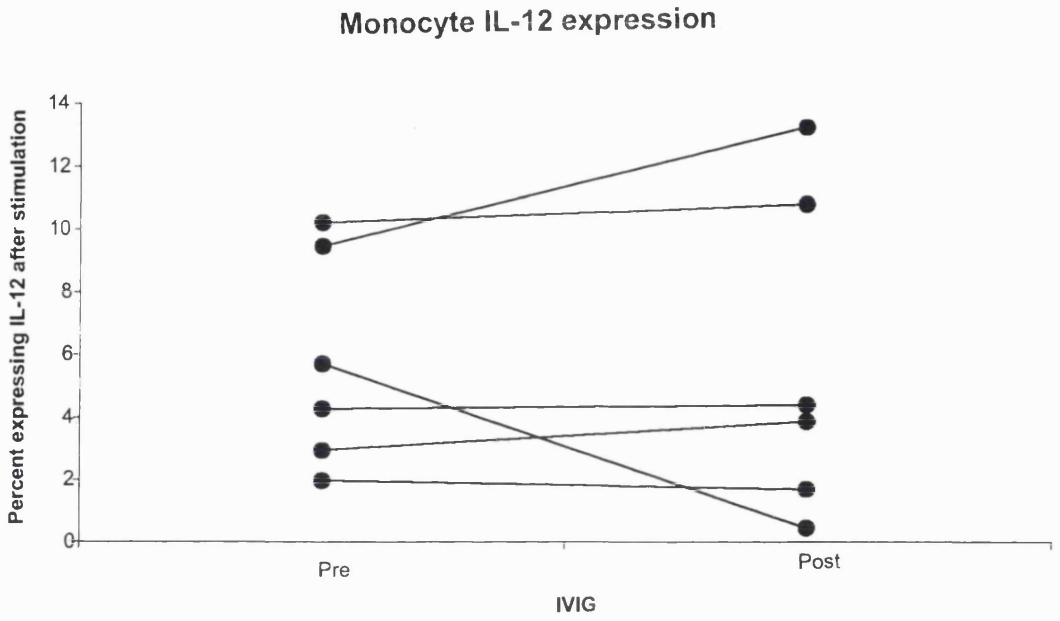


Figure 5.3 Effects of replacement dose IVIG on monocyte IL-12 expression.
There was no significant effect of IVIG at replacement dose on IL-12 expression following stimulation *in vitro* with LPS.

surface CD16 expression, NK cells in this experiment were defined as $CD3^{-}CD16^{+}$. Samples were taken from six CVID patients immediately before and after receiving their routine IVIG replacement therapy. The results are shown in Figure 5.4. As can be seen from the figure, NK cell expression of IFN-γ ranged from <10% in one individual to approximately 70% of NK cells expressing IFN-γ in other individuals. IVIG however, had no effect on the percentage of NK cells positive for IFN-γ (Mean±SEM of 37.3±10.0% before IVIG, 38.7±9.0 following IVIG, p=not significant by Student's paired t-test).

5.1.2 Effects of high-dose IVIG in vivo

Intravenous immunoglobulin is used at two very different dosages in clinical practice: replacement dose IVIG is prescribed at 200-400 mg/kg, given every 2-4 weeks to replace immunoglobulins in patients with intrinsic deficiencies of IgG. High-dose IVIG is prescribed at 1-2 g/kg, usually given over 2-5 days in divided doses. Whether or not the treatment is repeated depends on the underlying disorder. This hdIVIG is given as an immunomodulatory agent, as well as making use of Fc- and Fab-dependent mechanisms outlined in Chapter 1. Having established that replacement-dose IVIG also had immunomodulatory properties in CVID patients (but not in the small number of XLA patients studied), it was decided to investigate the effects of hdIVIG, using the same assay system.

The patients receiving hdIVIG were part of a study sponsored by Grupo Grifols, (trial number IG706), of the effects of hdIVIG on severe atopic eczema, initially designed by Dr Stephen Jolles, and undertaken between the Departments of Clinical Immunology and Dermatology of the Royal Free Hospital, and Grupo Grifols SA, the manufacturers of the IVIG product 'Alphaglobin'. It is not part of this thesis to report all of the details of hdIVIG therapy in this study, but only to examine the effects of hdIVIG given *in vivo* on intracellular cytokines measured following *ex vivo* stimulation with PMA and ionomycin.

Four patients were initially enrolled, over the course of one year; all were male, age range 19-40. All had severe atopic eczema, unresponsive to conventional therapy with topical and oral steroids and allergen avoidance. All had a history of atopic eczema, as defined

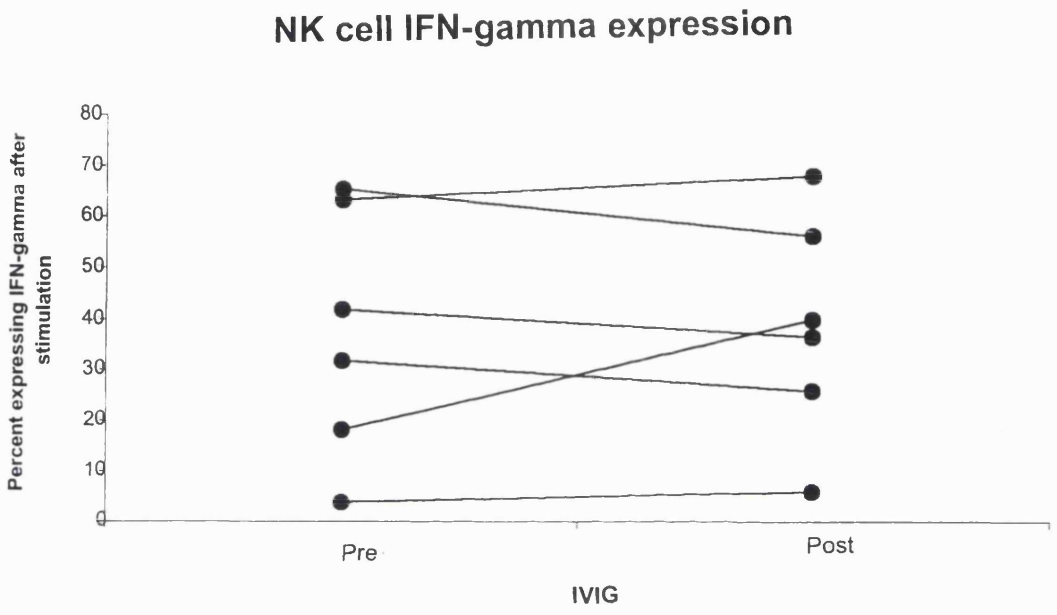


Figure 5.4 Effects of replacement-dose IVIG therapy on IFN-γ expression in 6 patients with CVID.

by Hanifin & Rajka (1980), severe enough to score ≥8 on the Rajka & Langeland (1989) scale. All treatments were unchanged during the lead-in period of the study. Patients received Alphaglobin at a dose of 2 g/kg body weight, given over 5 days for the first treatment. If well tolerated the treatment was subsequently given over two days. The treatment was repeated every month for a period of six months. Samples were taken seven days prior to first-dose IVIG, then immediately before ('pre' samples), and immediately after ('post' samples) each infusion. The 'post' samples were either taken from the contra-lateral arm or following a 10 ml washout sample of blood which was discarded. Intracellular cytokines were measured in whole-blood samples using the method described in Chapter 3. A normal control subject was bled each time a trial patient was bled to act as a control for the assay. The cytokines IFN-γ, TNF-α and IL-4 as well as the activation marker CD69 were measured in $CD4^+$ and $CD8^+$ cells on each occasion.

The results for one representative patient (Patient 05) are shown in Figure 5.5. As can be appreciated from Figure 5.5, it is difficult to determine the effects of IVIG on individual cytokines; there are occasions where activation marker expression is lower following hdIVIG (e.g. post #2) and times when CD69 expression increased following hdIVIG (e.g. post #3). IFN-γ expression did not parallel CD69 expression, indicating that there were genuine differences in cytokine expression being demonstrated, rather than changes in the frozen aliquots of stimulants, since the CD69-stained cells and IFN-stained cells were taken from the same culture tube.

To determine the immediate effects of hdIVIG on cytokine expression, the percentage change of expression due to the infusion was calculated on each occasion from the results 'pre' and 'post' each infusion:

$$\Delta Percent = \frac{\%\,Post}{\%\,\mathrm{Pr}\,e} \times 100\%$$

These results are plotted in Figure 5.6.

As can be seen in Figure 5.6, there was no consistent effect of hdIVIG on cytokine or CD69 expression during the course of the infusion itself. There was no difference in cytokine expression pattern between responders and non-responders.

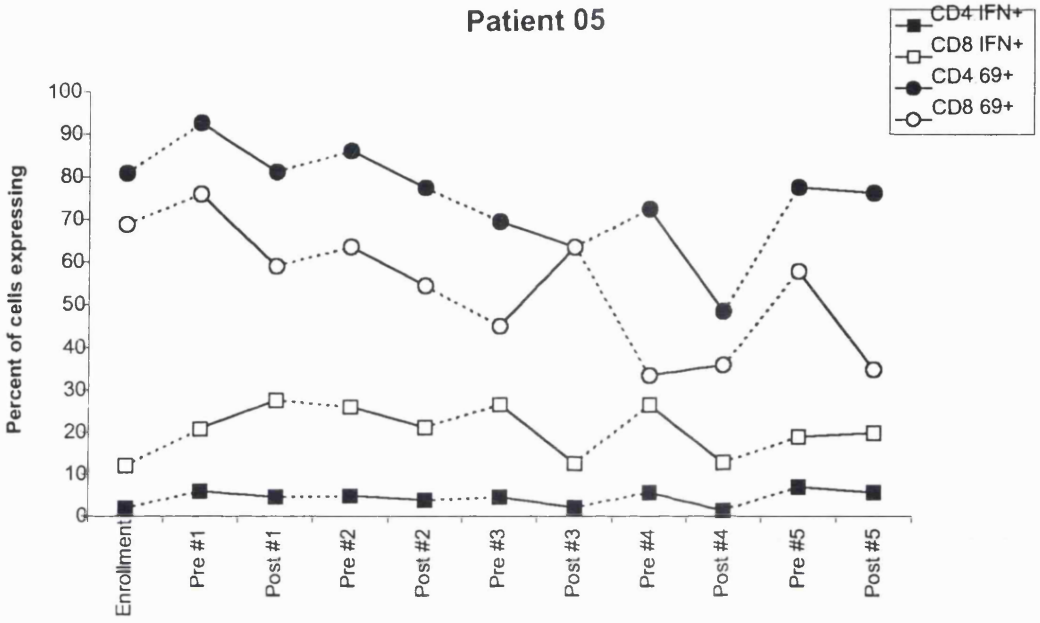


Figure 5.5 CD69 and IFN-γ expression in $CD4^+$ and $CD8^+$ cells from Patient 05 before ('pre') and after ('post') each of five treatments with hdIVIG given every month. Dotted lines indicate the period between infusions; solid lines represent the period during an infusion.

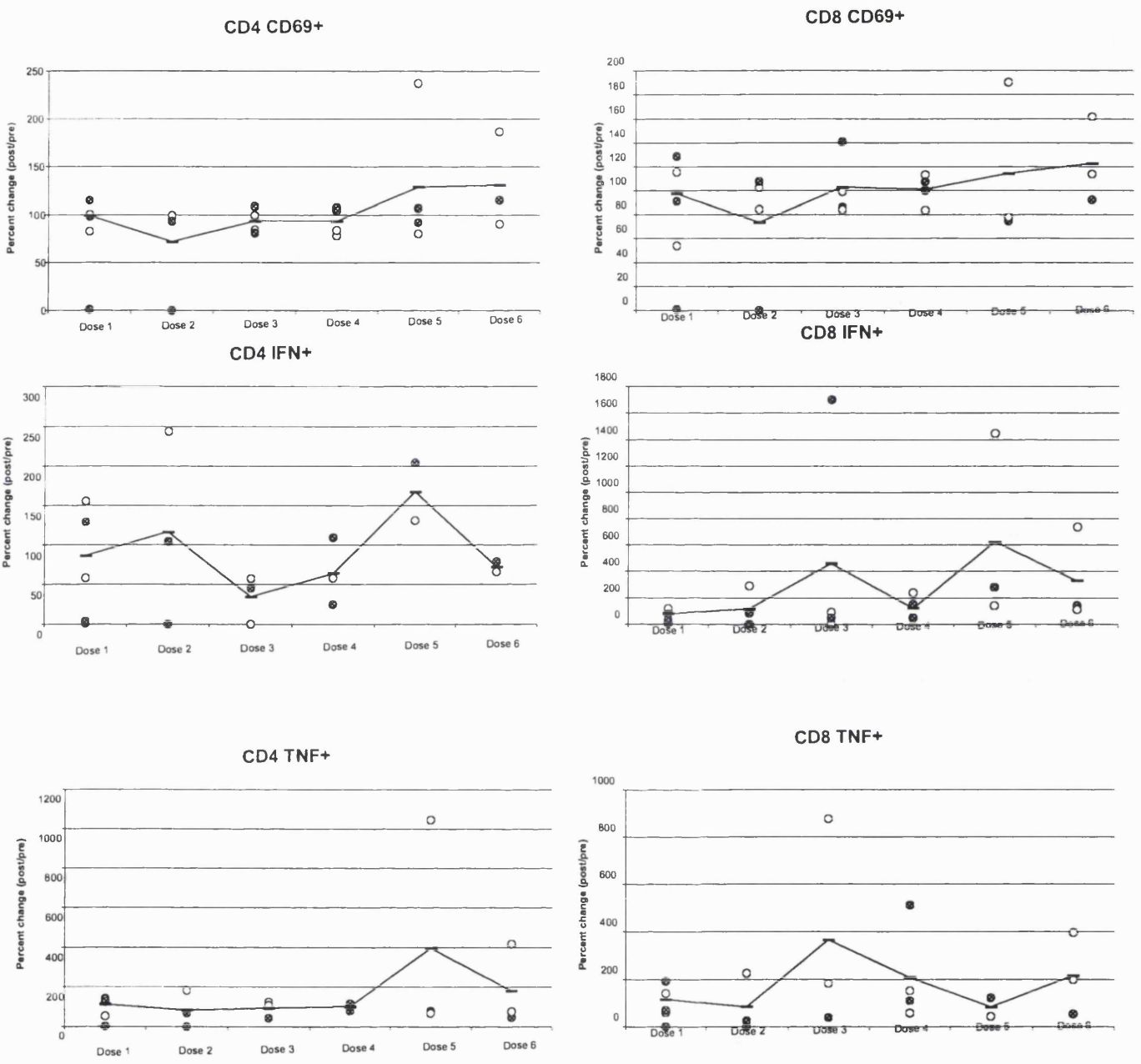


Figure 5.6 Plots of the change in cytokine (or CD69) expression due to the infusion during the course of an hdIVIG treatment (lasting from 2-5 days) for four patients with severe atopic eczema. Hence if the post level was unchanged from the pre level, a value of 100% would be plotted. Individual symbols represent individual patients. Solid symbols indicate patients who were deemed to have responded to hdIVIG at the end of the trial by reducing their skin score to <100; empty symbols indicate non-responders. Horizontal bars indicate the geometric mean.

The values for IL-4 expression fluctuated wildly, principally because of the combination of the large CoV of this assay combined with intrinsic low expression levels of IL-4 (e.g. <1% of cells positive for IL-4). For this reason, the IL-4 results were not analysed and are not shown here.

A variety of analytical approaches, parametric (analysis-of-variance) and non-parametric (Wilcoxon rank sum test) were used and failed to demonstrate any statistical significance between pre and post IVIG cytokine expression levels shown in Figure 5.6. Although this may seem to contradict the findings in the replacement dose experiment (section 5.1.1.1) it should be borne in mind that this hdIVIG experiment did not examine IL-2 levels, only involved four patients, and did not examine $CD8^{+}28^{-}$ cells (where increase in TNF-α expression was demonstrated in CVID).

A comparison was then made between the change in cytokine expression before each treatment episode related to the initial expression prior to commencement of hdIVIG therapy (Fig 5.7). To emphasise: Figure 5.6 shows the change in cytokine before and after *each* monthly infusion. Figure 5.7 shows the change relative to baseline (the pretreatment cytokine expression). The percentage change of the 'pre' reading for infusion *n* (Pre_n) relative to initial baseline cytokine level (the reading at 'pre' first ever infusion (Pre_1):

$$\Delta Percent = \frac{\%\,\mathrm{Pr}\,e_n}{\%\,\mathrm{Pr}\,e_1} \times 100\%$$

Analysis of the long-term effects of hdIVIG on cytokine expression is hampered by a number of factors. One factor was the small number of patients. Another was the large variation between subjects as well as between dosage points, making formal statistical comparison difficult. Since the first data point is defined as 100% (and hence a standard deviation of 0) there is a further reduction in time-points to compare, i.e. comparison can only be made dose #2 to dose #3, #4, #5 and #6, rather than dose #1 to #2, #3, etc. The presence of zero values greatly complicated the analysis. On two occasions serum samples only were accidentally taken from the patients. Occasionally results were excluded from the analysis when comparisons with baseline values gave 'infinite' or 'zero-

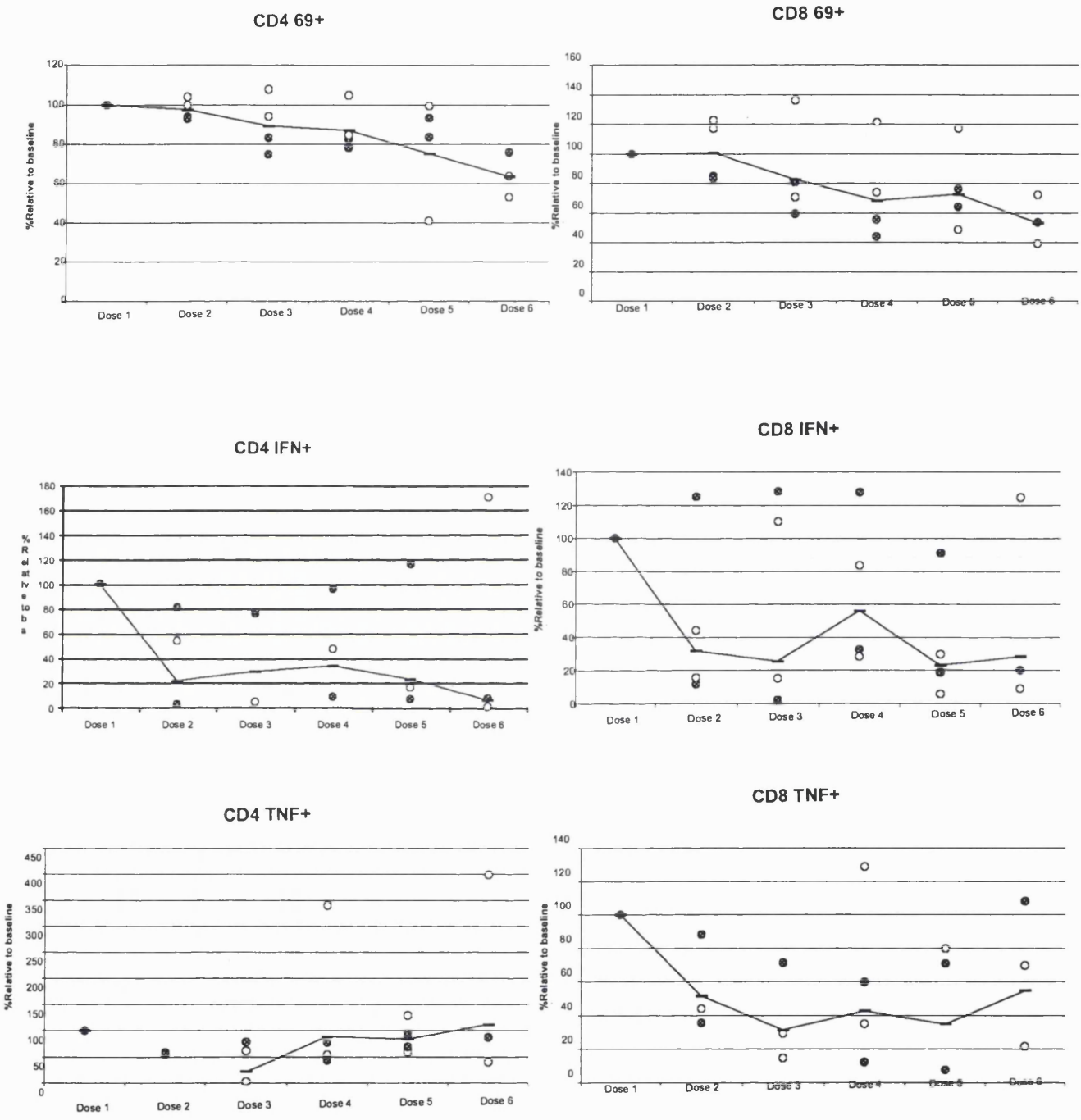


Figure 5.7 Effect of hdIVIG on CD69, IFN-γ and TNF-α expression in CD4 and CD8 cells in comparison to baseline expression before first infusion (defined as 100%). Solid symbols represent responders; empty symbols represent non-responders. Horizontal bars mark geometric mean of all four patients.

divided-by-zero' results. These were deliberately excluded from the analysis and defined as 'missing data'. The presence of missing or zero-value data precluded analysis using traditional analysis-of-variance procedures, which could have distinguished variation between subjects from variation due to an effect of hdIVIG. Given these limitations, analysis therefore was by visual examination of the data alone unless stated otherwise.

CD69 expression in both $CD4^+$ and $CD8^+$ cells declined during the six months of hdIVIG therapy to approximately 60% of baseline values. A paired t-test comparing dose point #2 with #6 did demonstrate that these differences were statistically significant, $p=0.024$ for $CD4^+$ cells and $p=0.021$ for $CD8^+$ cells. This demonstrates that long-term treatment with hdIVIG can restrict the ability of lymphocytes to respond to mitogens, and may therefore restrict antigen-specific responses as well.

IFN-γ expression was also reduced with time and number of treatments, in both $CD4^+$ and $CD8^+$ cells. One responder failed to reduce IFN-γ expression with time of therapy in either CD4 or CD8 cells, but the other responder had a marked decline to <20% of baseline values. Non-responders also reduced IFN-γ expression over time.

TNF-α expression over time of treatment was less effected by hdIVIG, particularly in $CD4^+$ cells where there was essentially no change from baseline, however the geometric mean CD8 TNF-α expression fell to around 40% of baseline values even after one month of treatment, an effect which was sustained for at least six months.

These findings taken together suggest that hdIVIG exerts an anti-inflammatory effect, reducing the ability of cells to express activation markers following mitogenic stimuli (and hence presumably the ability to respond to antigen) and to reduce the percentage of cells producing pro-inflammatory cytokines (IFN-γ and TNF-α).

5.2 Immunomodulation by ciprofloxacin *in vitro*

In addition to examining the effects of IVIG as an immunomodulatory agent, the *in vitro* effects of a range of concentrations of ciprofloxacin were examined using the whole-blood intracellular cytokine assay on cultured whole-blood samples.

Blood was taken from four healthy normal volunteers and aliquots (250 µl) were cultured with ciprofloxacin diluted in RPMI1640 (250 µl) for 18 h at 37°C in a humid 5% CO_2 atmosphere, using 5 ml polystyrene round bottomed tubes as in the earlier studies. Monensin alone, or monensin, PMA and ionomycin was then added (250 µl) and the cells were incubated for a further 2 h at 37°C. Following incubation, red cells were lysed, and the remaining cells were fixed, permeabilised and stained for IL-2 and TNF-α as described previously. The results are shown in Figure 5.8:

Following overnight incubation the expression levels of TNF-α or IL-2, even in the absence of ciprofloxacin was extremely low (<10% of $CD4^+$ or $CD8^+$ cells). There was no clear-cut effect of ciprofloxacin on expression of these two cytokines, either at concentrations reflecting normal therapeutic doses (~10 ng/ml) or even at extremely high concentrations (100 ng/ml). This impression was confirmed by two-way analysis of variance (Microsoft Excel, analysis not shown) which failed to demonstrate any statistically significant effects of ciprofloxacin on TNF-α or IL-2 expression.

Although this finding may appear to contrast with previous studies, which have examined the amount of IL-2 in the supernatants of cells cultured with ciprofloxacin, and found that ciprofloxacin increased IL-2 production (Riesbeck & Forsgren, 1990), there are important differences. The whole-blood intracellular cytokine technique does not examine the quantity of cytokine produced per cell, nor does it estimate the actual production of cytokine *in vivo*. Rather, it is a measure of the potential of that cell to produce cytokine in cognate interactions when stimulated during the antigen-specific recognition process. Putting all this together, this suggests that ciprofloxacin increases the output of IL-2 per cell, rather than altering cells to an IL-2-producing phenotype .

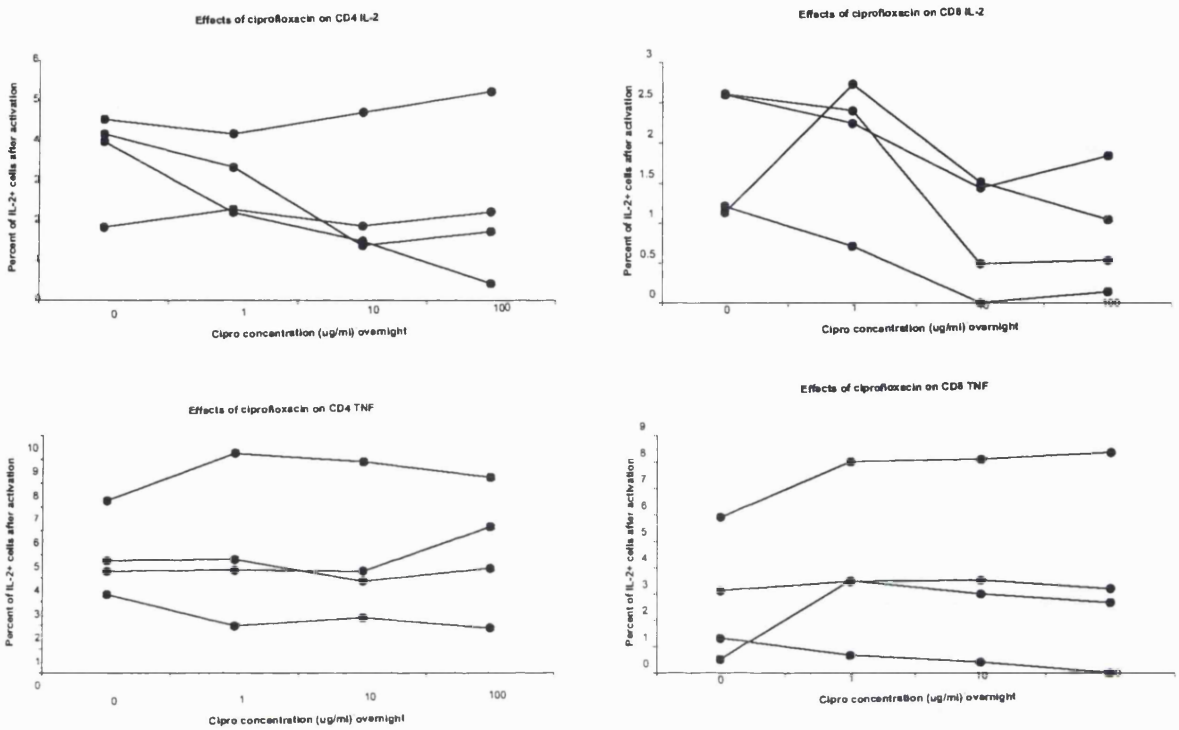


Figure 5.8

Dose-response curves of the *in vitro* effects of ciprofloxacin on IL-2 and TNF-α expression in four normal donors following an 18 h pre incubation with ciprofloxacin followed by stimulation with PMA & ionomycin for 2 h (TNF-α) or 4 h (IL-2).

5.3 Discussion

The aims of this section of the study were to examine the effects of two specific immunomodulatory agents on intracellular cytokine expression under a variety of conditions. The first part examined the effects of IVIG, initially *in vitro* and then *in vivo*. The *in vitro* study showed that IVIG had no effect on cytokine expression in T cells from normal individuals, yet did demonstrate a statistically significant reduction in IFN-γ expression in $CD4^+$ T cells from patients with CVID. This may reflect the increased expression of IFN-γ in CVID lymphocytes (North *et al*, 1996 and 1998) particularly in $CD8^+$ T cells. The abnormally high expression of IFN-γ expression in $CD8^+$ cell in CVID may partly account for why ciprofloxacin was not able to reduce IFN-γ expression in $CD8^+$ T cells, yet still does not explain the differences in ciprofloxacin response between normal and CVID $CD4^+$ T cells. A study using fluorescence microscopy to identify cytokine positive cells following culture with PMA and ionomycin did not demonstrate any effect of IVIG on IFN-γ expression (Andersson *et al*, 1993 & 1994). However, that study did not identify lymphocyte subsets, so changes within individual lymphocyte subpopulations would not have been seen. When the same workers used anti-CD3 to activate lymphocytes however, a reduction in IFN-γ expression with IVIG was seen, although only one IgG concentration (6 mg/ml) was tested.

In conclusion, *in vitro* IVIG appears to have an IFN-γ-reducing effect on CVID $CD4^+$ T cells and not on normal subject's T cells; the reason for this is not clear, but may reflect abnormal cytokine regulation in CVID.

Examination of the *in vivo* effects of replacement-dose IVIG showed no effect on the percentage of T lymphocytes that become activated (and hence express CD69) following stimulation with PMA and ionomycin. Also, no change in IFN-γ expression was identified. It is possible that no effect was seen in CVID because these patients are over-producing IFN-γ and the dose of IVIG was insufficient to reduce this. It may also be that different effects would be seen in CVID patients given their first ever dose of IVIG; all the CVID patients in this study were long-term recipients of IVIG therapy. It would

be important in future studies to determine the *in vivo* effects on IVIG-naive patients. Increases in IFN-γ following replacement dose IVIG *in vivo* were claimed in an early study examining lymphocyte cytokine production by immunofluorescence microscopy, but no data were shown to support this (Ling *et al*, 1993).

The effects of single doses of *in vivo* IVIG on cytokine production were shown by Aukrust *et al* (1994) who measured TNF-α in plasma after the infusion and demonstrated an increase in plasma TNF-α and TNF-R. However, Farber *et al* (1994) had previously shown that the transient increase in plasma TNF-α is only seen when the IVIG is given too rapidly and an adverse reaction has occured. Uneventful infusions were not associated with a rise in plasma TNF-α. Secreted TNF-α was not examined by Farber *et al* (1994), and a closer parallel can be drawn with Maksymowych's work (1996) who studied the *in vivo* effects of high-dose IVIG on LPS-induced TNF-α secretion in culture in a trial of hdIVIG for rheumatoid arthritis. In three out of four patients there was an increase in TNF-α secretion by LPS-stimulated PBMC following IVIG therapy.

Replacement dose IVIG did not affect monocyte IL-12 expression following LPS-stimulation in this study. The findings contrast with related studies of high-dose IVIG in mice in which has IVIG has been shown to down-regulate Fc receptors (FcR) on monocytes by cross-linking, resulting in a reduction in IL-12 production (Ma *et al*, 1998). In our study of replacement dose IVIG, presumably either the IgG dose was not high enough to produce this effect, or human monocyte IL-12 regulation may differ from that in mice. The effect of high-dose IVIG on monocyte IL-12 production was not assessed in the present study.

Replacement dose IVIG did not affect NK cell IFN-γ expression in this study. This may have been due to the short time interval between samples, in that there might not have been time for immunomodulation to occur. The CVID patients involved had been on long-term IVIG therapy, and IVIG-naive patients were not tested. Alternatively, IVIG may just not modulate IFN-γ expression in NK cells. There are no reports of IVIG modulating NK cell cytokine expression to my knowledge.
In the present study, at high dose, IVIG was able to down-regulate the ability of lymphocytes to be activated by PMA and ionomycin, and reduced IFN-γ expression and

perhaps also TNF-α (IL-2 was not measured). The effects were not seen immediately following the infusion, but one month later when the patient next attended for IVIG therapy. There was no evidence to support cytokine modulation having an effect on the eczema, since one patient who down-regulated IFN-γ responded to therapy and one did not. Similarly, a patient who did not respond to IVIG still down-regulated IFN-γ as did another responder.

There are few reports of the effects of hdIVIG on cytokine expression in disease. Apart from the effects on LPS-induced TNF-α production reported by Masymowych *et al*, (1996), there has been a study of cytokines and high-dose IVIG, which examined IL-10 in Kawasaki disease (Noh *et al*, 1998). Plasma IL-10 levels were reduced following therapy in patients receiving hdIVIG.

The mechanism of immunomodulation by IVIG is not clear. Of course IVIG preparations themselves may contain cytokines. Significant quantities of TGF-β1 and TGF-β2, but not IL-10, IL-6 or TNF-α have been reported in commercial IVIG preparations (Kekow *et al*, 1998). Antibodies to cytokines have also been reported in IVIG, including antibodies directed against: IL-1α, TNF-α (Abe et al, 1989) and IL-8 (Reitamo et al, 1993). Other non-immunoglobulin components in IVIG can also exert an immunomodulatory effect; both maltose and sucrose, at concentrations found in commercial IVIG preparations, have been shown to have an anti-proliferative effect (Alder et al, 1996).

There is also evidence that one mechanism for the effect of IVIG on cytokine expression may be Fc mediated. Intact immunoglobulin, but not $F(ab')_2$ *in vitro* could induce TNF-α expression in culture supernatants (Toungouz et al, 1995). However, the effects are complex, since the same group (Toungouz et al, 1996) demonstrated that this effect was due to anti-IFN-γ (but not anti-TNF-α) antibodies present within the IVIG. The effect could be duplicated by addition of anti-IFN-γ instead of IVIG. Such anti-IFN-γ antibodies have been shown to be present at higher concentrations in IVIG preparations high in specific antibodies to hepatitis B and cytomegalovirus (Denys et al, 1997), suggesting that they are an autoimmune response to viral infection.

Another possible mechanism for the immunomodulatory effects of IVIG on lymphocytes is due in part to indirect effects on accessory cells. The *in vitro* reduction of cytokine production by lymphocytes co-cultured with IVIG is dependent upon accessory cells (Skansén-Saphir et al, 1998).

This work in this section is the first to demonstrate that IVIG *in vivo* increases the potential of a subset of CD8 T cells to make TNF-α and CD4 lymphocytes to make IL-2. The increase in TNF-α synthetic capacity by IVIG is supported clinically by the finding that concurrent infections in IVIG recipients results in an adverse reactions (Jolles & Hill, 1998), and that increases in plasma TNF-α have been noted following adverse rections (Farber et al, 1994). In these cases, IVIG may be reducing the TNF-α secretion threshold of lymphocytes already partially activated by the infection. In contrast, IVIG has been shown to down-regulate TNF-α expression in lymphocytres from patients infected with HIV, and to increase membrane-bound p75 TNF receptors (Aukrust et al, 1997). However, patients with HIV may have an increased amount of TNF-α secretion compared with that produced by uninfected individuals, and the effects of IVIG in this context may not be found in other clinical situations.

It is notable that increases in IL-2 and TNF-α expression following IVIG occurred mostly in the CD28- subset. This population of cells is expanded in CVID (North et al, 1994) and may represent 'end-stage' cells entering the apoptotic process (Borthwick et al, 1996). The CD28- population in this study were activated to a greater extent than the CD28+ population (as shown by CD69 expression) in normals, but not in CVID. This failure of CVID CD8+28- cells to be activated despite high cytokine production may be fundamental to the disease state. The $CD8^{+}28^{-}$ population in normal individuals is dominated by expanded clones of cells (Mugnaini *et al*, 1999) and the kinetics of response of these cells may be different in CVID compared with normal subjects.

There is a general clinical impression that IVIG is 'anti-inflammatory' (discussed in Chapter 1), yet comparing pre and post-IVIG samples this effect was not seen at either replacement or high dose. However, comparison of pre-IVIG samples taken serially during long-term hdIVIG therapy in the eczema patients did show anti-inflammatory effects.

There are two possible general conclusions from this series of experiments. One is that replacement dose IVIG is not anti-inflammatory, and may be contributing to the granuloma formation that occurs in some patients with IVIG. The increased level of TNF-α expression post infusion may be transitory, and may not be as deleterious as first appears, since IVIG has been shown to protect cell cultures from TNF-α induced cell death (Stangel *et al,* 1997). The second possibility is that IVIG would reduce IFN-γ expression in CVID patients, but the dose is simply not high enough. I suggest that dramatic reductions in IFN-γ expression in the eczema patients on high dose therapy might be obtained in CVID patients treated with an increased dosage of IVIG up to 2 g/kg instead of the standard 200-400 mg/kg. This hypothesis could readily be tested in the future, since it would not expose CVID patients to additional drugs, and they could remain on the same IVIG product throughout the trial.

With regard to the 'Th2-like' diseases, there is one case report of high-dose IVIG in atopic eczema which examined intracellular IL-4 expression in $CD4^+$ cells during treatment, using an indirect staining technique on PBMCs. There was an increase in IL-4 expression immediately following the treatment and a subsequent decline by two weeks after IVIG (Jolles et al, 1999). The technique used in that study was more sensitive for IL-4 than the whole blood technique described in Chapter 3. The intracellular IFN-γ expression in hdIVIG has also been determined in PBMC using an indirect method, and again a decline in the percentage of IFN-γ+ cells was seen following hdIVIG, accompanied by an improvement in the clinical status of the patient (Jolles, 1996). An increase in Th2 and a fall in Th1 cytokine expression may seem to run counter to the concept of a Th2 disease improving. However, this simplistic approach ignores the kinetics of cytokine change in that the experiments described in this Chapter show that changes in IFN-γ expression occur within a month of hdIVIG therapy, not within hours of infusion. The IL-4 work by Jolles (1996) and Jolles *et al* (1999) also confirms this slow cytokine response to IVIG. Another important issue is that atopic eczema, although said to be 'Th2-like', does not mean that all T cells in the body are Th2 biased. The whole blood assay examined the response of all T cells to mitogen; an antigen-specific assay may have shown a different response. In addition, it may be that the polarised view of Th1 *or* Th2 does not pertain to humans so much as to mice (as discussed in Mosmann &

Sad, 1996). In humans, most antigen-specific T cells are Th0 and the present study may reflect a general decline in proliferative ability mediated by IVIG, rather than a cytokine-polarised result *per se*. In addition, the dichotomy of an immune response into Th1/Th2 may be overly simplistic.

6 Role of monocytes and IL-12 in IFN-γ induction in CVID

The experiments in Chapter 5 examining the effects of *in vitro* IVIG on cytokine expression demonstrated an interesting effect, which merited further investigation. Namely: blood samples from normal subjects, when incubated overnight displayed lower levels of activation marker and cytokine expression the following day (seen in Figure 5.1), compared with blood analysed immediately after sampling (seen in Figure 4.2). In contrast, blood from CVID patients did not display this effect to such an extent (also in Figure 4.2).

Several lines of evidence suggested that monocytes are playing an important role this phenomenon. Firstly, the whole-blood technique involves culture of all blood cells, including monocytes, in the proportions found in peripheral blood *in vivo*. Secondly, studies by our group demonstrated that a greater proportion of monocytes from patients with CVID express IL-12 on activation in comparison to normal controls (Cambronero, Sewell, *et al*, 2000). If monocytes from CVID patients are spontaneously producing IL-12 during the overnight incubation step, this could both lower the activation threshold of the lymphocytes, and increase the potential to express IFN-γ. To address this question, two approaches were taken. Firstly, intracellular cytokines were determined in PBMCs cultured in the presence or absence of monocytes. Secondly, intracellular cytokines were examined in PBMCs cultured with anti-IL-12 blocking antibody.

6.1 Monocyte depletion experiments

To determine the effects of monocytes on the expression of T cell IFN-γ in CVID, blood was taken from six patients and PBMC were isolated using density gradient centrifugation described in Chapter 2. Intracellular IFN-γ was determined in the

lymphocytes within these PBMC using the PBMC intracellular cytokine technique described in Chapter 2. Prior to IFN-γ determination, the PBMCs were divided four ways:

(A) Analysed immediately	(B) Monocytes depleted, then analysed immediately
(C) Incubated at 37°C overnight then analysed	(D) Monocytes depleted, incubated at 37°C overnight before analysis

Monocytes were depleted by an adherence step: PBMC at 1 × 10^6/ml in RMPI1640 supplemented with 10%FCS L-glutamine and antibiotics (as detailed in Chapter 2) were incubated at 37°C in tissue culture grade plastic petri dishes (Nunclon) for 30 min. Non-adherent cells were removed by pipette, the dish washed twice with medium, and the washings were pooled with the non-adherent cells. An aliquot of the cells was stained for $CD3^+$ and $CD14^+$ cells before and after the monocyte depletion step, and examined using standard flow cytometry protocols. Monocytes were always depleted by 50-70%. The total cell concentration was adjusted to 1 × 10^6/ml prior either to immediate intracellular IFN-γ analysis or overnight incubation at 37°C, and subsequent IFN-γ analysis. The results are shown in Figure 6.1.

IFN-γ expression was significantly increased by overnight incubation prior to PMA & ionomycin stimulation in $CD8^+$ cells (mean±SEM of 12.4±4.1% to 28.4±3.5%, p=0.017 by Student's t-test) and $CD8^+28^-$ cells (12.7±6.6% to 50.6±2.1%, p=0.007), as well as the minority population of $CD4^+28^-$ cells (5.8±4.2% to 30.8±3.5%, p=0.018). Although there was a trend towards increased IFN-γ expression in the other subsets, these did not reach statistical significance.

Monocyte depletion did not affect intracellular IFN-γ when cells were stimulated with PMA and ionomycin immediately after sampling.

However, monocyte depletion followed by overnight incubation significantly reduced IFN-γ expression in $CD8^+$ cells from a mean of 28.4±3.5% to 12.6±2.5% (p=0.005).

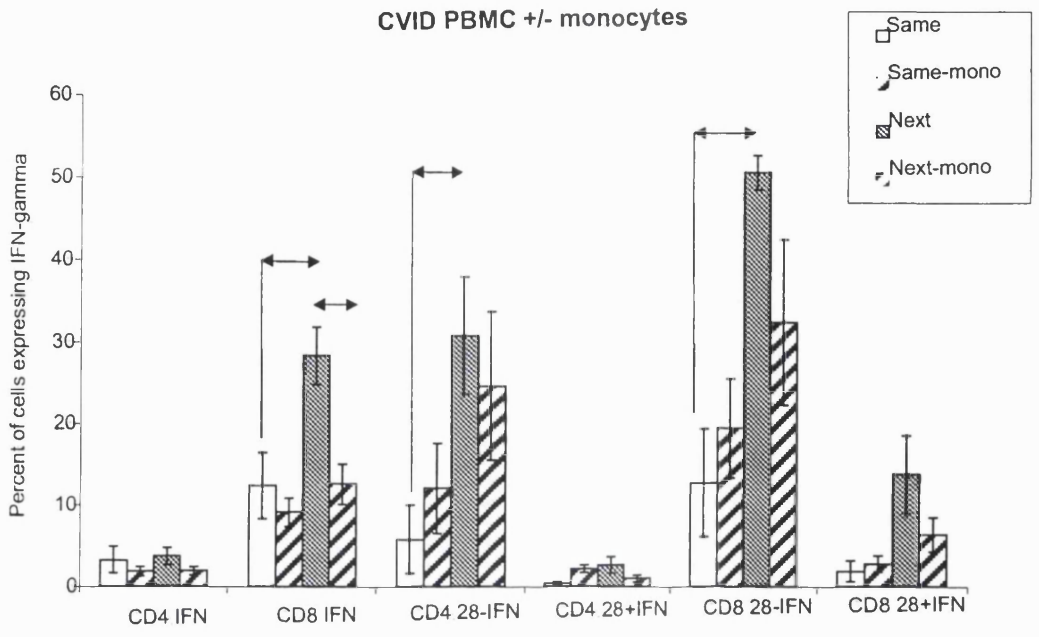


Figure 6.1

Effects of overnight incubation prior to stimulation with PMA and ionomycin on intracellular IFN-γ expression in PBMC, and monocyte-depleted PBMC from patients with CVID. Same = cells analysed immediately, Next = cells incubated overnight before stimulation, -mono = monocyte depleted. Statistically significant comparisons ($p<0.05$ by Student's paired t-test) illustrated with arrows.

There was a downward trend in IFN-γ expression in other cell populations but this was not statistically significant.

6.2 IL-12 blocking experiments

In order to establish the role of IL-12 in the IFN-γ over-expression in CVID, an IL-12 blocking experiment was performed. In this instance, the whole blood technique was used, in order to include monocytes in the cultures in their most physiological state. Blood was taken from CVID patients (n=6) and normal controls (n=4) and again divided into four experimental systems: the first division analysed IFN-γ expression in lymphocytes immediately after sampling, and the second analysed IFN-γ following an overnight incubation at 37°C diluted 1:1 with RPMI1640. Each of these divisions was further subdivided into those with anti-IL-12 monoclonal antibody, and those without. The samples incubated overnight therefore contained IL-12 blocking antibody during the both the overnight phase and during stimulation with PMA and ionomycin.

The blocking antibody used was C8.6 (Pharmingen International, USA), which reacts with human IL-12 p40 monomers, homodimers, and p70 heterodimers (but not the p35 subunit alone). Biologically active forms of IL-12 are therefore inhibited, and C8.6 has been shown to inhibit IL-12 mediated IFN-γ induction, mitogenic effects of IL-12 on PHA blasts, and to inhibit IL-12 mediated enhancement of NK cell-mediated cytotoxicity (D'Andrea *et al*, 1992 and 1995). Azide-free monoclonal antibody was used in this experiment, with a certified endotoxin level of ≤0.01 ng/μg. The blocking antibody was used at a final concentration of 10 μg/ml as this concentration completely abolished biological effects of IL-12 in the D'Andrea studies.

The influence of C8.6 on the expression of IFN-γ in $CD4^+$ and $CD8^+$ cells and their CD28 -/+ subsets is shown in Figure 6.2. When comparisons were made either of all subjects together, or subdivided into normal controls and CVID patients, there was no statistically significant effect of IL-12 blockade on IFN-γ expression in any cell subset. The increase in IFN-γ expression in $CD8^+$ cells incubated overnight was still apparent,

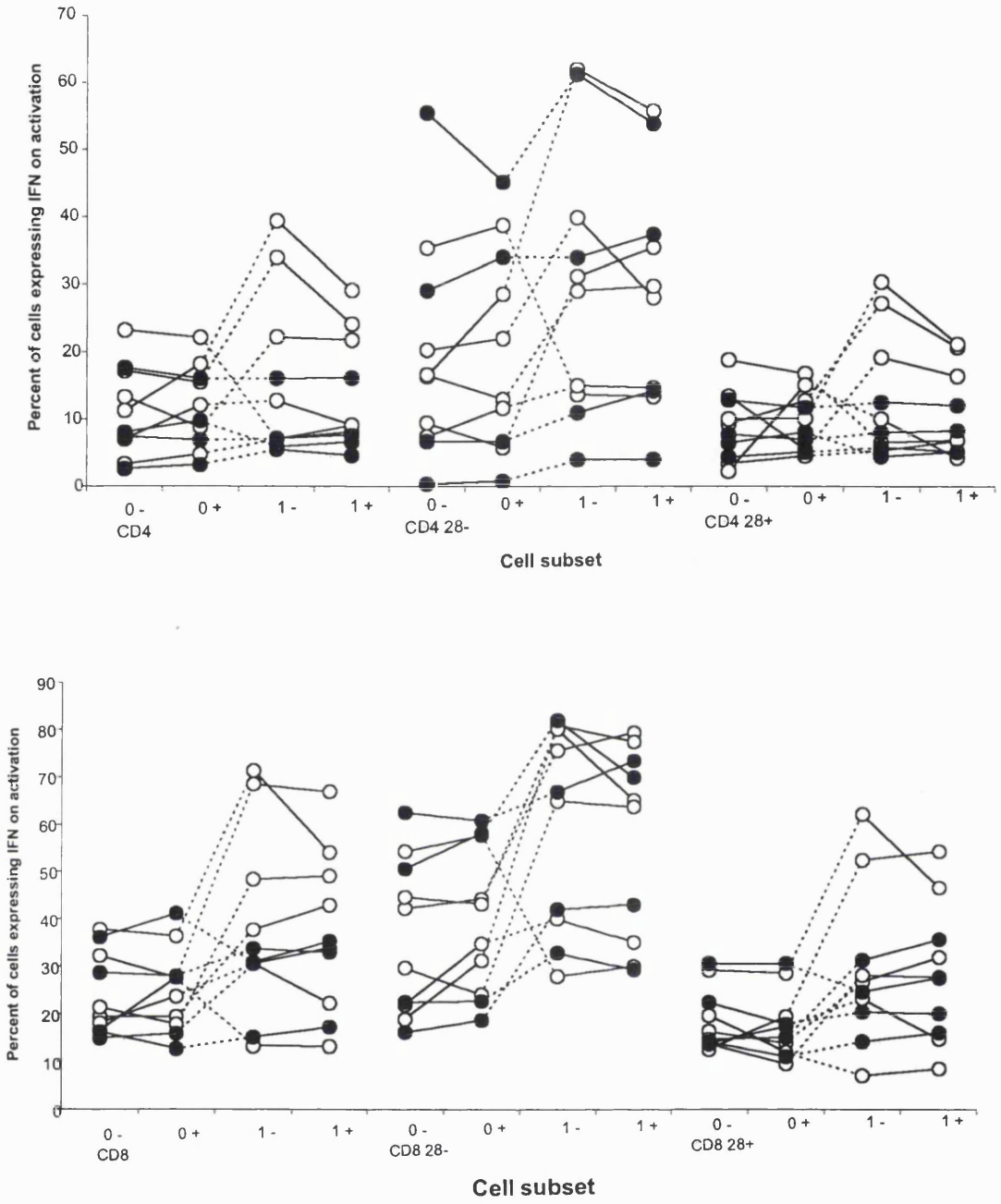


Figure 6.2 The effects of C8.6 IL-12 blocking antibody on IFN-γ expression in $CD4^+$ (top) cells and $CD8^+$ (bottom) and their CD28 -/+ subsets in normal subjects (•) and patients with CVID (o). 0 and 1 indicate samples analysed on day 0 (i.e. immediately after sampling) or day 1 (i.e. after overnight incubation at 37°C; the presence or absence of C8.6 is indicated by + or -, respectively. Lines connect samples from the same subject within a cell subset. Dotted lines distinguish samples from same subject analysed on day 0 compared with day 1.

yet was not affected by the presence of C8.6, seen by examination of the slope of the lines connecting points plotted at 1- and 1+. There were three CVID patients who showed a small decrease in IFN-γ expression in the presence of C8.6 overnight, but this difference was not statistically significant.

This data argues that the concentration of anti-IL-12 used was insufficient to block IL-12 effects in this study, or that IL-12 has little effect under these conditions. Since there was no discernible effect it was not possible to titrate the antibody concentration. However, 10 μg/ml of blocking antibody worked very effectively in both studies by D'Andrea *et al* (1992, 1995), and is generally considered a high concentration of antibody in blocking experiments.

6.3 Discussion

The aim of this part of the study was to examine the role that monocytes play in the abnormal IFN-γ expression seen in CVID, given that (1) the change in CVID T cell IFN-γ expression was different to that in normal T cells when whole blood samples (containing monocytes and lymphocytes) were incubated overnight prior to stimulation with PMA and ionomycin, and (2) previous research had shown an increased percentage of IL-12+ monocytes following activation with LPS in CVID compared with the percentage of IL-12+ monocytes after stimulation found in normal controls (Cambronero, Sewell *et al* 2000).

Several pieces of evidence have suggested that monocyte defects may play a role in the pathogenesis of CVID. Studies from Vienna in the 1980s showed that monocytes from CVID patients pulsed with *E. coli* were unable to stimulate autologous T cells as much as monocytes from healthy normal controls (Eibl *et al*, 1982), despite equal uptake of *E. coli* by CVID monocytes. Monocytes (as adherent cells) from CVID patients were unable to restore the defective anti-CD3-mediated proliferation and CD25 expression of T cells from CVID patients (Fiedler *et al*, 1987). Monocytes from normal donors were able to restore CVID T cell proliferation to normal levels however. Work by Aukrust *et al* (1994c) demonstrated that monocytes from CVID generated significantly greater quantities of reactive oxygen species following stimulation with PMA or zymozan. A

factor in the CVID patient serum enhanced this reactive oxygen species release, and was related to serum neopterin levels. This was not seen in XLA, and was thought to represent *in vivo* activation of monocytes in CVID. Aberrant monocyte activation in CVID has been suggested as the cause of the generation of granulomas in CVID, and patients with CVID and non-caseating granulomas were shown to have defective T cell proliferation in response to mitogens (Mechanic *et al*, 1998).

Incubating PBMC blood from CVID patients overnight before activating the lymphocytes with PMA & ionomycin resulted in significantly increased IFN-γ expression in $CD8^+$ cells, $CD8^+28^-$ cells and $CD4^+28^-$ cells. When monocytes were depleted before the overnight culture, this increase in $CD8^+$ cell IFN-γ expression was not seen.

Given the finding of elevated monocyte IL-12 expression in CVID by Cambronero, Sewell *et al* (2000), these results can be interpreted as follows: the high IFN-γ expression in CVID is due in part to high monocyte IL-12 expression. There is no effect on lymphocyte IFN-γ expression when cells are stimulated the same day immediately following monocyte depletion, since the lymphocytes in this case are still in their *in vivo* state, having received IL-12 signals *in vivo*. When incubated overnight, the IL-12 over-expressing monocytes present in the culture tubes result in higher IFN-γ expression in lymphocytes. This occurs particularly in $CD8^+$ cells. If cells are incubated overnight without monocytes, there is no longer over-expression of IL-12, and hence there is no longer elevated lymphocyte IFN-γ expression. It is perhaps significant that this effect was still noticeable despite the relatively poor percentage depletion of monocytes, suggesting that there is a critical threshold of IL-12 required to 'overdrive' the lymphocyte IFN-γ expression.

However, inhibiting IL-12 with a monoclonal antibody active against the biologically active p35/p40 heterodimer form of IL-12 did not block the effect of overnight incubation in enhancing IFN-γ expression, except in a few individuals (indicated by downward sloping lines in Figure 6.2). Since IFN-γ induction is mediated by other monocyte cytokines apart from IL-12, it was reasoned that other cytokines may be playing a role. It is clear that the induction of IFN-γ in lymphocytes is not solely dependent on IL-12, for example IL-18 is synergistic with IL-12 in IFN-γ induction

(reviewed in Okamura *et al*, 1998), yet IL-18 alone does not induce the development of Th1 cells.

7 T lymphocyte subsets and activation status in CVID

So far, this study has concentrated on using a specially-developed whole blood technique that demonstrated that IFN-γ expression in CVID is aberrantly elevated, confirming findings by North *et al* (1996). In addition, the effects of IVIG on cytokine expression in CVID were investigated and differential effects were found on different cell populations in CVID (Chapter 5). The contributions of monocytes and IL-12 towards these cytokine abnormalities were examined in Chapter 6. I now turn to the question of whether there are differences in the proportions of populations of T lymphocytes capable of producing cytokines in CVID compared with normal subjects, and ask whether any of these populations are abnormally activated in the peripheral blood *in vivo*. Although many studies (summarised in Chapter 1) have examined some of these issues, the present study was an opportunity to use four-colour flow cytometry to examine the size and activation status of more T cell subpopulations than has previously been reported.

7.1 Method

Blood (5 ml) was obtained from patients with CVID (n=15), XLA (n=4) and normal controls (n=12). Patient samples were taken immediately prior to routine IVIG infusion therapy. All samples were collected into EDTA using a standard Monovette collection system (Sarstedt). Samples were analysed within four hours of collection using a standard no-wash whole-blood staining technique. In brief, aliquots of blood (50 µl) were added to cocktails of monoclonal antibodies directly conjugated to fluorochromes in 5 ml polystyrene tubes (Greiner) at previously determined saturating concentrations (data not shown). Cells were stained for 15 min at room temperature in the dark. Red cells were then lysed by the addition of Optilyse C (250 µl), for 15 min. The lysis process was then arrested by the addition of phosphate buffered saline (250 µl) and acquisition on an Epics XL flow cytometer (Beckman Coulter) was performed within two hours. Immediately before flow cytometry, FlowCount beads (50 µl, Beckman Coulter) were added to each tube to provide absolute cell counts as detailed in Chapter 2. A minimum of 10 000 events in a lymphocyte forward/side scatter region were collected, and list mode analysis was performed off-line using WinList 4 (Verity).

Monoclonal antibody combinations for four-colour staining were chosen to identify several subpopulations of T cells. In each staining tube used, the following combinations of surface markers were examined: in FL1 activation status (either CD25 or HLA-DR), in FL2 CD28, in FL3 CD45RA or CD45RO, in FL4 CD8 or CD4 as shown in Table 7.1. Therefore eight tubes were used to stain for surface markers, and a ninth tube was used with mouse isotype-matched control antibodies of irrelevant specificity (for all four FL1-FL4 fluorochromes).

Tube	FL1	FL2	FL3	FL4
1	CD25	CD28	CD45RA	CD4
2	CD25	CD28	CD45RA	CD8
3	CD25	CD28	CD45RO	CD4
4	CD25	CD28	CD45RO	CD8
5	HLA-DR	CD28	CD45RA	CD4
6	HLA-DR	CD28	CD45RA	CD8
7	HLA-DR	CD28	CD45RO	CD4
8	HLA-DR	CD28	CD45RO	CD8

Table 7.1 monoclonal antibody staining combinations used

Cells were first 'immunogated' according to the expression of CD4 or CD8 using a CD4 (or 8) fluorescence versus sidescatter plot. Examination of the contents of this gate in a forward/side scatter plot ensured, particularly for CD4+ cells, that only cells with lymphocyte (as opposed to monocyte) size characteristics were counted. Dot-plots of events from the immunogate were then derived based on CD28- and CD28+ cells and CD45RA or RO expression against each activation marker (CD25 or HLA-DR). In addition, a plot of FL1 against sidescatter was used to define a FlowCount bead region for calibration purposes. Absolute counts were derived using an Excel 97 spreadsheet (Microsoft).

The arithmetic mean, median and skewness (a measure of how symmetrical the data is about the mean, symmetrical=0) were calculated for both absolute cell concentrations and percentages of all cell subpopulations (data not shown) to confirm that the data were normally distributed (arithmetic mean≈median, skewness≈0). Since all populations were

approximately normally distributed, comparisons between patient groups were made using Student's unpaired t test.

The CVID patients were subdivided on clinical criteria and absolute CD4 lymphocyte count into two groups, those with CVID A type characteristics (principally granulomas and CD4 counts <500/μl) (n=5) and those with the milder type non-A CVID (i.e. CVID B or C according to the Bryant *et al*, (1990) classification) (n=10). The use of IL-2 and anti-IgM stimulated immunoglobulin levels to divide patients into Type A, B or C CVID (Bryant *et al*, 1990) was not performed in this study.

7.2 CD28 Expression

The absolute concentrations of CD4 and CD8 T cells in normals and patients, as well as their subdivision into CD28-/+ subsets are shown in Figure 7.1. The only significant difference in absolute CD4 or CD8 T cell counts between normals and CVID patients lies within the CVID Type A group, characterised by low CD4 counts (mean±SEM 802±57/μl versus 343±20/μl, $p<0.0001$). In addition, there were significantly less CD8+ 28+ cells in CVID patients of the severe type A group (normals 309±36/μl versus 95±24/μl in CVID Type A, $p<0.0001$), and there were significantly more CD8+28- cells in the non-type A CVID patients than in normals (403±94/μl in non-Type A versus 179±36/μl in normals, $p=0.05$).

In percentage terms, the proportions of CD28+ cells are illustrated in Figure 7.2. The CVID patients had proportionally more CD8+28- cells (51.2% in CVID compared with 34.2% in normal subjects, $p=0.025$) as shown by the reduction in CD28+ cells in Figure 7.2. Although the subset is very much a minority one, there was a larger percentage of CD28- cells in the $CD4^+$ subset in CVID (4.9±1.3% in normals versus 10±1.6% of $CD4^+$ T cells in CVID, $p=0.002$). There were no significant differences in any T cell subset between normal donors and XLA patients in terms of CD28 expression, either in percentage or in absolute counts.

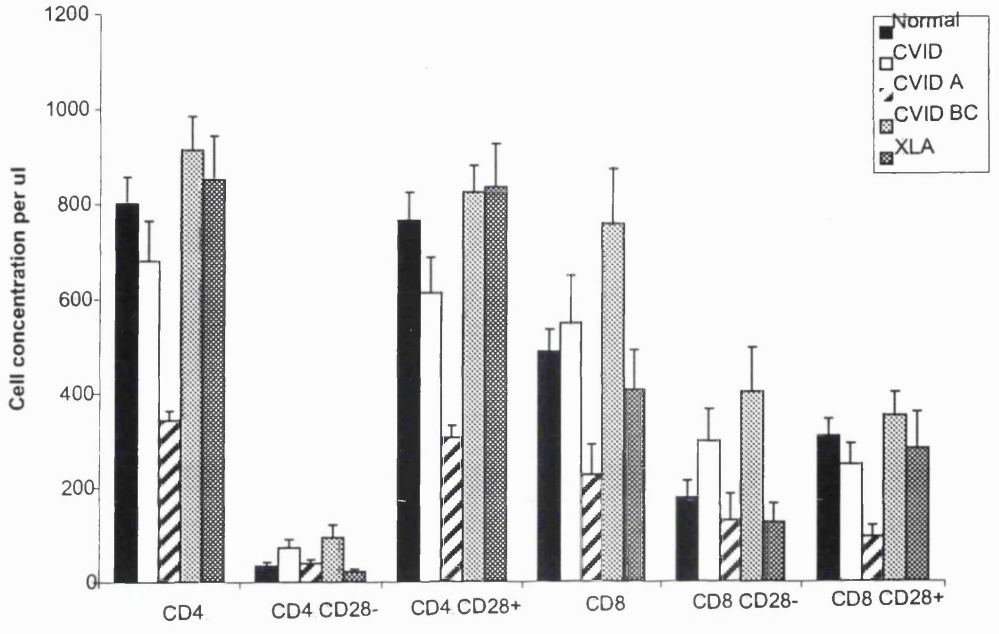


Figure 7.1 Absolute T cell counts in CVID, XLA and normal subjects according to CD4, CD8 and CD28 expression.

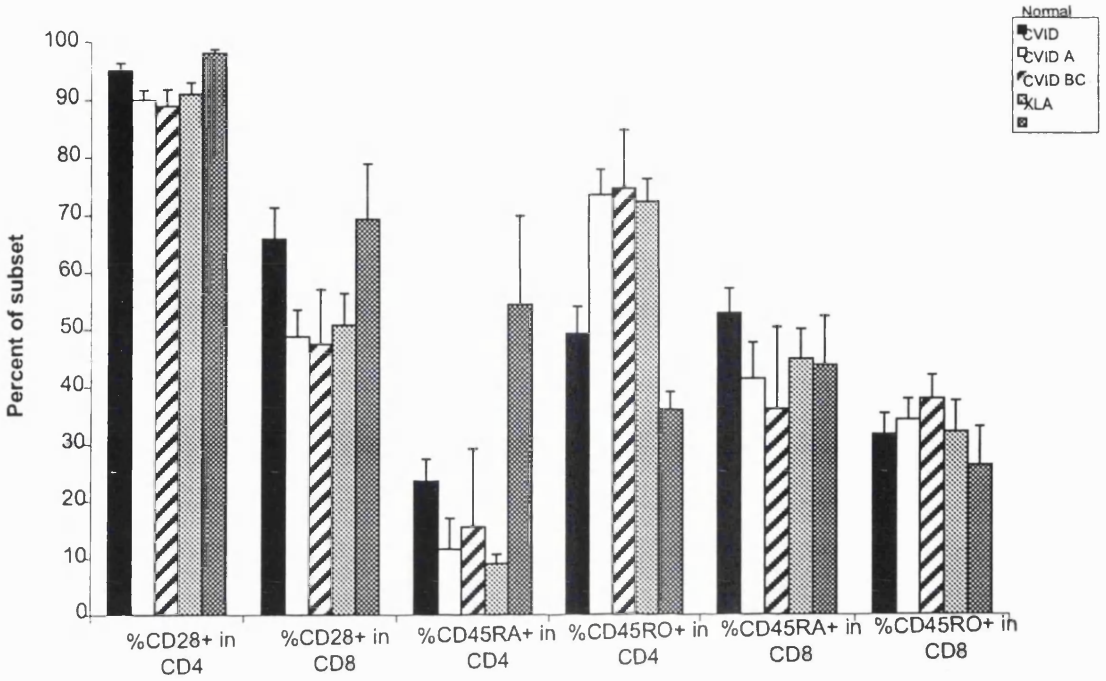


Figure 7.2 Percentage of T lymphocyte subsets expressing CD28, CD45RA or CD45RO

7.3 CD45RA and RO Expression

Within the CD4 populations, there were significantly fewer CD45RA cells in the CVID group as a whole (70±20/μl, p=0.016) and within each CVID subgroup (Type A 51±45/μl, p=0.036; Type B/C 83±16/μl, p=0.026) compared with these cell populations in normal controls (196±42/μl)(see Figure 7.3). There appeared to be an increased number of CD4+45RA+ cells in XLA patients, but this was not statistically significant. In CVID patients, the CD4+45RO+ numbers were not significantly different to normal except in the subgroups of CVID, where there were fewer RO+ cells in the type A group (253±36/μl compared with 389±47/μl in normal subjects, p=0.035), and more in the milder type B/C forms (662±68/μl, p=0.005).

Within the CD8 populations, there were fewer differences between patients and normal controls. There were fewer CD8+45RA+ cells in the type A CVID patients only (81±36/μl compared with 266±38 in normal subjects, p=0.003), with a similar CD28± distribution to normal subjects. There was a trend to an increase in CD8+CD45RO+ cells in non-type A CVID, but this did not reach significance.

The relative proportions of CD45RA+ and CD45RO+ cells are shown in percentage terms in Figure 7.2, to the right of the CD28 percentages. The percentage of CD4 cells which were CD45RO+ was much lower in normal subjects (49±5%) than in CVID 73±4%, p=0.001). Interestingly, only in the non-Type A CVID patients was the converse true, i.e. a relative decrease in the percentage of CD4+ cells which were CD45RA+ (9±2%, compared with 23±4% in normal controls, p=0.003).

7.4 Triple expression of CD4 (or 8), CD45RA (or RO), and CD28

Absolute counts of the subdivision of CD4 (or CD8) into CD45RA+ and CD45RO+ cells based on CD28 expression is shown in Figure 7.4. Within the CD4+ population, there were significantly fewer CD4+CD45RA+CD28+ cells in CVID (61±17/μl compared with 187±41/μl in normal controls, p=0.013). Concentrations of CD4+CD45RO+CD28+ cells were similar in CVID compared to levels seen in normal controls. However there were fewer such cells in type A CVID (235±37/μl compared with 373±49/μl in normal subjects, p=0.04) and more in non-type A CVID (598±53/μl,

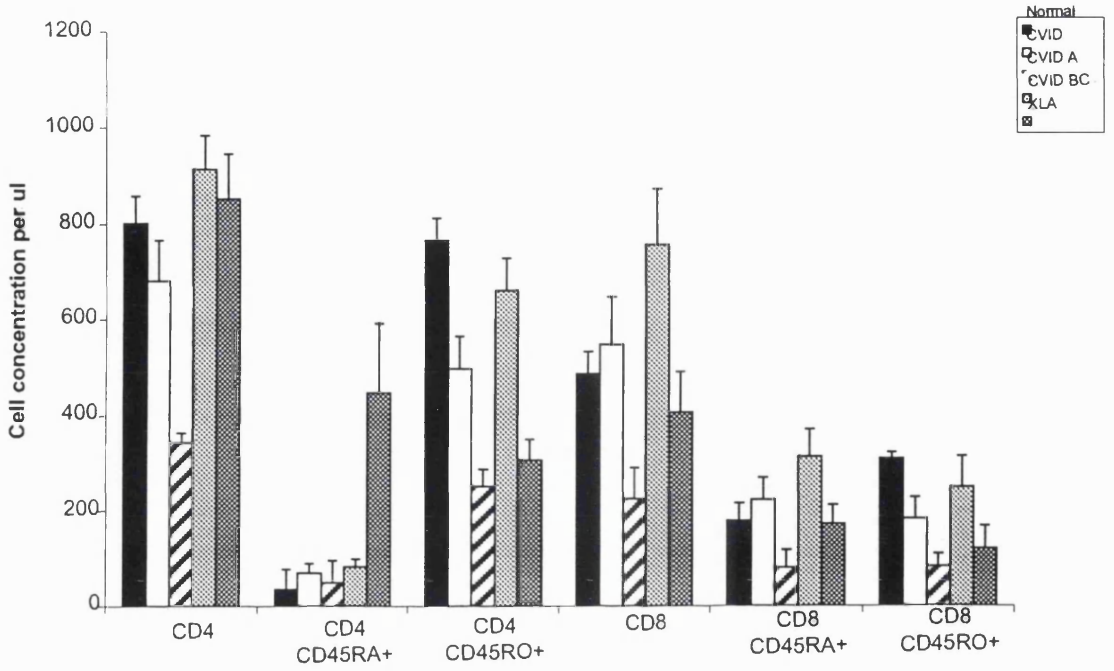


Figure 7.3 Absolute concentrations of T cell subsets in normals, CVID, CVID subsets and XLA according to CD45 antigen expression

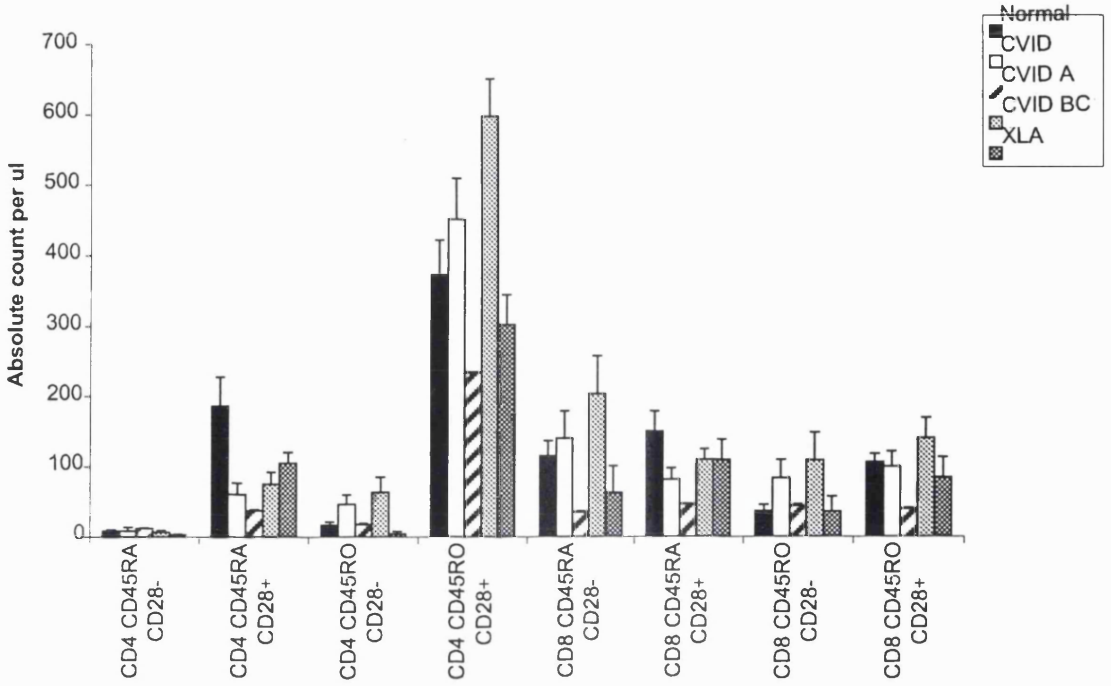


Figure 7.4 Absolute counts of T lymphocyte subsets according to CD4 or CD8, CD45RA or RO, and CD28 expression

p=0.006). Apart from significantly fewer CD4+CD45RA+CD28- cells in XLA patients (3.1±1.5/μl) compared with that seen in normal subjects (9.0±1.5/μl, p=0.02) there were no other significant CD4/45/28 subset differences between normal subjects and XLA patients.

Within the CD8+ population, CD8+CD45RA+28+ cells were similar in CVID as a whole compared to normal subjects. However type A CVID had lower levels (81.9±17/ul compared with 151±29/μl in normal controls, p=0.032). CD8+CD45RO+CD28+ cells were much lower in CVID type A than in normal controls (39.2±6.6 compared with 107±12/μl in normal controls, p<0.0001), yet there was no significant difference between normal subjects and CVID as a whole, or non-type A CVID. No differences were found between XLA patients and normal controls in terms of CD8/CD45/CD28 populations.

7.5 Relative proportions of CD45RA/CD45RO/CD28 expressing populations

Interesting differences in percentage terms could be seen between patient groups and normal controls when the expression of CD28 was examined in relation to CD45RA/RO subsets. The expression of CD28 on CD45RA+ and CD45RO+ cells is shown in Figure 7.5. Within CVID as a whole (17.4±4.3%, p=0.021), and CVID Type A (25.3±7.2%, p=0.044) there were a greater percentage of CD4+RA+ cells which were CD28- than in normal subjects (6.0±1.1%). Similarly, in CD8+RA+ cells, there was a significantly greater percentage of CD28- cells in CVID (62.8±5.1%, p=0.04) than in normals (44.6±6.6%). There were no significant differences in terms of CD28 expression in CD45RO subsets of CD4 or CD8 cells between normal subjects and CVID patients. Also, there were no differences in CD28 expression between normal subjects and CVID patients in any of the CD45 subsets (Figure 7.5).
Examining the CD28- subset from the opposite direction, namely asking what is the proportion of CD28- cells which are CD45RA+ and what is the proportion of CD28- cells which are CD45RO+ is shown in Figure 7.6

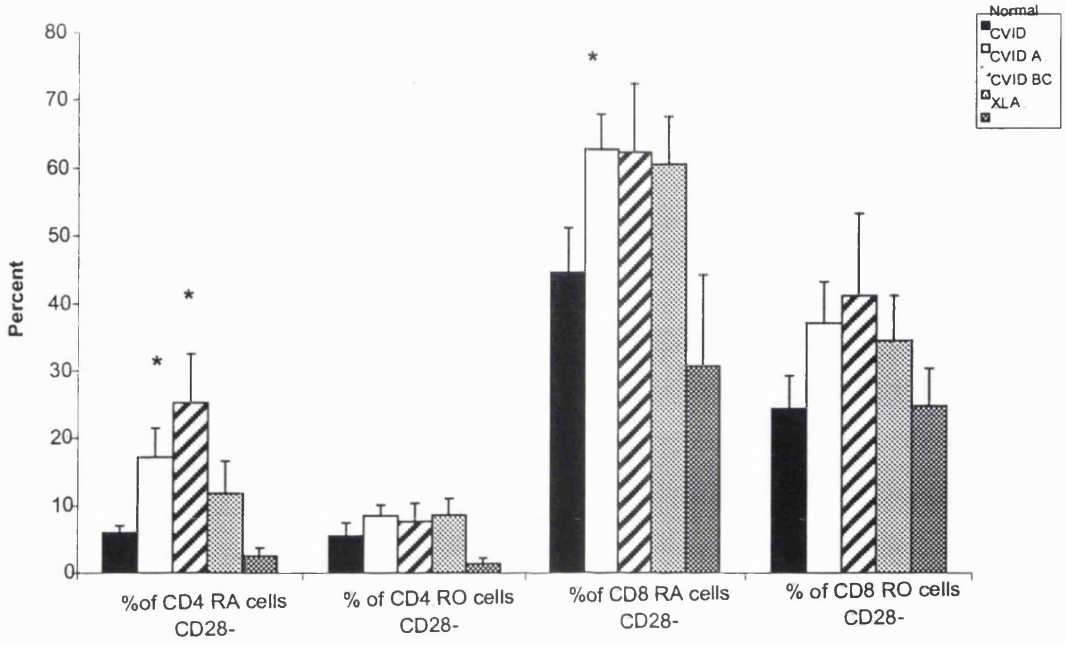


Figure 7.5 Percentage of CD28- cells within CD45RA and CD45RO cells. *=p<0.05

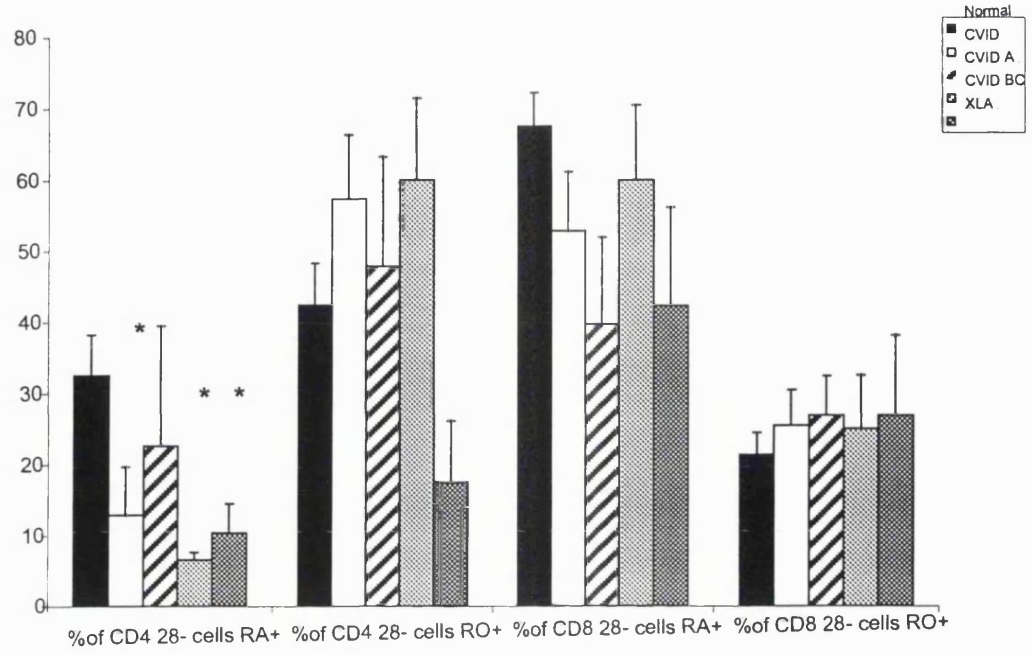


Figure 7.6 Expression of CD45RA and CD45RO within CD28- cells. *=p<0.05 compared with normal subjects

There was a significantly lower proportion of CD4+28- cells which were CD45RA+ in CVID as a whole (12.9±6.8%, p=0.036) or in non-Type A CVID patients (6.6±1.1%, p=0.001) than in normal controls (32.6±5.6%). Interestingly, there were also significantly fewer CD4+CD28- cells in XLA patients which were CD45RA+ (10.4±4.1%, p=0.008). There were no significant differences in the CD8+ population.

7.6 CD25 expression

CD25, the IL-2Rα chain, is normally expressed on only a minority of peripheral blood cells *in vivo*. Figure 7.7 shows the percentage of cells expressing CD25 in the various T cell subsets defined by CD4 or 8, CD45RA or RO, and CD28. For the sake of clarity, since this approach generates such a large number of comparisons, the means and standard errors and accompanying t-test probabilities are given in Table 7.2, rather than mentioned explicitly in the text.

In all subpopulations, and in all patient groups, CD25 expression was extremely low (~1%), making valid comparisons between groups difficult. Within the CD4 populations (Figure 7.5) no patient group expressed higher levels of CD25 than normals (although CD28- subpopulations of CD4 cells showed a trend towards greater expression in CVID type A, this was associated with wide variation, and did not reach statistical significance). In general, XLA patients had similar levels of CD25 expression to normals and CVID patients actually had lower levels than normal subjects.

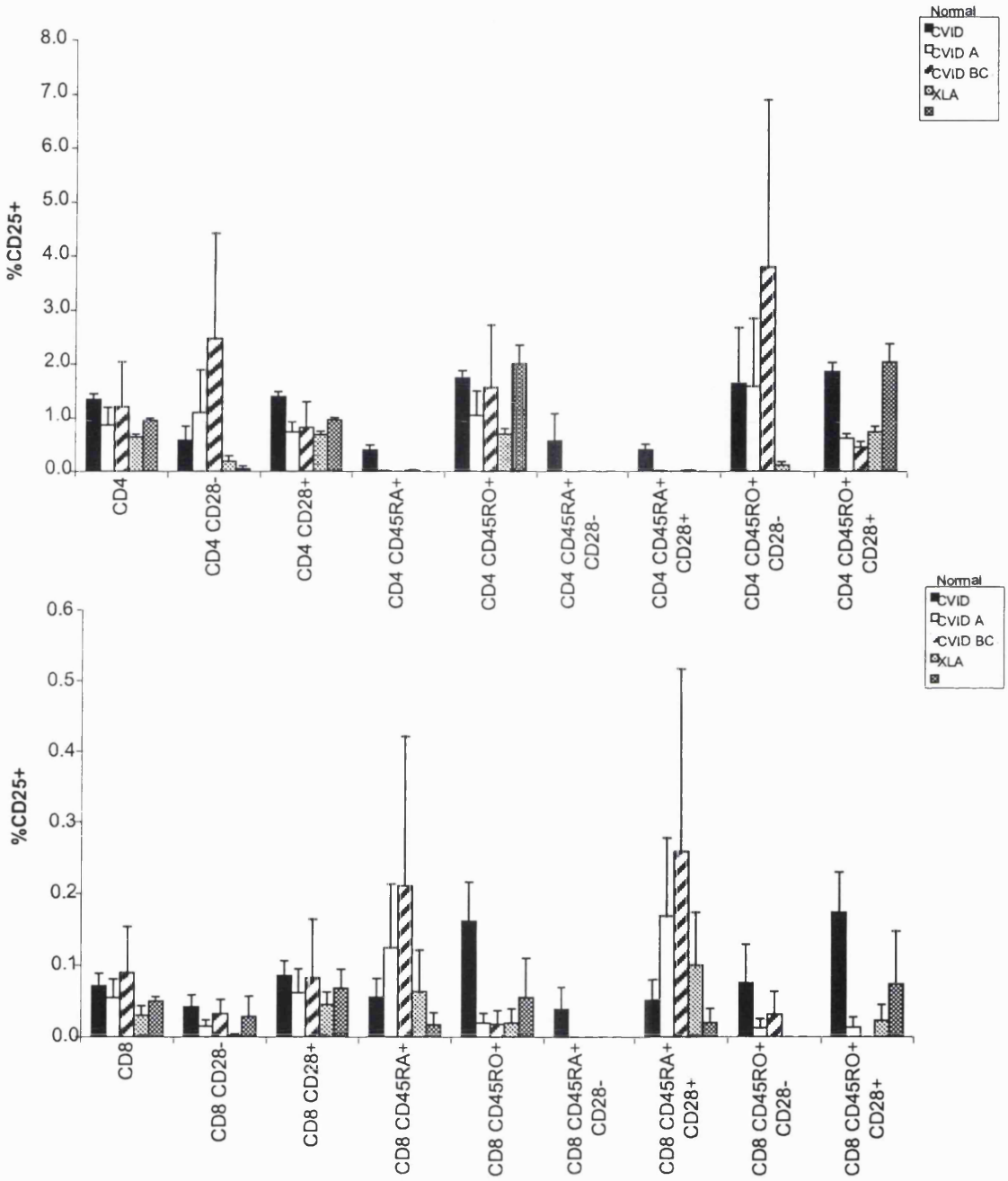


Figure 7.7 Percentage of CD4 cells (top) and CD8 cells (bottom) expressing CD25 *in vivo* in normal subjects, CVID and XLA

Population	Normal	CVID	CVID A	CVID BC	XLA	Compared with normal controls, p=			
						CVID	CVID A	CVID BC	XLA
CD4	1.35±0.1	0.87±0.32	1.21±0.8	0.64±0.05	0.95±0.05	-	-	<0.0001	0.004
CD4 28-	0.58±0.26	1.1±0.8	2.47±2	0.19±0.11	0.05±0.05	-	-	-	-
CD4 28+	1.4±0.1	0.74±0.18	0.83±0.5	0.69±0.06	0.96±0.04	0.005	-	<0.0001	0.001
CD4 45RA+	0.4±0.1	0.01±0.01	0±0	0.02±0.02	0±0	0.002	0.002	0.003	0.002
CD4 45RO+	1.75±0.14	1.05±0.46	1.56±1.2	0.7±0.11	2.01±0.35	-	-	<0.0001	-
CD4 45 RA+28-	0.57±0.5	0±0	0±0	0±0	0±0	-	-	-	-
CD4 45RA+ 28+	0.4±0.1	0.01±0.01	0±0	0.02±0.02	0±0	0.003	0.002	0.003	0.002
CD4 45RO+28-	1.64±1.0	1.59±1.27	3.8±3	0.11±0.07	0±0	-	-	-	-
CD4 45RO+28+	1.86±0.17	0.63±0.08	0.45±0.1	0.74±0.11	2.04±0.34	<0.0001	<0.0001	<0.0001	-
CD8	0.07±0.02	0.05±0.03	0.09±0.06	0.03±0.01	0.05±0.01	-	-	-	-
CD8 28-	0.04±0.02	0.01±0.01	0.03±0.02	0±0	0.03±0.03	-	-	0.042	-
CD8 28+	0.09±0.02	0.06±0.03	0.08±0.08	0.05±0.02	0.07±0.03	-	-	-	-
CD8 45RA+	0.06±0.03	0.12±0.09	0.21±0.21	0.06±0.06	0.02±0.02	-	-	-	-
CD8 45RO+	0.16±0.05	0.02±0.01	0.02±0.02	0.02±0.02	0.06±0.06	0.026	0.027	0.029	-
CD8 45 RA+28-	0.04±0.03	0±0	0.26±0.26	0±0	0±0	-	-	-	-
CD8 45RA+ 28+	0.05±0.03	0.17±0.11	0.03±0.03	0.1±0.07	0.02±0.02	-	-	-	-
CD8 45RO+28-	0.08±0.05	0.01±0.01	0±0	0±0	0±0	-	-	-	-
CD8 45RO+28+	0.17±0.06	0.01±0.01	0±0	0.02±0.02	0.07±0.07	0.017	0.011	0.026	-

Table 7.2 Percentage of T lymphocytes expressing CD25 defined by CD4 or 8, CD45RA or RO, and CD28. The mean±SEM is shown. Significant p values are shown for comparisons of normal subjects with patient groups by Student's t-test. - =not significant

7.7 HLA-DR Expression

HLA-DR is expressed at low levels *in vivo*, and is an example of an activation marker expressed relatively late after the initial stimulus (Oshima & Eckels, 1990).
The expression of HLA-DR in the present study was considerably greater than CD25 expression, making statistical comparisons between groups more meaningful. Again, comparisons of the many figures involved are shown in tabular form, in Table 7.3 and illustrated in Figure 7.8.

Within the CD4 subset, the percentage HLA-DR expression was increased in CVID (5.8±0.8%, p=0.03) compared with that in normal controls (3.8±0.3%), and as the majority of CD4 cells are CD28+, the expression of HLA-DR on CD4+28+ cells (4.5±0.6, p=0.04) was also significantly elevated compared with that in normal controls (3.2±0.3). An unexpected finding was the considerably *higher* expression of HLA-DR on the minority population of CD4+28- cells (76.9±11.4%, p=0.013)and on CD4+45RO+ cells (9.1±0.9%, p=0.029) in XLA compared with levels seen in normal controls (where 24.8±4.7% of CD4+28- cells, and 6.2±0.6% of CD4+45RO+ cells were HLA-DR+).

HLA-DR expression was similar between patient groups save for minor differences between CD8+CD28+ DR expression in CVID patients as a whole (7.3±1.3%), who had higher levels than normals (4.2±0.6%, p=0.039). Interestingly, in CD8+ cells, HLA-DR expression was considerably *lower* in XLA in CD8+RA+28+ cells (0.6±0.3%) than normal controls (2.7±0.6%, p=0.038).

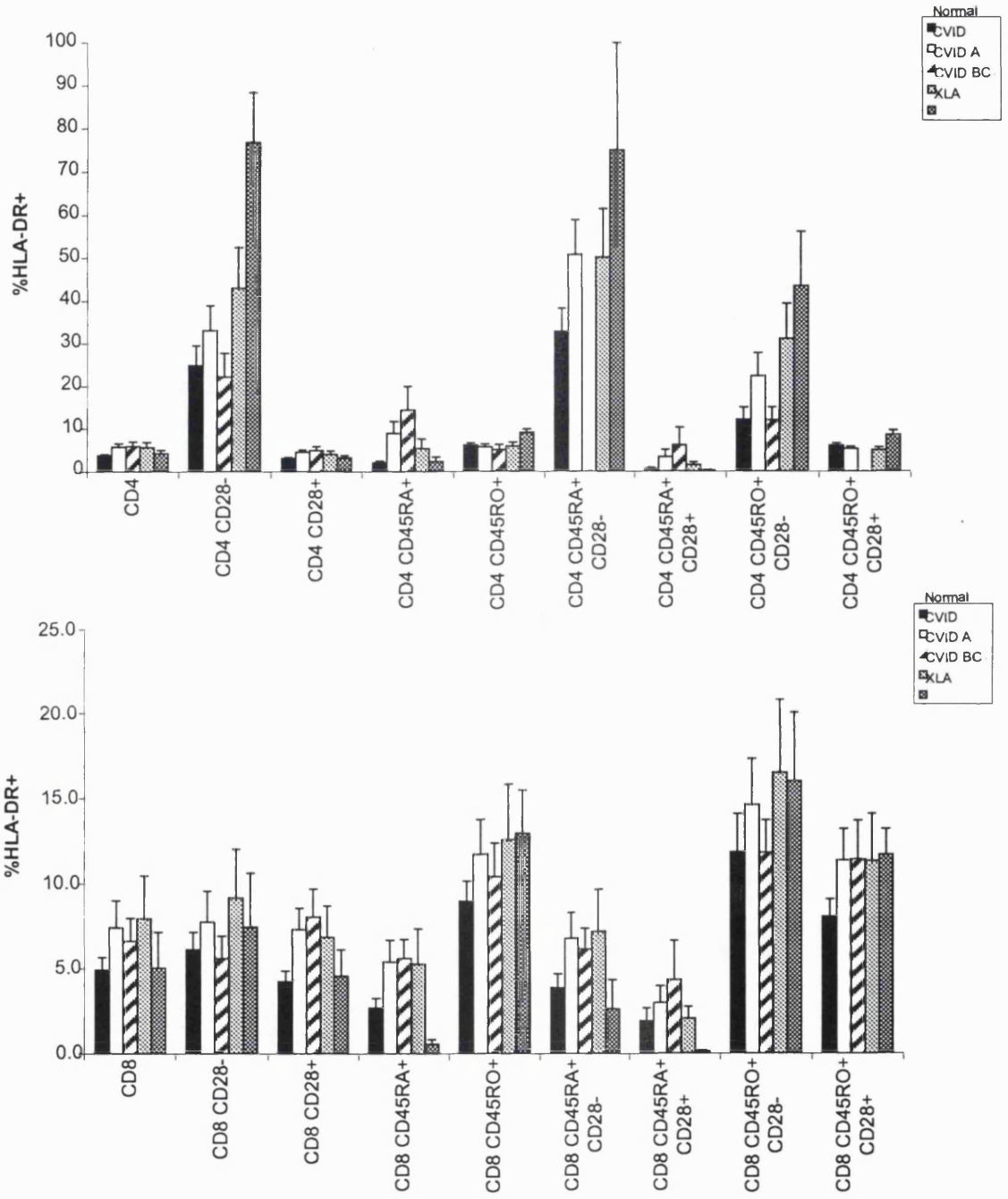


Figure 7.8

Percentage of CD4 cells (top) and CD8 cells (bottom) expressing HLA-DR *in vivo* in normal subjects, CVID and XLA

Population	Normal	CVID	CVID A	CVID BC	XLA	Compared with normal controls, p=			
						CVID	CVID A	CVID BC	XLA
CD4	3.8±0.26	5.8±0.8	5.9±1.1	5.7±1.2	1.3±1.2	0.026	-	-	-
CD4 28-	24.9±4.7	33.0±5.8	22.3±5.5	43.1±9.5	76.9±9.5	-	-	-	0.013
CD4 28+	3.21±0.27	4.5±0.6	5.0±0.9	4.1±0.8	3.3±0.8	0.043	-	-	-
CD4 45RA+	2.1±0.4	9.0±2.8	14.4±5.7	5.4±2.3	2.4±2.3	0.029	-	-	-
CD4 45RO+	6.21±0.56	5.8±0.8	5.2±1.2	5.9±1.0	9.1±1.0	-	-	-	0.029
CD4 45 RA+28-	32.8±5.4	50.9±8	0±0	50.2±11.2	75±11	-	-	-	-
CD4 45RA+ 28+	0.66±0.2	3.4±1.8	6.1±4.2	1.6±0.7	0.24±0.66	-	-	-	-
CD4 45RO+28-	12.2±2.9	22.3±5.4	12.0±3.1	31±8	43±8	-	-	-	-
CD4 45RO+28+	6.0±0.5	5.3±0.5	0±0	4.9±0.7	8.6±0.7	-	-	-	-
CD8	4.9±0.7	7.4±1.6	6.6±1.4	7.9±2.5	5.0±2.5	-	-	-	-
CD8 28-	6.1±1.1	7.7±1.8	5.6±1.3	9.1±2.9	7.5±2.9	-	-	-	-
CD8 28+	4.23±0.6	7.3±1.3	8.0±1.6	6.9±1.8	4.6±1.8	0.039	-	-	-
CD8 45RA+	2.7±0.6	5.4±1.3	5.6±1.1	5.3±2.1	0.7±2	-	-	-	0.004
CD8 45RO+	9.0±1.2	11.7±2.1	10.4±2.0	12.6±3.3	12.9±3.3	-	-	-	-
CD8 45 RA+28-	3.9±0.8	6.8±1.5	6.2±1.2	7.2±2.5	2.6±0.7	-	-	-	-
CD8 45RA+ 28+	1.9±0.8	3.0±1.0	4.3±2.3	2.1±0.7	0.11±0.7	-	-	-	0.038
CD8 45RO+28-	11.9±2.3	14.6±2.7	11.8±1.9	16.5±4.3	16.0±4.3	-	-	-	-
CD8 45RO+28+	8.0±1.0	11.3±1.8	11.4±2.3	11.3±2.8	11.6±2.8	-	-	-	-

Table 7.3 Percentage of T lymphocytes expressing HLA-DR defined by CD4 or 8, CD45RA or RO, and CD28. The mean±SEM is shown.

Significant p values are shown for comparisons of normal subjects with patient groups by student's t-test. - =not significant

7.8 Co-expression of CD28 and CTLA-4 (CD152)

This study has confirmed the finding of others (North *et al*, 1998) of a greater proportion of CD28- cells within the CD8+ subset in CVID, compared with normal subjects. In addition, the CD28- subsets of cells in CVID express IFN-γ differently to normal subjects, although in this experiment CD28- cells did not express either CD25 or HLA-DR differently to normal subjects.

Given these abnormalities of CD28- cells in CVID, the question then arose as to whether the CD28- cells might be negative because they have been activated and are expressing CTLA-4 (CD152) and have gone on to down regulate CD28 expression. Whole blood samples from normal subjects (n=3) and CVID patients (n=4) were therefore simultaneously stained for expression of CD28 (FL1), CD152 (FL2), CD8 (FL3) and CD3 (FL4). The CD152/PE monoclonal antibody was from Immunotech. The percentage of cells expressing CD152/CD28 within CD4 and CD8 subsets are shown in Figure 7.9.

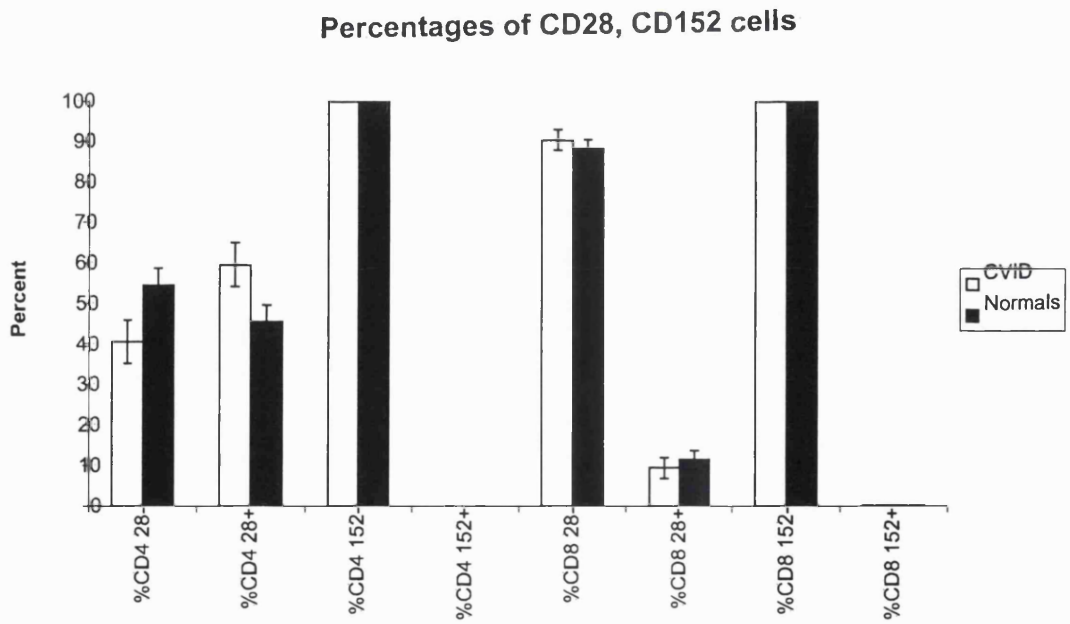
Neither normal subjects nor CVID patients expressed any CD152 in this study, even using up to 20 µl of monoclonal antibody per 50 µl of cells (4-times the recommended concentration) on any cells (CD28+ or CD28-). (Figure 7.9 represents cells stained with the four-fold increase in anti-CD152 antibody). Although this figure does not demonstrate any difference in the proportions of CD8+28- cells in CVID compared to normal subjects, this probably reflects the small numbers of subjects compared (3 normal subjects and 4 patients).

7.9 Discussion

Although numerous studies have examined T cell phenotypes in CVID (e.g. Spickett *et al*, 1990; Spickett *et al*, 1992) the present study is the first to use the resolving powers of four-colour flow cytometry to clearly distinguish lymphocyte phenotypes in CVID.

The finding that the most extreme abnormalities in this study were found in the Type A group, chosen because they had a combination of proven granulomatous disease and low

Figure 7.9 Expression of CD28 and CD152 (CTLA-4) on CD4 and CD8 T cells in CVID and normal subjects



CD4 T cell counts (<500/ul) confirmed the earlier findings regarding this subgroup of patients, namely low numbers of CD45 RA+ cells (Bryant *et al*, 1990; Farrant *et al*, 1994). The new finding here is that this can be now be seen in the light of previous reports which found that in CVID there is an expansion of the CD8+28- population (North *et al*, 1994). Simultaneous examination of CD45RA and CD28 in the present study has shown that there are fewer CD4+45RA+28+ cells in CVID. In addition, when relative proportions of cells are compared, a higher percentage of CD45RA+ T cells from CVID patients are CD28-, in both the CD4+ and CD8+ populations. With CD45RO+ cells however, this is not the case. In both the CVID patients and normal individuals, in the CD4 population, the proportion of CD28- cells is roughly equal in CD45RA+ and CD45RO+ populations; in the CD8 population most CD28- cells were RA+. However, the absolute numbers of any RO+ population is greater than the RA+ population in CVID. In CVID, there are more CD28- cells in the CD8+RA+ population than the CD8+RO+ population. Viewed from another perspective, more CD28- cells are RA+ than RO+ in CVID, even in the CD4 population, but particularly in the CD8 population.

It should be borne in mind that certain other studies have not distinguished CD45RA+ cells from CD45RO- cells (e.g.Jaffe *et al*, 1993), this could be misleading, since the distribution of CD45RA and RO do not have strictly reciprocal expression (Salmon *et al*, 1989). In addition, some cells are neither CD45RO+ nor CD45RA+. In the present study, the selection of staining antibodies positively identified CD45RA+ cells or CD45RO+ cells; rare double positive cells would 'count twice'.

Although formal typing of CVID patients into the three groups (Types A, B and C) using the Bryant technique (1990) (and subsequently investigated by Spickett *et al*, 1994) was not performed in the present study, similar abnormalities as reported by Spickett *et al* (1994) were confirmed in the most severely affected (Type A) patients. This Type A subgroup of CVID patients has also been found to have other phenotypic abnormalities, such as an expansion of CD8+ cells (Wright *et al*, 1990) which tend to be CD57+ and have deficient IL-2 yet elevated IFN-g production (Jaffe *et al*, 1993). No patient in the present study had elevated levels of $CD8^+$ T cells, and $CD57^+CD8^+$ cells were not examined.

Although examination of lymphocytes from a large series of CVID patients using 2-colour flow cytometry failed to reveal differences in CD25 or HLA-DR expression (Spickett *et al*, 1991), the techniques used may not have been sufficiently sensitive given the limitations of 2-colour compared with 4-colour techniques. Using only 2-colour flow cytometry to detect low levels of activation marker expression has been shown to be less accurate than multi-parameter flow cytometry (reviewed in Amlot *et al*, 1996).

The discrepancy of expression of CD25 and HLA-DR in the present study is interesting, since both markers are often viewed as 'activation antigens' (Amlot *et al*, 1996). Although CD25 is a relatively 'early' activation marker, expressed rapidly after the initial stimulus (Jackson *et al*, 1990), HLA-DR is a 'late' activation marker, appearing several days after the initial stimulus (Oshima & Eckels, 1990). The discrepancy in activation shown here may indicate an aberrant pattern of activation in CVID, particularly since CD152 (CTLA-4) was not expressed on either CD28- or CD28+ cells *in vivo*. Studies of soluble activation markers, such as soluble CD25 and beta-2 microglobulin, have shown an increase in serum levels in CVID (North *et al*, 1991b). This may indicate that activated cells are either rapidly sequestered *in vivo*, and/or that activation is occuring in a separate immunological 'compartment' e.g. the lymph nodes, spleen, or in granulomas and these cells are not reaching the peripheral blood.

The role of CD28- cells is now becoming better understood. There is an increase in CD8+28- with age (Fagnoni *et al*, 1996), and it could be argued that future work should use an older cohort of normal control subjects than was available in the present study. Other studies have found that CD8+28+ cells preferentially migrate to the gut (Arosa *et al*, 1998) which contains predominantly CD28- cells (Ohteki *et al*, 1993). This presumably indicates that there is differentiation of CD28+ to CD28- cells at this site. Given the inflammatory bowel condition that is frequently present in CVID (Cunningham-Rundles & Bodian, 1999) this may account for the source of CD28- cells in CVID.

A study by Nociari *et al* (1999) has determined the memory status of CD28- cells in detail. That study did not find a preferential accumulation of CD45RO+ cells in the CD8+28- population except in normal subjects <25 years old. The present study did not find an increase in the proportion of RO+ cells in normals in the CD8+28- population (see figure 7.5), in fact of the CD8+28- cells the majority were CD45RA+ (68±5%)

compared with CD45RO+ (21±3%, $p<0.001$). This was also the case in CVID and XLA subjects. An interesting possibility raised by the Nociari (1999) study is that CD28-45RA+ cells are derived from CD45RO+ cells, since the CD28-RA+ cells were found to be both CD25+ and HLA-DR+ (a feature of recently primed cells), and to be IFN-γ+ (also a feature of primed memory T cells (Salmon *et al*, 1994)) and had similar length telomeres to their CD45RA- counterparts. The present study showed that CVID CD8+28-RA+ cells did not express CD25, but did express HLA-DR, at similar levels to that seen in normal controls. The finding that in CVID there are similar numbers of CD8+28-RA+ cells as in normal control subjects (shown in Figure 7.4) suggests that this reversion mechanism can still operate in CVID. However, in the severe Type A group there were significantly fewer CD8+28-RA+ cells (35±8/μl) compared with concentrations of these cells in normal controls (115±22/μl, $p=0.004$) suggesting that in the clinically more severe form of CVID there is a defect in reversion of CD45RO+ to CD45RA+ cells.

Similar reversion mechanisms for CD4+ cells have also been proposed (Nociari *et al*, 1999). The finding that CD8+28- population contains a predominance of persistently expanded clones which are not expressing activation antigens (Mugnaini *et al*, 1999) reinforces the possibility that in the Type A severe form of CVID with significantly reduced numbers of CD8+28- cells (Figure 7.1) there may be loss of a valuable repertoire of cells.

8 General discussion & future work

The aims of this study were to:

- develop a whole-blood, small sample volume assay for intracellular cytokines, which could be used to investigate common variable immunodeficiency and be adaptable for routine clinical use.
- validate the assay using a range of diseases thought to have immunoregulatory abnormalities
- examine the immunomodulatory properties of the therapeutic interventions of intravenous immunoglobulin and ciprofloxacin on intracellular cytokine expression
- examine the role of the monocyte in the immunoregulatory abnormalities of CVID
- examine the role of T cell activation in the immunoregulatory abnormalities of CVID

The overall aim was to use data obtained using the new intracellular cytokine method to construct a hypothesis for cytokine mediated dysregulation in common variable immunodeficiency and explain how this immunoregulation is altered by therapeutic interventions.

The development of a whole blood intracellular cytokine assay was carried out, and resulted in a rapid culture technique, 2 h for IFN-γ and TNF-α, and 4 h for IL-2, which has now been published (Sewell et al, 1997). One limitation of the assay was the inability to reliably detect major IL-4 expression. Others have noted this difficulty (e.g. Ferry *et al*, 1997). Other techniques for IL-4 staining have been proposed, such as using PBMC rather than whole blood, using an indirect staining technique (Jolles *et al*, 1999), and using very high concentrations of mitogens, in the presence of numerous cytokines for many hours of culture (Pharmingen Catalogue 1998). These approaches are counter to the original aim of the present study, which was to develop a rapid, small volume culture technique for intracellular cytokines. Although it was difficult to monitor meaningful changes in IL-4 expression in eczematous patients receiving hdIVIG therapy (Chapter 5), the method was still sensitive enough to demonstrate a profound 'Th2' bias in a child with hyper-IgE syndrome (Chapter 4).

The whole blood intracellular cytokine technique itself was reasonably stable in terms of intra- and inter-assay variation, considering the variability of proliferative response in biological systems. Steps to attempt to reduce the variability included freezing multiple aliquots of mitogens, and strict use of the same batch of monoclonal antibodies in for individual experiments. Further developments could include performing each assay in triplicate - although this might be prohibitive in terms of reagent costs. A possible solution to this could be to miniaturise the assay even further, so the present total volume of reagents could be used for more replicates. Preliminary work has shown that this approach is possible, but the data is not yet robust enough to present. The manufacture of automated sample loaders for flow cytometers which can accommodate culture trays as well as individual tubes, as well as centrifuges which can be loaded with culture trays shows the direction for the future automation of this type of assay.

Further to the attempts to reduce the variability of the intracellular cytokine assay were the attempts to develop a reference preparation, which could act as an internal control for cytokine expression in the culture system. Such standards now exist for flow cytometry (Prof G Janossy, personal communication), but the stabilising solutions now used in the current clinical flow cytometry national external quality assurance scheme (NEQAS) are only suitable for fixed (but not permeabilised) cells. In the present study, efforts were made without success to use either a cell line or frozen lymphocytes. A new cell product, HiCK2 cells (Pharmingen), have been marketed for the standardisation of intracellular cytokine assays, but samples were not available to include in this thesis. This would be an area of useful future work, especially if the assay was shown to be helpful in monitoring therapy for example. Two other whole blood intracellular cytokine methods have been proposed, but with important limitations, as discussed in Chapter 4. For example, the technique of Ferry *et al* (1997) uses similar stimulants but with but with a much longer (10 h) incubation period. The method of Jason & Larned (1997) utilised very high PMA concentrations for long culture periods which makes discrimination of $CD4^+$ cells impossible.

The second aim was to validate the assay in a variety of clinical circumstances. Cytokine regulatory abnormalities were demonstrated in several conditions known to be diseases

of immunoregulation, but with smaller quantities of blood and in a shorter period than has previously been possible. The advantages of the four colour flow cytometer used in this study was that individual lymphocyte subsets producing cytokine could be examined in more detail than has previously been described. This is an important advance, since abnormalities of cytokine regulation between normal subjects and patients can sometimes only be seen when small subsets of cells are examined, rather than CD4 or CD8 lymphocytes as a whole. The differences in IFN-γ expression in the CD8 CD28+ cells of patients with CVID and normal controls is a good example. The work shown here examined some additional subsets, which have not previously been well characterised. The CD4+7- T cell, for example, may be particularly relevant to the pathogenesis of diseases such as atopic eczema. Further work could look much more closely at the cytokine regulation of this small population of cells. Work already underway by Professor George Janossy's group at the Royal Free Hospital is using intracellular cytokine expression of CD4 and CD8 cells subdivided into CD45RA and CD45RO subsets to monitor the immune reconstitution of patients with HIV receiving highly active anti-retroviral therapy (Richard Tilling, personal communication).

Intracellular cytokine techniques are now being used to investigate immunoregulatory abnormalities in a wide range of disorders, including multiple sclerosis (Becher *et al*, 1999), asthma (Kon *et al*, 1998), HIV infection (Estcourt *et al*, 1997) and Graves disease (Roura-Mir *et al*, 1997), although apart from the studies by Ferry *et al* (1997) in atopic eczema and Sewell *et al* (1999) in CVID these studies have used PBMC rather than whole blood samples.

A potential criticism of the intracellular cytokine technique in investigating disease is that it 'only' demonstrates cells producing cytokine in response to a mitogenic stimulus with PMA and ionomycin. Previous work, for example North *et al* (1996) showed these activators to be the most suitable for the cytokines we had reagents to detect, Direct stimulation with individual antigens could be a possible way forward, but is limited, particularly in diseases such as CVID which have an extremely low antigen-specific precursor frequency (Kondratenko et al, 1997). Another limitation of the antigen-specific approach is the form of antigen to be presented. Even immunodominant peptides may not be presented in an entirely physiological manner in the test tube, and so fail to stimulate the very cells they would be used to investigate. A compromise approach may

be more useful in the future: incubating with individual antigens for the first part of the culture, and then adding mitogens to supraphysiologically stimulate responding cells. In this way the appropriate cells would respond in a way which could be detected, whereas the physiological response might be below the detection threshold of the system used. A variant of this approach is now being used at the Royal Free Hospital in the investigation of the effects of monocytes on lymphocyte cytokine regulation, for example, by culturing first with LPS in the absence of monensin to allow release of monocyte-derived cytokines, and then with PMA and ionomycin in the presence of monensin to see what effect this has on lymphocyte IFN-γ expression. Another area currently being investigated is of using an initial culture period with a cytokine under investigation, for example IL-18, followed by a mitogenic stimulus, for example with anti-CD3 and anti-CD28, or PMA and ionomycin (Margaret North, personal communication).

A novel approach, short of direct stimulation with antigens, was attempted in the present study, namely, the identification of antigen-specific cells by means of the variable region family of the T cell receptor expressed on the cell. Obviously, this approach can only be used for V-beta-restricted antigens, but certain melanoma antigens do have this property. Unfortunately, appropriate V-beta specific monoclonals staining cells sufficiently brightly to isolate by flow cytometric analysis were not available in this study. However, this is an exciting area for future work, particularly since various cytokine therapies are now being used in clinical trials of melanomas. The ability to monitor cytokine regulation directly within a major subset of antigen-specific lymphocytes would be a great step forward.

Using the intracellular cytokine technique to monitor immunomodulatory therapies was the third aim of this study. The experiment used to investigate the IL-2 expression following *in vitro* ciprofloxacin was a useful example of what is and what is not assessed using this technique. Numerous earlier studies have shown that ciprofloxacin can increase the production of IL-2 by lymphocytes *in vitro* (e.g. Riesbeck & Forsgren, 1990; Riesbeck, 1994). These studies have examined IL-2 in lymphocyte culture supernatants. The intracellular cytokine assay did not demonstrate any statistically significant effect of ciprofloxacin, although there was a trend towards a reduction in the percentage of lymphocytes which make IL-2 with increasing concentrations of ciprofloxacin (Chapter 5). The important point here is that the intracellular cytokine technique does not measure

the output of cytokine per cell, rather the percentage (or absolute number) of lymphocytes that stain positive for cytokine. For cell surface antigens, it is possible to quantify the amount of antigen per cell using standardised fluorescent beads (Ivory, 1996). This may be a possible area of future development for the whole blood assay. It may be that for certain studies, a combination of the intracellular cytokine assay (determining how many cells make a given cytokine) combined with measurements of cytokine within lymphocyte culture supernatants (determining how much cytokine per cell is being secreted) may be necessary. These two variables may not move in parallel (Ivory, 1997) and the question of the regulation of cytokine secretion *in vivo* is an important one for future work.

The ability of the assay to investigate cytokine production rapidly using small quantities of blood facilitated the investigation of the immunomodulatory effects of IVIG in common variable immunodeficiency. No previous study had been able to show in such detail how cytokine regulation may be modulated by IVIG. Previous work had either examined the effects of IVIG on cytokine expression *in vitro*, as discussed in detail in Chapter 1, and had demonstrated that IVIG may decrease IL-1 (Iwata *et al*, 1987), decrease IL-6 (Andersson *et al*, 1990), and decrease TNF-α (Abe *et al*, 1989) in culture supernatants. Using intracellular techniques, Andersson *et al* (1990) demonstrated a reduction in the numbers of IL-3, IL-4, IL-5, TNF-β and GM-CSF producing cells, and no effect on IL-2, IL-10, TNF-α and IFN-γ producing cells in co-culture experiments with IVIG. The *in vivo* effects of IVIG on cytokines have only been demonstrated by Ling *et al* (1993) who showed an increase in plasma IL-6 and IFN-γ levels post-infusion. Although Aukrust *et al* (1994b) showed that IVIG could increase plasma TNF-α levels, Farber *et al* (1994) demonstrated that this was only a feature of adverse reactions to IVIG infusions.

In the present study, it was shown that IVIG infusions given at replacement dose to CVID patients lead to an increase in TNF-α production by $CD8^+$ T cells, and an increase in IL-2 production by $CD4^+$ T cells; IFN-γ expression was not affected. This runs counter to the clinical perception of IVIG as an 'anti-inflammatory agent', reinforced by accounts of IVIG reducing antigen-dependent and antigen-independent T cell responses *in vitro* (van Schaik *et al*, 1992). The increase in TNF-α and IL-2

immediately post-IVIG infusion were not seen in the present study when IVIG was given at high dose to patients with severe atopic eczema. This may be due to immunoregulatory differences in CVID patients compared with eczema patients, since CVID patients have an increased background level of IFN-γ production by $CD8^+$ T cells (North *et al*, 1996), or it may be a dose effect. Normal volunteers did not receive IVIG at either replacement dose or high-dose in the present study. The long-term effects of hdIVIG given to eczema patients (Chapter 5) showed a statistically significant reduction in the activation of T cells following mitogenic stimulation (in terms of expression of the activation antigen CD69), and suggestions of a reduction in IFN-γ and TNF-α expression.

Additional information came from the experiments on the *in vitro* effects of IVIG on IFN-γ production in normal subjects, CVID and eczema patients. Although IVIG *in vitro* did not influence IFN-γ expression in normal subjects, at high IgG concentrations IFN-γ was reduced in CVID patients only. This should be seen in the context of the cytokine expression studies in Chapter 4, confirming that IFN-γ expression is higher in CVID than in normals before therapy. It is not known whether the IFN-γ expression in CVID patients who have never received IVIG are increased or decreased compared with normal subjects. Totally IVIG-naive CVID patients were unfortunately not available in the present study, but would be an important area for future work, to determine whether the IFN-γ abnormalities in CVID are a result of the disease, or the treatment itself. In the high-dose IVIG experiment, prolonged use of hdIVIG reduced IFN-γ and TNF-α expression, although more subjects will be needed to confirm this statistically.

There is therefore a suggestion that high dose IVIG could be used in CVID to reduce the proportion of peripheral blood cells which can be stimulated to secrete pro-inflammatory cytokines. This would be an important area for further work. The concentrations of IgG, which had the greatest effect in the in vitro study, were equivalent to 20 g/l, which is readily obtainable in clinical practice. The high-dose therapy given to the eczema patients was only given at monthly intervals. Consideration should be given to a study of monthly high-dose IVIG therapy in CVID patients with granulomas and other inflammatory manifestations. The overall IgG dose would be greater, but the

dosage interval could be extended beyond the current three-week replacement-dose average. Patients in the trial would continue to be treated with their existing IVIG preparations, so exposing each individual only to the risks of high-dose versus low-dose therapy. This contrasts with the ethical dilemmas of introducing the potential risks of IVIG therapy to IVIG-naive patients, for example in trials of IVIG for systemic vasculitis. Obviously further ground work is still needed, particularly confirming the *in vitro* and the high-dose effects, but this could be an exciting therapeutic possibility in the future.

Further work in our laboratory investigating the expression of IL-12 in monocytes using a modification of the whole blood intracellular cytokine technique gave rise to the fourth aim of this study, namely to examine the role of the monocyte in the cytokine abnormalities found in CVID. Since the finding of elevated serum IL-12 levels in CVID (Raga et al, 1998) and our finding of elevated monocyte intracellular IL-12 expression in CVID (Cambronero, Sewell, et al, manuscript submitted), we theorised that the increased propensity of CVID patient's monocytes to make IL-12 could be partially responsible for the elevated IFN-γ expression by CVID patient's lymphocytes. A series of monocyte depletion experiments confirmed that reducing the concentration of monocytes in overnight cultures with CVID patient lymphocytes did indeed reduce IFN-γ expression. Attempts were then made to block biologically active IL-12 in these cultures using a new monoclonal antibody, C8.6, which only binds the p75 heterodimeric form of IL-12. Although lymphocytes from some of the patients tested showed a reduction in lymphocyte IFN-γ expression, there was no overall effect. It could be argued that this was because the C8.6 used was ineffective, since a titration response could not be obtained. However, very large concentrations of blocking antibody were employed which are effective in other culture systems, and for the same time period as the depletion experiments above, which argues against saturation of the antibodies which could happen during prolonged culture. A reasonable hypothesis is that other IFN-γ promoting cytokines are produced in excess by CVID monocytes compared with monocytes from normal control subjects. IL-18 is an obvious candidate, and future work should examine monocyte IL-18 production in CVID once directly-conjugated anti-IL-18 monoclonal antibodies become available for use in intracellular cytokine assays.

The finding of similar NK cell IFN-γ expression in CVID and normal controls (Cambronero, Sewell *et al*, 2000) may argue against IL-18 being the sole monocyte cytokine responsible for driving IFN-γ over-expression in CVID, since the prime inducer of IFN-γ in NK cells is IL-18. Alternatively, counter-regulatory systems may be effective in controlling NK but not lymphocyte IFN-γ expression in CVID.

Of therapeutic interest would be further studies of the effects of IVIG on NK and particularly monocyte cytokine expression. The experiments shown here of the *in vivo* effects of IVIG on these cells were at replacement-dose. These should be repeated both in patients receiving high-dose IVIG, and should also examine monocyte IL-12 expression.

The fifth aim of the study, to investigate T cell phenotypes and activation marker expression in CVID sprang directly from the findings of cytokine abnormalities within CVID T cell subsets. What is different about these T cell subsets that results in increased IFN-γ expression compared with normal cells? Several earlier studies have examined some of these issues, but none has done so with the ability to distinguish small lymphocyte subsets afforded by four-colour flow cytometry.

Several earlier findings were confirmed using this approach, such as the reduction in CD4 cells, and particularly CD45RA+ cells in CVID, and the increased proportion of CD8+28- cells that is seen not only in CVID, but also in numerous other chronic inflammatory conditions. In terms of activation marker expression, although CD25 was not found to be increased on CVID T cells, the statistically significant reduction in CD25 expression in CVID was unexpected, but might not have been detectable in earlier two-colour studies (Spickett et al, 1991). However, this should be seen in the context of the extremely low levels of CD25 expression in peripheral blood in all subject groups studied here. The finding of increased soluble CD25 in CVID (North et al, 1991b) may explain the lower cell-surface CD25 expression in CVID, indicating CD25 that has been shed into the plasma. Similar phenomena have been shown for L-selectin in CVID (Zhang *et al*, 1996; Nordøy *et al*, 1998).

HLA-DR is expressed at higher levels than CD25 even in the normal subjects. In particular, DR was expressed at higher levels on 28- cells than CD28+ cells, and at higher levels on RO+ than RA+ cells (Chapter 7). Patients with CVID were found to express higher levels of DR than normal subjects, particularly on CD28+ cells and on RA+ cells, suggesting an aberrant pattern of activation compared with that normally seen. Other studies have demonstrated abnormal activation in CVID (and importantly not in XLA), including increased levels of soluble CD25 and CD8 (North *et al*, 1991b) and increased neopterin levels (Aukrust *et al*, 1992) - an indicator of elevated lymphocyte activation *in vivo*.

Further investigation of the differences between CD28- and CD28+ cells in CVID could be directed at examination of the relative senescence of these cells between normal volunteers and CVID patients. Now that telomere length, and telomerase activity, can be readily determined in small numbers of cells (Bryant *et al*, 1997), a useful experiment would be to isolate CD8+28- and CD8+28+ cells by immunomagnetic beads and to determine their telomere length and telomerase activities. The expansion of CD28- cells in CVID may indicate an expansion of 'suppressive' cells (Azuma *et al*, 1993). Although CD28- cells have been thought to be 'senescent' and antigen unresponsive (Borthwick *et al*, 1994) there is increasing evidence, discussed more fully in Chapter 7, that CD28- cells may represent the majority of antigen-responsive cells (Mugnaini *et al*, 1999) and may also have reverted from CD45RO+ cells, perhaps in the gut (Nociari *et al*, 1999).

A further area to consider in the context of explaining some of the lymphocyte subset abnormalities occurring in CVID springs from the recent work by Inglesias et al (1999) who demonstrated that CD4+CD45RA+ cells in CVID express more CD95 (*fas*) than normal subjects (but not less *Bcl*-2 or Bax), and that this results in increased spontaneous apoptosis in the depleted CD4+45RA+ subpopulation and presumably was the cause of death of the cells already depleted. In addition, Saxon et al (1995) showed that B cells from a subset of CVID patients over-express CD95, and thus are more prone to spontaneous apoptosis.

The possibility of the further involvement of IL-12 and apoptosis in the pathogenesis of CVID was recently suggested by the work of Tarrant *et al* (1999). Their work examined a

different Th1-mediated disease, experimental autoimmune uveitis (EAU), and the role of IL-12 and Th1 cells in its induction. Expecting to find that exogeneous IL-12 would make EAU worse, the authors were surprised to find that giving IL-12 to mice early in the course of the retinal protein immunisation required to induce EAU, resulted in failure to develop the disease. In addition, although there was hyperinduction of IFN-γ during the IL-12 administration period, afterwards antigen-specific IFN-γ levels were reduced, as were IL-5 production and serum antibody levels. Even more remarkably, they were able to demonstrate enhanced apoptosis of antigen-specific T cells by the IL-12 therapy, mediated in part by induction of nitric oxide (Tarrant *et al*, 1999). It is tempting to speculate that similar phenomena are occurring in CVID, but with a perpetual IL-12 drive from the monocyte abnormalities, resulting in perpetual IFN-γ induction, antibody deficiency and increased apoptosis of antigen-specific CD45RA+ T cells.

Given the finding in the present study and elsewhere of the importance of the monocyte in the immunoregulatory abnormalities of CVID, a further explanation for the immunomodulatory properties of IVIG comes to light. One of the factors controlling monocyte IL-12 production is now thought to be the degree of cross-linking of monocyte surface FcR. Certainly in murine systems, IL-12 production is inhibited by cross-linking of FcR gamma I receptors (Ma et al, 1998). This inhibition occurs via IL-10 dependent and independent pathways, and probably results from inhibition of NFkB. It would seem logical therefore that increasing serum IgG concentrations by increasing the dose of IVIG for example, would tend to result in more FcR cross-linking, and reduced monocyte IL-12 production. This in turn might be expected to reduce IFN-γ induction by T cells, as well as other cytokines driven by IL-12 and IFN-γ, such as TNF-α.

A report of the modulation of FcR expression may therefore be of benefit in the therapeutic immunomodulation of CVID patients. It is now known that FcR expression is modulated by dopamine (Gomez *et al*, 1999). In experimental settings, dopamine agonists (bromocryptine, leuprolide and pergolide) have been shown to up-regulate FcR expression, particularly those operating through D2 receptors (Gomez *et al*, 1999). These agents have been used clinically for other indications for many years. An obvious experiment would be to attempt to upregulate FcR in CVID patients using dopamine

agonists, hence increasing the degree of FcR cross-linking, which would reduce IL-12 production and pro-inflammatory cytokine drive. Before attempting such a trial, the whole-blood intracellular cytokine assay could be used to monitor IL-12 and IFN-γ expression in patients already receiving these drugs (and also patients receiving dopaminergic antagonists) for currently licensed therapeutic indications. If an effect on IL-12/IFN-γ expression was shown, then a trial of such agents (probably as an adjunct to existing therapy) in CVID could be considered. Not only might this be of use in CVID, but also in many other disorders where immunomodulation of IL-12/IFN-γ would be appropriate, especially as splenic macrophage abnormalities have been demonstrated in end-stage renal disease (Ruiz *et al*, 1990) and cirrhosis (Gomez *et al*, 1994) for example.

In conclusion, this study has shown that not only can abnormalities of cytokine expression be demonstrated in CVID, but that the drugs currently used to manage the disease may be modulating these cytokines.

Speculative model of the mechanisms of cytokine dysregulation and lymphocyte phenotype abnormalities in CVID

Based on the work presented in this study and additional evidence from other studies cited above, the following speculative mechanism is proposed as a model to explain some of the abnormalities of cytokine regulation in CVID, and to suggest possible ways in which current and proposed therapeutic interventions could act. Further experimental work is also suggested. The model is outlined in Figure 8.1; references in the text to 'Box' refer to a numbered box in this figure.

The initial 'insult' in CVID has not been established (Box 1), but is likely to involve both genetic (as discussed in Webster *et al*, 1997) and environmental factors concerned with the recognition and successful clearance of pathogens, particularly encapsulated bacteria, from mucosal surfaces. It is not yet clear whether selective IgA deficiency, for example, may predispose to CVID (as reviewed in Burrows & Cooper, 1997), but absence of an

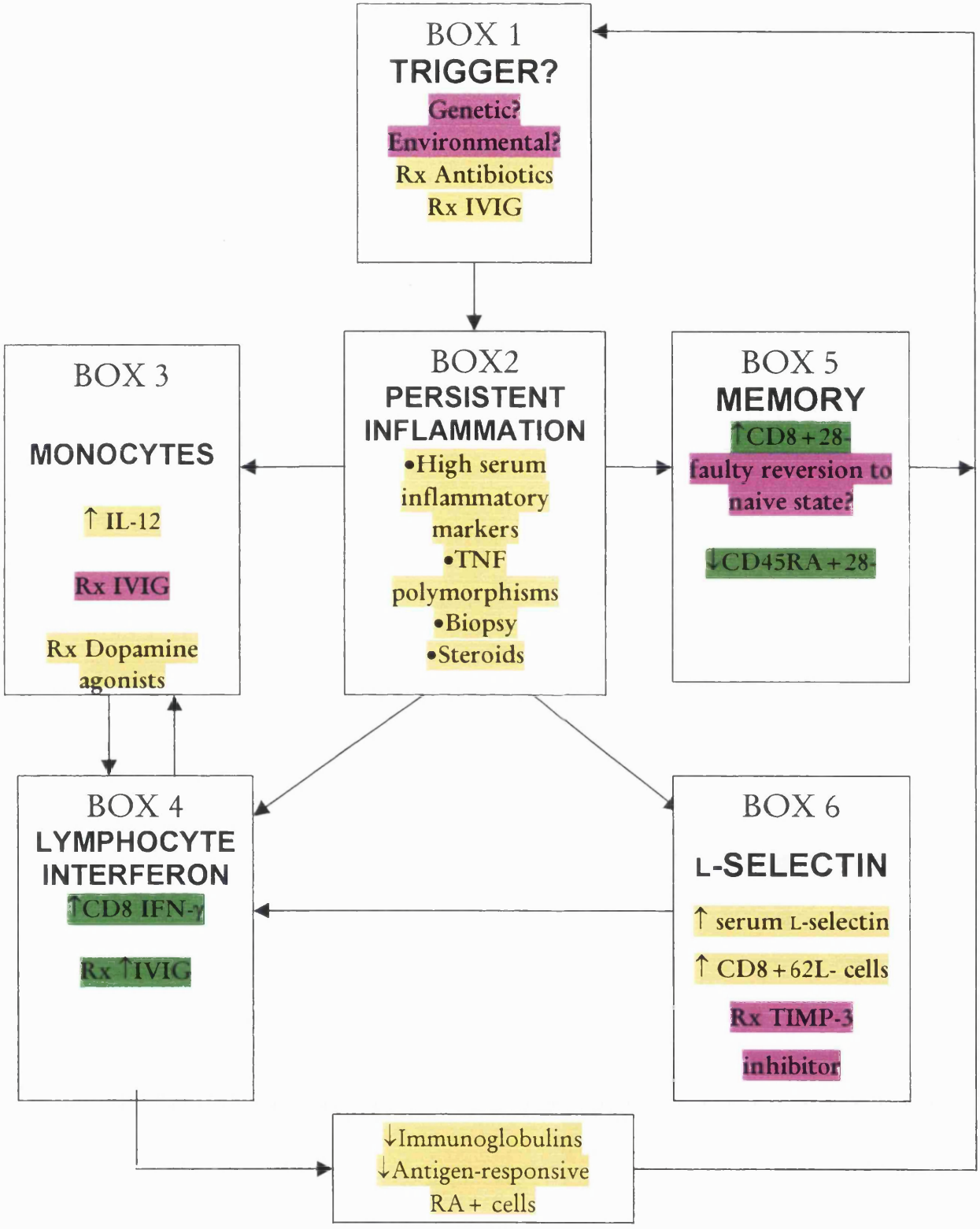


Figure 8.1 A speculative model of the mechanisms of cytokine dysregulation and lymphocyte phenotype abnormalities in CVID, with areas for potential therapeutic intervention. Evidence is colour-coded as: data from thesis, data from literature, speculation. ↑ increased, ↓ decreased, Rx possible therapeutic intervention

immunoglobulin isotype important in mucosal defence would seem to be a logical risk factor for recurrent mucosal infections. Therapeutic interventions in Box 1 may include preventing/treating bacterial infections with prophylactic antibiotics, or altering bacterial adherence, and of course replacement IVIG to replace immunoglobulins and neutralise pathogens.

Having established an infection, it seems that in CVID something further goes wrong with the resolution of the immune response (Box 2). Several lines of evidence suggest this, including the presence of increased inflammatory markers in the serum, even in uninfected patients (North *et al*, 1991b; Zhang *et* al, 1996; Nordøy *et al*, 1998). In the gut, this may be particularly important, given the role of the intestinal mucosa and associated lymphoid tissues as a fundamental component of the immune system (Kohne *et al*, 1996). Indeed, many patients with CVID have unexplained diarrhoea and malabsorption in the absence of an identifiable pathogen (Teahon *et al*, 1993). In a study of the enteropathy of primary hypogammaglobulinaemia, 80% of patients had evidence of microscopic colitis with increased intraepithelial lymphocytes (Teahon *et al*, 1993). Therapeutic interventions in Box 2 would include anti-inflammatory agents such as corticosteroids. These are currently used in the management of CVID, particularly Type A CVID with granulomatous manifestations (Spickett *et al*, 1996). However the extensive side-effect profile, combined with their very general immunosuppressive actions makes longterm therapy in immunodeficiency unsatisfactory.

It is suggested that the presence of increased inflammation in CVID may in its turn have fundamental effects on immune regulation. A greater percentage of monocytes from patients with CVID have been shown to express IL-12 following stimulation with LPS than monocytes from normal controls (Cambronero, Sewell, *et al*, 2000) (Box 3). The monocyte depletion experiments described in Chapter 6 suggest that in CVID, monocytes play an important role in the abnormally high expression of IFN-γ in $CD8^+$ T cells. The IL-12 blocking experiments in Chapter 6 also suggested that although IL-12 may play a part in the high IFN-γ production, (an)other cytokines may also be involved. One obvious possibility to investigate in the future would be IL-18. However, it is not

clear in which cell the primary defect lies, since it is conceivable that abnormally high IFN-γ levels produced by lymphocytes could, by positive-feedback mechanisms, be causing increased monocyte IL-12 expression in CVID.

The therapeutic implications of Box 3 are that various interventions could be used to reduce monocyte IL-12 over-expression, as discussed earlier. Interventions could include increasing the cross-linker of FcR, IgG, by increasing the dose of IVIG from replacement (200-400 mg/kg) to high (1-2 g/kg) dose. Alternatively the expression of FcR on the monocyte surface could be increased, for example by using dopamine agonists.

Box 4 details the effects of the inflammation on CD8 (and CD4) cells, namely the increase in IFN-γ expression. The effects of increased IFN-γ may be widespread, but importantly in this model may include induction of apoptosis of CD45RA+ cells (Tarrant *et al*, 1999), alteration of IgG subclass ratios (Snapper *et al*, 1988), B cell antigen-nonresponsiveness (Duggan-Keen *et al*, 1990) and hypogammaglobulinaemia by down regulation of Fc^{γ}R-IIb (Snapper *et al*, 1989). Therapeutic interventions in Box 4 include increasing the dose of IVIG from replacement- to high-dose, since this was shown in Chapter 5 to reduce IFN-γ expression by CD8+ T cells.

Box 5 attempts to account for some of the abnormalities of lymphocyte subpopulations found in Chapter 7. A key finding in CVID, as well as in other disorders with an chronic inflammatory component, has been the increase in CD8+28- cells. As discussed in Chapter 7, CD8+28+ cells are attracted to sites of inflammation, where a switch to CD8+28- cells has been proposed. This switch may have important effects, since CD28- cells form a memory compartment consisting of an set of expanded clones derived from CD28+ cells (Mugnaini *et al*, 1999). Another key aspect of the CD28- population is that in normal individuals, clones may switch back to a 'naive' CD45RA+ phenotype (discussed in Mugnaini *et al*, 1999). In Chapter 7 it was shown that this population is very

low in the severe Type A form of CVID, and therefore presumably represents a failure of CD28- cells to revert to a CD45RA+ phenotype. There are currently no specific therapeutic maneuvers which are known or suggested to interfere with the processes in Box 5, however resolution of the inflammatory changes in Box 2 may be of some benefit.

A further implication of chronic inflammation in CVID is the effects on L-selectin (CD62L) expression on T lymphocytes, shown in Box 6. Inflammatory mediators such as arachidonic acid metabolites, e.g. 5-oxo-6,8,11,14-eicosatetraenoic acid produced by bacterial infections activate enzyme(s) which cause cleavage of cell-surface L-selectin from neutrophils and lymphocytes (Powell *et al*, 1999). C-reactive protein, induced as part of the acute phase response, can also cause L-selectin shedding (Zouki *et al*, 1997). This process presumably is active in CVID, since elevated serum levels of L-selectin, and increased numbers of $CD62L^-$ lymphocytes have been reported in CVID (Zhang *et al*, 1996; Nordøy *et al*, 1998).

Important aspects of increased CD62L- lymphocytes are that their activation requirements, and ability to enter the high endothelial venules of lymph nodes is restricted compared with CD62L+ cells (reviewed in Springer, 1994). This may account in part for the failure of CVID patients to respond to recall or neoantigens (Kondratenko *et al*, 1997. In addition, elevated levels of $CD8^+CD62L^-$ cells have been associated with the immunodeficiency seen in myeloma patients (Walchner & Wick, 1997). In addition, CD62L- T cells are Th1 biased, and produce IFN-γ (Kanegane *et al*, 1996). Blocking L-selectin with therapeutic monoclonal antibodies also increases IFN-γ production (Laskay *et al*, 1997). Interestingly, a potential link between Box 1 and Box 6 may be from studies in mice showing that infection with lymphocytic choriomeningitis virus results in an increase in CD62L- cells which persist for months following clearance of the virus (Christensen *et al*, 1996). Of these CD62L- cells, 30-40% are $IFN\text{-}\gamma^+$ whereas the CD62L+ cells do not produce IFN-γ. It is not known whether similar viruses may be responsible for CVID induction in humans.

An area for potential therapeutic intervention in Box 6 may be the finding that L-selectin shedding can be inhibited by experimental inhibitors of tissue (but not matrix) metalloproteinase inhibitors, such as Ro 31-9790 (Borland *et al*, 1999). This would be an exciting area for future research.

The model proposed here (illustrated in Figure 8.1) involves the development of cytokine regulatory abnormalities in CVID which affect both the size of various lymphocyte subpopulations and the levels of critical immune components such as immunoglobulin. Although these abnormalities may appear to be severe in individual patients, and hence require substantial intervention to correct, there is increasing evidence that cytokine regulation may exhibit certain features of non-linear (chaotic) systems (reviewed in Dagleish, 1999) whereby small changes in key cytokines can cause marked clinical changes (Marriott Westby & Dagleish, 1997).

One of the original aims of the present study was to develop a technique for the determination of intracellular cytokines which could eventually be used in clinical practice for the diagnosis and/or monitoring of disease. This work demonstrates that such a technique is not only feasible, but has actually been used to demonstrate abnormalities and responses to therapy in a number of immunoregulatory disorders. Future work needs to better define the conditions which could be usefully monitored using this approach. An example where more sensitive monitoring of cytokines in order to track disease activity is urgently needed is Wegener's granulomatosis.

An exciting possibility stemming from the present study is therefore that increasing the dose of IVIG, perhaps combined with additional means of modulating cytokine regulation suggested by the proposed model, may lead to a reduction in some of the complications of CVID.

W A Carrock Sewell, London 1999

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