HUMAN GM-CSF, IL-3 AND IL-5 RECEPTOR EXPRESSION AND THEIR FUNCTIONAL DOMAINS STUDIED WITH MONOCLONAL ANTIBODIES

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ABSTRACT

Human GM-CSF, IL-3 and IL-5 receptor expression and their functional domains studied with monoclonal antibodies

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Human IL-3, GM-CSF and IL-5 are a group of cytokines that are important both in haemopoiesis and inflammation. The receptors for GM-CSF, IL-3 and IL-5 are heterodimers that comprise a ligand-specific α-chain and a common β chain (β_c). These receptors belong to the cytokine receptor superfamily, but they are structurally and functionally more related to each other and thus make up a distinct subfamily. Although these receptor have been studied extensively over the past few years, some basic questions such as the overall pattern of expression, the mechanism of receptor activation and the functional domain or ligand binding sites of these receptor are still largely not known. Attempts to answer some of these questions will help to understand the biological and pathological roles of these cytokines, and more importantly will help to generate receptor antagonists that can be used for therapeutic application.

In this thesis, I investigate the α-chain and β_c of receptors for GM-CSF, IL-3 and IL-5 from several aspects and the overall aim is to define functional domains in these receptor subunits. I have generated 4 groups of monoclonal antibodies (MoAbs) which are directed to the each of the α-chains and to β_c respectively. With these MoAbs, the expression of these receptor subunits on human primary cells was examined. The CD34+ cells were shown to have relatively higher levels of IL-3Rα and to a lesser extent GM-CSFRα, but very limited β_c. I have shown for the first time in this thesis that human basophils have an extremely high level of IL-3Rα, a character that can be applied to purify basophils by means of anti-IL-3Rα MoAbs. The non-haemopoietic cells such as human endothelial cells were also explored for their cytokine receptor expression and were, surprisingly, found to express IL-3R α and β chains. In addition, I have also shown here that cytokine receptor expression on the cell surface is a dynamic process as the

expression of IL-3Rα on neutrophils and IL-3Rα and β_c on endothelial cells can be up-regulated by several other cytokines such as GM-CSF, TNF-α or IFN-γ. Of these MoAbs two neutralising ones were isolated. One of them, anti-IL-3Rα MoAb 7G3, could completely block the binding and function of IL-3. The other, anti-β_c MoAb QP1, could specifically inhibit the binding and function of all three cytokines. The mechanism of antagonising by both neutralising MoAbs was through binding to the functional domains of these receptors as the neutralising MoAbs and the appropriate cytokines demonstrated a direct competition of binding to each other. In order to identify the functional domains of these cytokine receptor subunits, I mapped the epiotpes of both neutralising MoAbs using several receptor mutants. Anti-IL-3Rα MoAb 7G3 was mapped to the *N*-terminal domain of IL-3Rα while anti-β_c MoAb QP1 to domain 4 of β_c, and more precisely a conformational region comprises several residues in the B'-C' and F'-G' loops.

The work presented in this thesis has implications in the research of cytokine receptors in several aspects. These MoAbs to GM-CSF, IL-3 and IL-5 receptor α and β chains offer a more direct and amenable approach to study cytokine receptor expression and regulation. The two neutralising MoAbs provide reagents for the functional analysis of these cytokines *in vitro*. They also open the potential for *in vivo* manipulation of these cytokine receptors in conditions such as allergy, and some leukaemias and lymphomas where cytokine overexpression or receptor aberrant expression may contribute to their pathogenesis. Finally, the epitope-mapping of the neutralising MoAbs gives strong evidence that the *N*-terminal domain of IL-3Rα and the domain 4 of β_c are functional domains involved in ligand binding and receptor activation. By analogy, it may also imply that the *N*-terminal domain of GM-CSFRα and IL-5Rα, and the domain 4 of receptor subunits analogous are also functional domains. In addition, the two neutralising MoAbs may provide a practical approach for the determination of the structure of receptor binding sites by co-crystallisation.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university of other tertiary institute and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for loan and photocopying if accepted for the award of the degree.

Qiyu Sun

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MONOCLONAL ANTIBODIES ARISING

Name	Antigen	Isotype	FACS	I.P.	Western blot	Blocking
6H6	IL-3Rα	IgG_1	+	+	+	-
***7G3**	IL-3Rα	IgG_{2a}	+	+	+	+
***9F5**	IL-3Rα	IgG_1	+	+	+	-
4A5	GM-CSFRα	IgG_1	+	+	-	-
4C11	GM-CSFRα	IgG_1	-	-	+	-
4H1	GM-CSFRα	IgG_1	+	+	+	-
6E10	GM-CSFRα	IgG_1	+	+	+	-
8D10	GM-CSFRα	IgG_1	-	-	+	-
8E3	GM-CSFRα	IgG_1	+	+	+	-
8G6	GM-CSFRα	IgG_1	+	+	+	-
***A14**	IL-5Rα	IgG_1	+	+	+	-
1C1	β_c	IgG_1	+	+	+	-
1E3	β_c	IgG_1	+	+	-	-
***3D7**	β_c	IgG_1	+	+	+	-
3H5	β_c	IgG_1	+	+	+	-
4F3	β_c	IgG_1	+	+	-	-
4H1	β_c	IgG_1	+	+	+	-
5F9	β_c	IgG_1	+	+	-	-
8B8	β_c	IgG_1	+	+	+	-
8E4	β_c	IgG_1	+	+	+	-
QP1	β_c	IgG_1	+	+	+	+
QP2	β_c	IgG_1	+	+	+	+

* commercially available through **PharMingen**, San Diego, CA 92121, USA.

ABBREVIATIONS

ALL	Acute Lymphoblastic leukaemia
AML	Acute myeloid leukaemia
β_c	β-chain
BM	Bone marrow)
BSA	Bovine Serum Albumin
CD	Cluster Designation or Cluster of Differentiation
cDNA	Complimentary DNA
CM	Conditioned media
CNTF	Ciliary neurotrophic factor
CSF	Colony stimulating factor
DMEM	Dulbeccos modification of Eagles media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDC-P1	Factor-dependent cell line (Paterson Institute #1)
FITC	Fluorescein isothiocyanate
FL	Logarithmic fluorescence Intensity
FSC	Forward light scatter
G418	Geneticin
G-CSF	Granulocyte-CSF
GM-CSF	Granulocyte/Macrophage-CSF
HA	Hyaluronic acid
HAT	Hypoxanthine-Aminopterin-Thymidine
HBSS	Hank's Balanced Salt Solution
HCM	Hybridoma growth media

HLA	Human Leucocyte antigen
HSC	Haemopoietic stem cell
HUVEC	Human umbilical vein endothelial cells
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG1	Immunoglobulin gamma-1 isotype
IgG2a	Immunoglobulin gamma-2A isotype
IL	Interleukin
IMDM	Iscoves Modified Dulbecco's Medium
kD	Kilodalton
LPS	Lipopolysaccharide
M	Molar
MoAb	Monoclonal Antibody
MHC	Major histocompatibility complex
min	Minute
mRNA	Messenger RNA
NP-40	Nonidet P40
O.D.	Optical density
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phyco-erythrin
R	Receptor
Rα	Receptor α-chain
SD	Standard deviation
TNFα	Tumour Necrosis Factor-α

CHAPTER I

INTRODUCTION

1.1 Preface

IL-3, GM-CSF and IL-5 are a group of cytokines that have growing importance both in haemopoiesis and inflammation. They function through binding to their cell surface receptors that consist of a ligand-specific α chain and a common β chain (β_c). The overall aim of this thesis was to define functional epitopes of the receptor for IL-3, GM-CSF and IL-5 by means of monoclonal antibodies (MoAbs). Specifically, the focus of this study was to:

1) develop of MoAbs for each receptor chain as specific tools, and
2) apply these MoAbs to determine receptor expression on primary cells and their regulation by other cytokines, and
3) identify MoAbs with antagonistic activities to facilitate the functional analysis of these cytokines and their receptors, and
4) use these MoAbs to define the functional domains of α and β chains of the GM-CSF, IL-3 and IL-5 receptors that are important for receptor binding and function.

To perform these studies, it is necessary to develop new tools, unavailable elsewhere, namely MoAbs specific for each receptor chains. The availability of these MoAbs makes it possible to study the mechanism of receptor activation by analysing the α and β chains association under various conditions. The neutralising MoAbs themselves may also have potential for clinical application in antagonising the overstimulation by these cytokines in certain disorders such as allergy and some leukaemias. Importantly, the mapping of functional MoAbs provides more precise information, at least in part, over the receptor modelling, in defining the IL-3 binding site on the IL-3R α chain and ligand interacting sites on the β chains. In the long term the antagonistic MoAbs described here may provide an alternative approach to determine the structure of the ligand

binding site by the co-crystallization of the antagonistic MoAbs with the appropriate receptor subunit.

1.2 IL-3, GM-CSF and IL-5

IL-3, GM-CSF and IL-5 are closely related in terms of their gene structure and location, their biological activities and more significantly, their cell surface receptors. Although there is limited amino acid sequence similarity among these three cytokines, they exhibit a similar tertiary structure with 4 α-helices in a two up two down configuation (Diedrichs *et al.*, 1991; Milburn *et al.*, 1993; Olins *et al.*, 1995).

1.2.1 IL-3

Human IL-3 is a 133 amino acid glycoprotein with a molecular weight ranging from 15 to 33 kDa depending on its glycosylation state. IL-3 is produced by stimulated T lymphocytes (Niemyer *et al.*, 1989) and mast cells (Plaut *et al.*, 1989) and its production is under tight regulatory control in T cells. The gene for human IL-3 has been cloned (Fung *et al.*, 1984; Yokota *et al.*, 1984) and mapped to chromosome 5 (Yang *et al.*, 1986) band q23-31 by somatic cell hybrid analysis and *in situ* chromosomal hybridization (Le Beau *et al.*, 1987).

The biological activity of IL-3 is pleiotropic. Firstly, IL-3 plays a stimulating role in haemopoiesis. It stimulates the proliferation and differentiation of multipotential haemopoietic progenitors as well as lineage-committed progenitors including those for neutrophil, eosinophil, monocytes, basophil, megakaryocyte, erythroid cell and B lymphocyte (Clark *et al.*, 1987; Sieff *et al.*, 1987; Lopez *et al.*, 1988; Metcalf, 1991; Haak-Frendscho *et al.*, 1988; Elliott *et al.*, 1989; Saeland *et al.*, 1993). Therefore, IL-3 may also be regarded as a "multipotential colony stimulating factor" (Multi-CSF). Secondly, IL-3 is also involved in inflammation by stimulating the maturation and functional activation of effector cells. It activates eosinophils by inducing phagocytosis,

degranulation, proteoglycan production and up-regulation of the expression of adhesion molecules (Lopez *et al.,* 1988; Fujisawa *et al.,* 1990; Sadeghi *et al.,* 1992; Czech *et al.*, 1993). IL-3 also stimulates the histamine release from basophils (Haak-Frendsho *et al.,* 1988; Lopez *et al.*,1990). On monocytes, IL-3 promotes its adhesion to endothelium and expression of MHC class II molecules (Elliott *et al.,* 1990; Sadeghi *et al.,* 1992). More recently, IL-3 has also been shown to amplify the GM-CSF stimulated MHC class II expression on neutrophils (Smith *et al.,* 1995). In addition, cells other than those of haemopoietic origin can also respond to IL-3. For instance, IL-3 can enhance IL-6 and IL-8 production from human umbilical vein endothelial cells (HUVEC) (Korpelainen *et al.,* 1993; 1995).

1.2.2 GM-CSF

Human GM-CSF is a 127 amino acid protein that is extensively modified by the addition of both N- and O-linked carbohydrate (Lusis *et al.,* 1981; Donahue *et al.,* 1986) to produce a 20 to 30 kDa glycoprotein. The cDNA that encodes a GM-CSF precursor protein of 144 amino acids has been isolated from libraries made from human T-cell line mRNA and peripheral blood T lymphocyte mRNA (Wong *et al.,* 1985; Lee *et al.,* 1985; Cantrell *et al.,* 1985). The gene for GM-CSF is only 10 Kb from the IL-3 gene on chromosome 5 (Miyatake *et al.*, 1985). GM-CSF is predominantly produced by T lymphocytes, although some other cells such as macrophages, fibroblasts and endothelial cells may also express small amounts (reviewed in Gasson, 1991).

The biological activities of GM-CSF are very similar to those of IL-3, but the functions of GM-CSF are more restricted to the granulocyte-macrophage lineage. From *in vitro* studies, GM-CSF supported haemopoiesis by stimulating all cells in the granulocyte, macrophage and eosinophil lineages, starting from multipotential progenitor (Sieff *et al.,* 1987; Tomonaga *et al.,* 1986; Metcalf *et al.,* 1986). It is important to note that GM-CSF is essential for the survival of haemopoietic progenitors (Metcalf and Merchav, 1982; Burgess *et al.,* 1982) as

well as mature cells such as granulocytes (DiPersio *et al.,* 1988), macrophages (Chen *et al.,* 1988), and eosinophils (Lopez *et al.,* 1986; Begley *et al.,* 1986).

GM-CSF is also an important regulator for inflammation through stimulation of the functional activity of mature haemopoietic and some non-haemopoietic cells. On neutrophils, GM-CSF primes their superoxide production, phagocytosis, lysozyme secretion (Weisbart *et al.,* 1987; Lindemann *et al.,* 1988), expression of adhesion molecules and adhesion to endothelial cell (Griffin *et al.,* 1990; Gamble *et al.,* 1990). GM-CSF also stimulates antibody-dependent cell-mediated cytotoxicity (ADCC) by neutrophils (Lopez *et al.,* 1983; Vadas *et al.,* 1983). On eosinophils, GM-CSF enhances ADCC and phagocytosis (Lopez *et al.,* 1986). In addition, GM-CSF also activates macrophages (Grabstein *et al.,* 1986), monocytes (Gamble *et al.,* 1989; Elliott *et al.,* 1990) and basophils (Haak-Frendscho *et al.,* 1988).

1.2.3 IL-5

Human IL-5 is a disulphide-linked homodimeric glycoprotein secreted mainly by T lymphocytes (Walker *et al.,* 1991). The mature human IL-5 monomer comprises 115 amino acids with molecular weight of 26 kDa for the dimer. In native conditions, it is usually modified by carbohydrate, which produces a molecular weight of 40 to 45 kDa materials, although the carbohydrate is not essential for the biological activities *in vitro* (Tominaga *et al.,* 1990). The human IL-5 gene has been cloned (Azuma *et al.,* 1986; Mizuta *et al.,* 1989) and found to contain four exons and three introns. The gene location is close to that of IL-3 and GM-CSF on chromosome 5 at q23.3-31. (Takahashi *et al.,* 1988).

Human IL-5 has a prime function on eosinophils. It induces the production of eosinophils from bone marrow by stimulating eosinophil precursors, either alone or in combination with a network of cytokine such as IL-3 and GM-CSF (reviewed by Sanderson, 1988; 1992;) . IL-5 also plays an important role in the functional activation of eosinophils. It can enhance IgA-induced eosinophil

degranulation (Fujisawa *et al.,* 1990), upregulate adhesion molecule integrin CD11b on human eosinophils (Walsh *et al.,* 1990). IL-5 also has activities on human basophils (reviewed by Denburg 1992). For example, IL-5 increases histamine production and leucotriene generation from basophil (Bischoff *et al.,* 1990). In addition, IL-5 may also have function on human B and T lymphocytes. It can augment Ig production of antigen-stimulated human B blasts in the presence of IL-2 and also increase the secretory form of μ-chain mRNA expression (Morikawa *et al.,* 1993). This finding provided evidence that IL-5 directly mediated human B-cell terminal differentiation. On human T cells, IL-5 appears to increase the IL-2Rα expression (Noma *et al.,* 1987), enhance generation of T killer cells in the presence of IL-3 (Aoki *et al.,* 1989), and augment cytotoxic T lymphocytes (CTL) generation in the presence of IL-4 (Nagasawa *et al.,* 1991).

1.2.4 The biological activities of IL-3, GM-CSF and IL-5 *in vivo*

Although the biological activities of IL-3, GM-CSF and IL-5 have been investigated extensively from *in vitro* studies, their functions *in vivo* still need further clarification. Very limited work has been done on *in vivo* activities of these cytokines in human. Administration of IL-3 to human induces temporary multilineage stimulation of haemopoiesis(Ganser *et al.*, 1990), but repeated use of IL-3 led to significant bone marrow fibrosis (Orazi *et al.*, 1992). In animal models, the above questions have been well addressed through injecting cytokines into the animal, and through transgenic mice carrying cytokine genes and mice with cytokine or receptor genes inactivated. When cytokines such as GM-CSF or IL-3 were repeatedly injected into mice, the recipient mice showed increases in progenitors as well as mature blood cells (Metcalf *et at.*, 1986; 1987). Transgenic mice carrying IL-3 gene (Chang *et al.*, 1989; Wong *et al.*, 1989) showed neutrophil infiltration in several organs or a clinical feature resemble chronic myeloid leukemia. Tansgneic mice carrying the GM-CSF gene (Lang *et al.*, 1987) underwent multi-organ macrophage invasion, and transgenic mice with the IL-5 gene (Dent *et al.*, 1990) suffered from lifelong eosinophilia. On the

other hand, mice with the knock-out of genes for GM-CSF (Dranoff *et al.*, 1994; Stanley *et al.*, 1994), or IL-3 (Mulligan RC, personal communication), or IL-3Rα (Hara et al., 1995), or the β_c for the GM-CSF, IL-3 and IL-5 receptors (Robb *et al.*, 1995; Nishinakamura *et al.*, 1995), or the β_c plus IL-3 (Nishinakamura *et al.*, 1996) undergo near normal development as well as normal haemopoiesis. Although the function of IL-3, GM-CSF and IL-5 may be different among different species, the above investigations suggest in general that these cytokines is not required for "steady-state" haemopoiesis. This may also suggest that the major roles of those cytokines may be in pathological conditions such as inflammation.

1.3 The receptors for IL-3, GM-CSF and IL-5

IL-3, GM-CSF and IL-5 function through binding to and activating their specific receptors that are cell-surface glycoproteins. The receptors consist of heterodimers comprising a ligand-specific α-chain and a common β_c. The α-chains bind their cognate cytokine with low affinity, whereas the β_c alone does not show any detectable binding to any of these cytokines, but is required, in association with the α-chains, to confer high affinity binding (Hayashida *et al.*, 1990; Gearing *et al.*, 1989; Tavernier *et al.*, 1991; Kitamura *et al.*, 1991). On the high affinity conversion, the β_c increases IL-3 affinity 500~1000 fold (Kitamura 1991; 1992), GM-CSF binding affinity 20~100 fold (Hayashida *et al.*, 1990) and IL-5 binding affinity 2~5 fold (Tavernier *et al.*, 1991, 1992). The β_c is essential for signal transduction (Kitamura *et al.*, 1991; Kitamura and Miyajima, 1992; Sakamaki *et al.*, 1992).

1.3.1 Structure and function

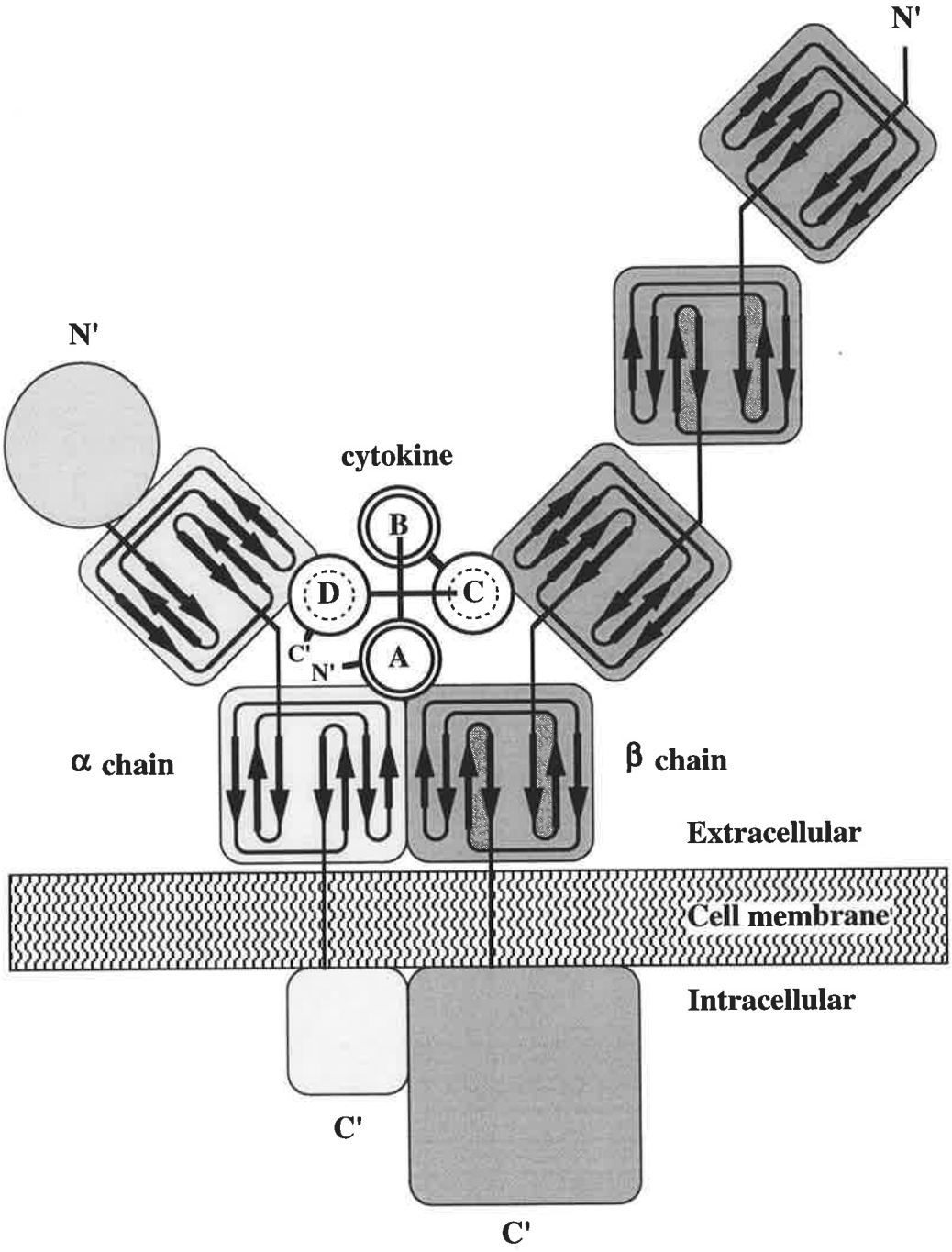
Structurally, both the α-chains and β_c for the IL-3, GM-CSF and IL-5 receptor belong to the type I cytokine receptor superfamily, which includes receptors for ciliary neurotrophic factor (CNTF), erythropoietin (EPO), G-GSF, growth hormone IL-2, IL-4, IL-6, IL-7,. IL-9, leukaemia inhibitory factor (LIF)

oncostatin M and prolactin (Bazan, 1990, Taga *et al.,* 1992). Members of this family are characterised by the sharing of a cytokine receptor module (CRM) in their extracellular regions, which contain four conserved cysteine residues and a unique Trp-Ser-Xaa-Ser (WSXWS) motif. Each CRM has 2 fibronectin-like domains of approximately 100 residues. In contrast, the cytoplasmic regions of this family are poorly conserved.

In the extracellular region of Figure 1-1, the α-chains of IL-3, GM-CSF and IL-5 receptors have an *N*-terminal domain and one copy of the CRM. The *N*-terminal domain is unique amongst the GM-CSF, IL-3 and IL-5 receptor α-chains, one of the characteristics that distinguishes them from other members of the cytokine receptor superfamily. The extracellular region of β_c contains four fibronectin-like domains, denoted domains 1, 2, 3, 4 from the *N*-terminus. They organise in pairs that correspond to cytokine receptor module (CRM) 1 and CRM 2 (Goodall *et al.,* 1993). The tertiary structure of the α and β chains of IL-3, GM-CSF and IL-5 receptors is unknown. The three dimensional structure of a soluble form of GH receptor and growth hormone binding protein has been elucidated by X-ray crystallography (de Vos *et al.,* 1992). To relate this to GM-CSF, IL-3 and IL-5 receptors, the sequences of the α- and β-chains for IL-3, GM-CSF and IL-5 receptors have been appropriately aligned with the GH receptor (Goodall *et al.,* 1993). Using the GH receptor as a model, the structures of the CRM of α-chain and CRMs of β_c for GM-CSF, IL-3 and IL-5 receptors were predicted as shown in figure 1-2 (Bagley CJ, personal communication). In that model, the α and β chains face each other and both of them have a contact region to the proposed ligand.

Although the model provides some predictions about the ligand-binding sites on the receptor molecule, the exact ligand binding regions on the receptor chains were largely unknown before the commencement of this study. To define such a ligand binding site on the receptor will provide insight for the mechanism of receptor binding and activation. It may also help in order to generate a

Figure 1-1 Diagram of the GM-CSF, IL-3 and IL-5 receptor-ligand complex. The receptor is a heterodimer consisting of an α-chain (light stippling) and the β-chain (heavy stippling). The CRMs are shown to consist of 2 domains, each of which contains a barrel of strands (heavy arrows) connected by loops. The α-chain has one copy of CRM and an additional N-terminal domain. The β-chain has two copies of CRM. Both the α- and β-chains have transmemberane and cytoplasmic domains. The ligand is represented as a four-helix bundle viewed end-on , with helices A and B entering the plane of the page and helices C and D leaving it.


N'
N'
cytokine
B
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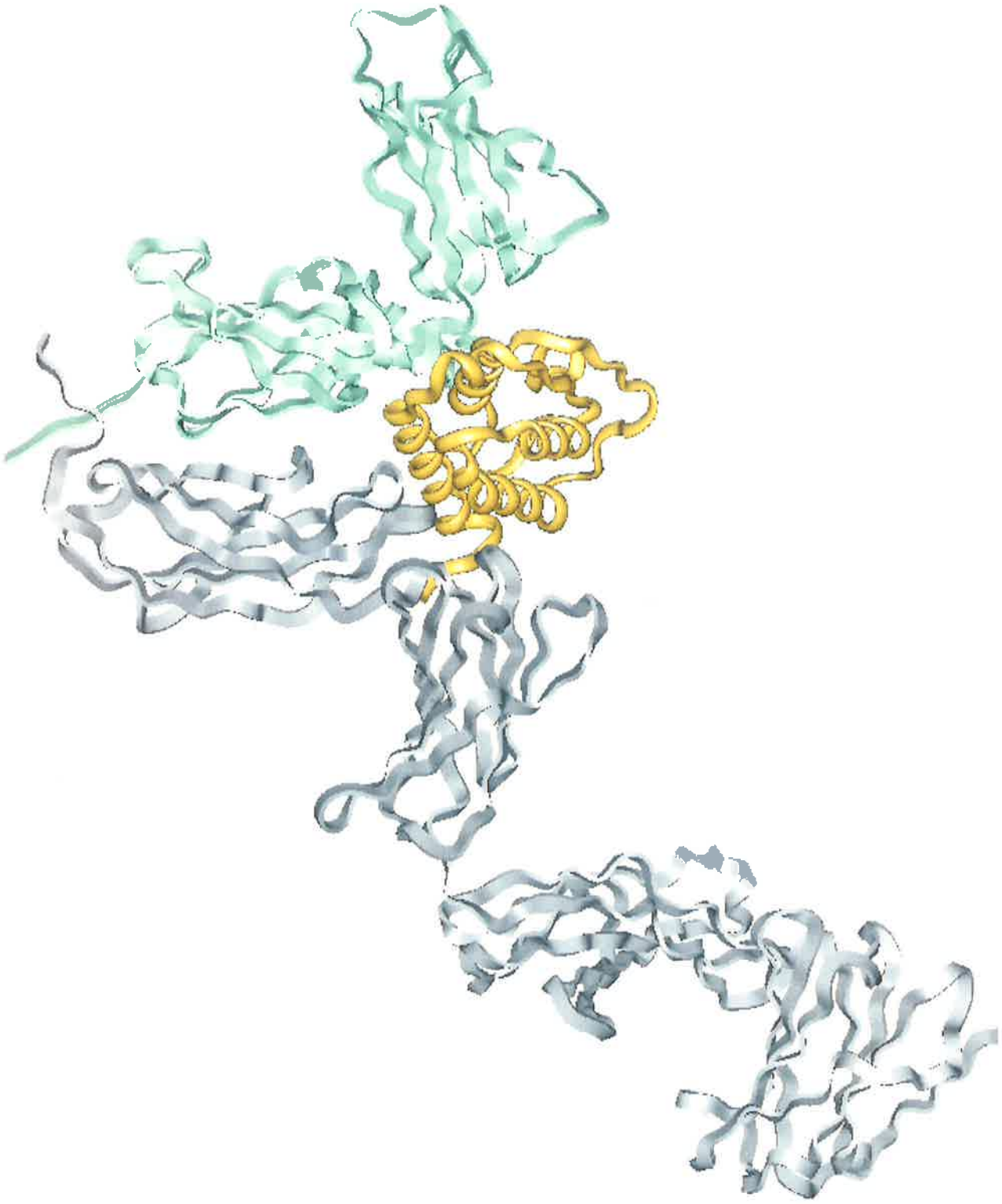
A
α chain
β chain
Extracellular
Cell membrane
Intracellular
C'
C'

Figure 1-2 A model illustrating the proposed structure of the ligand and receptor complex for GM-CSF, IL-3 and IL-5, based on the three dimensional structure of growth hormone and its binding protein (model generated by Dr C. Bagley). Green ribbon represent the CRM of α-chain and black one the CRM1 and CRM2 of the β-chain. Both the chains compose the receptor complex which interacts with the proposed ligand (GM-CSF in this case) that shown in orange ribbon.



synthetic molecule that may mimic the native ligand and exhibit agonistic or antagonistic activities. Recently several studies have been explored to define the ligand interacting site on the α and β_c of IL-3, GM-CSF and IL-5 receptors. These investigations have revealed that the *N*-terminal domain of the α-chain and the B'-C' and F'-G' loops in the domain 4 of β-chain are important regions for ligand binding and function. IL-3Rα-chain studies with MoAb (Sun *et al.*, 1996) or mutational analysis (Rapoport *et al.*, 1996; Barry *et al.*, 1997) have revealed that the N-terminal domains and the LSXWS motif are important for the IL-3 binding and function. Mutagenesis studies of GM-CSFR α-chain revealed that the paired cysteine residue and some residue in the WSXWS motif were essential for the ligand binding (Ronco *et al.*, 1984; Doshi *et al.*, 1994; Ronco *et al.*, 1994). In other studies with chimeric or mutant IL-5R α-chains, the *N*-terminal domain had been shown to be of importance in ligand interaction (Cornelis *et al.*, 1995). The β_c has also been investigated extensively. It has been shown that His^{367} of β_c is important for GM-CSF high affinity binding (Lock *et al.*, 1994), while other residues such as Tyr^{365} and Ile^{368} in the B'-C' (Woodcock *et al.*, 1994) and Tyr^{421} in the F'-G' loop (Woodcock *et al.*, 1996) are essential for the binding of 2 or 3 ligands.

Although many residues and motifs in the receptor α- and β- chains have been reported to be of importance in ligand binding and function, the overall pattern of ligand and receptor interaction for IL-3, GM-CSF and IL-5 still needs further characterisation.

1.3.2 Receptor activation and signal transduction

Receptor dimerisation/oligomerisation is generally believed to be important for many receptor systems such as G-CSFR, EPOR, IL-2R, IL-6R and c-kit (Lev *et al.*, 1992; Hiraoka *et al.*, 1994; Miura and Ihle, 1993; Davis *et al.*, 1993; Hibi *et al.*, 1990). Ligand-induced receptor dimerisation or oligomerisation appears to be a general feature of cytokine receptors (reviewed by Heldin, 1995). Once

cytokines bind to their cell-surface receptors, they induce receptor clustering followed by receptor activation and signalling.

The α and β chain dimerisation is also an essential step for the activation of IL-3, GM-CSF and IL-5 receptors. However, the pattern of receptor dimerisation among IL-3, GM-CSF and IL-5 receptors may be different. We have shown that the IL-3 receptor dimerisation is ligand-induced (Stomski *et al.*, 1996), while GM-CSF receptor dimerisation seems to occur without ligand (our unpublished observation).

Following the activation of the receptor for IL-3, GM-CSF and IL-5, the signalling is mediated by β_c through protein phosphorylation by associating tyrosine kinases. One of the earliest events after receptor activation is the induction of tyrosine phosphorylation of several proteins. Although neither the α or β chain in IL-3, GM-CSF and IL-5 receptor has intrinsic tyrosine kinase activity, rapid tyrosine phosphorylation of many cellular proteins has been described in human cells stimulated by IL-3 or GM-CSF (reviewed by Miyajima, 1993). Phosphorylation of proteins then lead to the activation of various signalling molecules that are part of the Ras (Satoh *et al.*, 1992; Duronio *et al.*, 1992) and the JAK-STAT (reviewed by Darnell *et al.*, 1994) pathways. In general, the Ras pathway seems responsible for the anti-apoptosis signals (Kinoshita *et al.*, 1995) while the JAK-STAT pathways lead to DNA synthesis (Muli *et al.*, 1996).

1.3.3 Receptor expression and distribution

The receptors for IL-3, GM-CSF and IL-5 are expressed and distributed extensively on a variety of cells covering both haemopoietic and nonhaemopoietic cells. Traditionally the receptor investigation was carried out by radiolabelled ligand binding assays. It has been shown that eosinophils express the high affinity receptor for all three ligands while monocytes only have GM-CSF and IL-5 receptors. The cross-competition ligand binding assays on monocytes and

eosinophils also gave the initial suggestion that the three ligands may share a receptor compartment. As for the neutrophils, they have high affinity receptor for GM-CSF (Lopez *et al.*, 1988), but when they were stimulated with GM-CSF, they also express high affinity IL-3 receptor (Smith *et al.*, 1995)

The development of MoAbs to the IL-3, GM-CSF and IL-5 receptor chains was essential to determine the expression, distribution and regulation of receptors in a ligand-independent manner. The separated receptor chains can specifically targeted and their regulation can also be quantified. MoAbs can also be applied to study the expression of receptors in earlier haemopoietic cells such as CD34 cells together with other cell surface markers. It has been shown that CD34 cells express more α-chains rather than β_c (Santo *et al.*, 1993; our observation), but β_c expression on the more mature cells such as monocytes, eosinophils and neutrophils is at a relatively high level (our observation).

More interestingly, with the combination of MoAb, ligand-binding studies and receptor mRNA assays, it has also been revealed that some endothelial cells can express IL-3 receptor (Colotta *et al.*, 1993; Korpelainen *et al.*, 1993), and some breast cancer cells express GM-CSF receptor (our unpublished observation). Although the significance of these phenomena still needs to be clarified, it extends the functions of IL-3, GM-CSF from haemopoiesis to inflammation and possibly to oncogenesis as well.

1.4 The pathological roles of IL-3, GM-CSF, IL-5 and their receptors

IL-3, GM-CSF and IL-5 may be involved in pathology through their increased or abnormal productions, or via the up-regulation of their receptor expression. The over production of these cytokines, or aberrant or abnormal expression of their receptors may have important implications. Several studies have suggested that the over-stimulating effect played important roles in the pathogenesis of certain diseases. For example, many studies have demonstrated that the mRNA levels

of IL-3, GM-CSF and IL-5 were increased at sites of allergic inflammation and that the presence of these cytokines correlates with tissue eosinophilia (Durham *et al.,* 1992; Corrigan *et al.,* 1992; Kay *et al.,* 1991; Walker *et al.,* 1991). The elevation of these cytokines may be due to the TH_2-type T-lymphocytes or eosinophils accumulation at the tissues (Moqbel *et al.,* 1991). In addition, allergic disorders may have distinct cytokine profiles over non-allergic ones (Hamilos *et al.,* 1995), which further suggests the roles of cytokines, in particular IL-5 and GM-CSF, in allergy.

IL-3 and GM-CSF have also had implication on the pathogenesis of leukaemia. Many *in vitro* studies suggest that both IL-3 and GM-CSF stimulate the leukaemic cell growth and mediate their survival. In acute lymphoblastic leukaemia (ALL), IL-3 and GM-CSF can stimulate the leukaemic cell growth *in vitro* (Wörmann *et al.,* 1989; Attias *et al.,* 1995). IL-3 and GM-CSF also markedly enhance acute myeloblastic leukaemia (AML) cell growth *in vitro,* especially in the presence of TNF (Elbaz *et al.,* 1994; Delwel *et al.,* 1992). The suppression of leukaemic cell growth by an IL-3 antagonist (Smit *et al.*, 1995) and the induction of apoptosis of leukaemic cells by a GM-CSF antagonist (Iversen *et al.,* 1996) give more evidence of the roles of cytokines in the pathogenesis of leukaemia. More ever, IL-3 has been shown to be a prime stimulator for the follicular B cell lymphomas (Clayberger *et al.,* 1992). In addition, the receptors for IL-3 and GM-CSF have been confirmed to be expressed on many primary leukaemic cells as well as the leukaemic cell lines (Gore *et al.,* 1994; Trentin *et al.,* 1994).

Although the precise mechanisms of the pathogenesis of these cytokines in the above disorders are still not clear, it is possible that we may be able to control these diseases by suppression of the overstimulation by the cytokines. One of the approaches is by manipulating the cytokine receptors. For instance, it may be possible to develop receptor antagonists to inhibit the cytokine signalling. In order to achieve this, it is important to understand in more detail the structure and function of these receptors. The antibodies developed in this thesis provides

new clues and tools with which to modulate the functions of GM-CSF, IL-3 and IL-5 receptors.

CHAPTER II

MATERIALS AND METHODS

2. 1 Tissue Culture Media And Reagents

2.1.1 Tissue Culture Reagents: RPMI-1640, HDMEM, F-12, FCS were purchased from Gibco Laboratories, Glen Waverley, Victoria, Australia.

Dulbecco's modified eagles medium (DMEM) was provided by MultiCel Pty Ltd, Castle Hill, NSW, Australia.

2.1.2 Hybridoma growth medium (HGM): Hybridoma growth medium was RPMI-1640 medium supplemented with 20% FCS, 10% NCTC-109 (Gibco, Caitherburg, MD), 120 μg/ml penicillin G, 160 μg/ml gentamicin, 2mM L-glutamine, 0.2% sodium bicarbonate.

2.1.3 J774 conditioned medium (J774-CM): J774 cell (Rathgen *et al.*, 1986) was grown in DMEM medium supplemented with 10% FCS and $5x10^{-5}$M β-mercaptoethanol (Sigma, St. Louis, MO). When the cells were about 90% confluent, LPS (Department of Immunology and Microbiology, University of Adelaide) was added to the final concentration of 10 μg/ml for 16 hours or overnight. The culture medium was changed and supernatant was harvested after 48 hours incubation. The J774 conditioned medium was filtered through a 0.22μM membrane. It was stored at 4°C for up to 6 months or frozen at -20°C until use.

2.2 Cytokines and other antibodies

EPO was purchased from Johnson & Johnson (Rantan, NJ)

G-CSF was obtained from Amgen (Thousand Oaks, CA).

GM-CSF was expressed in E.coli and purified in the laboratory by Dr C Bagley as described (Hercus *et al.*, 1994).

IFN-γ is a gift from Genentech, San Fransisco, CA

IL-3 is a gift from Genetics Institute, Cambridge, MA.

IL-4 is a gift from Immunex, WA

IL-5 was produced in Sf9 cells as detailed (Tavernier *et al.*, 1989)

TNF-α was a gift from Dr J. Gamble in the Hanson Centre for Cancer Research.

Anti-CD34 MoAb, 12.8, was a gift from Dr Andrews of Fred Hutchison Cancer Center, Seattle, WA

Anti-CD69 MoAb conjugated with PE was from Becton Dickinson, San Jose, CA

Antiphosphotyrosine MoAb, 3-356-10, was purchased from Boehringer Mannheim, Frankfurt, Germany

A negative control MoAb, 3D3 (anti-*salmonella* murine IgG1), was a gift from Dr L Ashman of Division of Haematology.

2.3 Cell lines and Human Primary Cells

The Chinese Hamster Ovary (CHO) cell Lines: The permanent CHO cell lines, F6 (expressing IL-3Rα, Sun *et al.*,, 1996), A9/C7 (expressing GM-CSFRα-chain, Hercus *et al.*,, 1994), A8 (expressing IL-5Rα) and CHOβ (expressing β_c) were developed by Dr Jo Woodcock in this Laboratory. For F6 cell, IL-3Rα cDNA was cloned into pcDNA/Neo (Invitrogen, San Diego, CA). Linearized plasmid constructs were introduced into CHO cells by electroporation (Bio-Rad Gene Pulser, North Ryde, NSW) at 1,300 V and 25 μF using $5x10^5$ CHO cells. The cell suspension was then cultured in F-12 medium with 10% FCS for two days before adding 350 μg/ml G418 (Geneticin, GIBCO Laboratories, Glen Waverley, Victoria, Australia). From the G418-resistant CHO colonies, the F6 cell line stably expressing 2 to $5x10^5$ IL-3Rα-chains per cell were created. A9/C7, A8, CHOβ cell lines were all produced using similar method as F6 cells.

COS cell transfectant: COS cells were maintained in RPMI-1640 medium supplemented with 10% FCS. Cells transiently expressing receptor subunit cDNA were developed by electroporation (Woodcock *et al.*, 1994). Briefly, $2x10^7$

cells in 0.8 ml ice-cold PBS was transfected with 10 μg of IL-3 or GM-CSF or IL-5 receptor α chain DNA in pcDM8, and/or 25 μg of pcDNA1 vector containing β_c chain at 500 μF and 300V. After transfection, cells were cultured for 48 to 72 hours before harvesting for experiment.

FDCP1 cells: A FDCP1 cell expressing IL-5Rα was a gift from Dr J Tavernier from Roche Research Ghent, Belgium. Cells were maintained in RPMI-1640 with 10% FCS (non-heat inactivated).

NS-1 cells: NS-1 cell, a myeloma cell line, was obtained from Dr P Simmons, Division of Haematology, Hanson Centre for Cancer Research. It was grown in RPMI-1640 medium containing 10% FCS before fusion.

MO7e cells: MO7e cells, a human megakaryoblastic cell line, were from Dr P Crozier, Aucland, New Zealand.

TF-1 and TF-1.8 cells: TF-1 cells (Kitamura *et al.*, 1989) and TF-1.8 cells (a gift from Dr J Tavernier from Roche Research Ghent, Belgium) were maintained in RPMI-1640 medium supplemented with 10% FCS and 2ng/ml GM-CSF.

Bone Marrow Mononuclear cells: Bone marrow was obtained from healthy volunteers from the Division of Haematology. Bone Marrow aspirate was diluted with Hanks Buffered Salt Solution (HBSS, KCl 5.4 mM, KH_2PO_4 0.4 mM, NaCl 136.9 mM, $NaHCO_3$ 4.2 mM, Na_2HPO_4 0.1 mM) and separated by lymphoprep (Nycomed, Oslo, Norway), gradient centrifugation. Red blood cells were lysed in ice-cold isotonic ammonium chloride solution. Bone marrow mononuclear cells were maintained in RPMI-1640 containing 10% FCS for up to 12 hours or RPMI-1640 plus 20% FCS for up to 24 hours at 4°C.

Neutrophils: Neutrophils were purified from peripheral blood of normal volunteers by dextran sedimentation (Dextran T-500, Pharmacia, Uppsala,

Sweden) and density gradient separation on lymphoprep (Nycomed, Oslo, Norway) followed by hypotonic lysis of erythrocytes as described for bone marrow cells. The cell preparation was resuspended in RPMI with 0.1% BSA (Boehringer Mannheim, Sydney, Australia). The purity of preparation was greater than 95% neutrophils as judged by morphologic examination of Wright's stained cytocentrifuge preparations. For higher purity, neutrophils were purified using Metrizamide (Nycomed, Oslo, Norway) multistep gradients, as described (Vadas *et al.*, 1979), and were greater than 98% pure. After purification, neutrophils were used fresh or cultured in 24-well (10^6 cells/well) tissue culture dish (Nunc, Roskilde, Denmark) in RPMI-1640 with 5% FCS in the presence or absence of 1 ng/ml GM-CSF.

Monocytes: Human monocyte purification was based on the protocol of Sanderson *et al.* (1977). Briefly, fresh blood from healthy donors was diluted with HBSS and underlayered with Lymphoprep before centrifuging in the Beckman J-6M/E at 1800 rpm for 25min. The layer containing mainly monocyte and lymphocyte was pooled and washed, and the platelets were removed by another spin. Then cells were resuspended in Elution Buffer (HBSS, FCS 0.1% , EDTA 0.01%, glucose 0.1%) and placed in a Beckman JE-6B elutriator using a Sanderson chamber, with rotor speed of 2050 rpm and a flow rate at 12 ml/min. After that, the lymphocytes were removed and monocytes were washed and resuspended in RPMI-1640 medium with 10% FCS. The preparation is more than 85% pure.

Eosinophils: Human eosinophils were purified from the peripheral blood of slightly eosinophilic volunteers after sedimentation through dextran and centrifugation through a discontinuous density gradient of hypertonic Metrizamide, as previously described (Vadas *et al.*, 1979). These were greater than 95% pure.

HUVEC: HUVECs were collected from umbilical vein within 24 hours of delivery. The cannulated vein was flushed with RPMI-1640 medium and then digested with collagenase for 13 minutes at 37°C. The cell preparation was washed and cultured in gelatin-coated plastic flasks in M199 medium (Cytosystem, Sydney, Australia) containing Earle's salts, 20% FCS and 20 µg/ml endothelial cell growth supplement.

2.4 MoAb Production

2.4.1 Immunisation

BALB/c mice were injected intraperitoneally with 10^7 cell transfectant or 5-10 µl purified protein in 0.5 ml PBS together with 50µg Adjuvant Peptides (Sigma, St. Louis, MO). The immunisation was repeated 3 to 4 times at two week intervals. Three weeks after the final intraperitoneal injection, mice were boosted with 10^6 cell transfectants or 5 µg protein in 0.2 ml PBS via the tail vein. Three days later, the mice were sacrificed and their spleenocytes were collected for the hybridoma fusion.

2.4.2 Hybridoma Fusion

The hybridoma fusion method was modified from that originally described by Köhler and Milstein (1975) and Gefter *et al.* (1977). Briefly, the splenocytes cell preparation was washed with serum-free RPMI-1640 medium followed by hypotonic lysis of erythrocytes using 0.2% sodium chloride solution. Splenocytes were counted and mixed with NS-1 cell at the ratio of 4:1 before centrifuging for 5 minutes at 400g. Then the cell pellet was resuspended gently over a period of one minute with 1 ml of polyethylene glycol-1500 (PEG, Boehringer Mannhein, Germany), prewarmed to 37°C. The PEG cell mixture was incubated at 37°C for a further minute followed by dilution with warm hybridoma growth medium 1 ml, 2 mls, 5 mls and 10 mls, all at 1 minute intervals. Then the cell mixture was placed at 37°C for a further 3 minutes before centrifuging for 5 minutes at 400g. The cells were gently resuspended in 200 ml of HGM supplemented with 20%

J774-CM and hypoxanthine/aminopterin/thymidine (HAT, Boehringer Mannheim, Germany) at a final concentration of 0.1 mM, 0.016 mM and 0.8 μM, respectively. The cell suspension was aliquoted at 200 μl/well over 10 to 11 flat bottomed 96-well plates (Nunc, Roskilde, Denmark) and cultured at 37°C.

After 8 to 12 days incubation or when the hybridoma clone was visible by the naked eye, 100 μl of supernatants were collected and screening was initiated

2.4.3 Screening for antibody-producing hybridoma

Three kinds of assays have been applied for the screening of hybridomas.

Rose Bengal colorimetric assay (RBA): RBA was set up as described (Lyons *et al.*, 1985). Round-bottomed 96-well plate (Nunc, Roskilde, Denmark) was coated with 100 μ/well goat anti-mouse Ig (G+M) antibody (Caltag Laboratories, San Francisco, CA) at 5 μg/ml at 4°C overnight. The plates were then washed with PBS before adding 100 μl/well hybridoma supernatant and incubating at 4°C for 60 minutes. The target cells, 2-5x10^5 in 100 μl of RPMI-1640 containing 10% FCS and 0.1% sodium azide, were added to the well and incubated at 4°C for 120 minutes. After incubation, the unbound cells were flicked out and 100 μl of Rose Bengal dye (0.25% in PBS) (Faulding, Australia) was added and culture for a further 15 minutes. Then the plates were washed and the bound cells were lysed with 100 μl 1% Sodium Dodecyl Sulphate. The dye released from the bound cells was analysed on Titertek Multiskan ELISA reader (BIO-RAD, model 3550) at the wave length of 540 nm (OD 540), which corresponded to the absorbency maximum of Rose Bengal. Wells having an OD 540 more than twice that of negative control well were considered positive.

Enzyme-linked immunosorbent assays (ELISA): A peroxidase based ELISA was also applied for the detection of MoAbs against soluble receptor. First the round bottomed 96-well plate (Nunc, Roskide, Denmark) was coated with soluble antigen at the concentration of 2 μg/ml in PBS at 4°C overnight. The plate was

washed followed by incubating with 50 µl hybridoma supernatant for a further 1 hour at room temperature. After washing off the unbound antibody, 50 µl of peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:4000 dilution, DAKO, Denmark) was added for a further 1 hour at room temperature. The plate was then washed as above, followed by the addition of peroxidase substrate solution, OPD (O-phenylene Diamine, H_2O_2, Sigma). Optical density (O.D.) of each well was measured using an O.D. plate reader at the absorption wave length of 490 nm. Wells with O.D. measuring more than twice that of the negative control well were considered positive.

Flow cytometer analysis: see 2.5

Once positive wells were located, a single cell cloning process was performed to ensure the monoclonality.

2.4.4 Single-cell clone

Single cell cloning was carried out by means of limiting dilution. A flat bottomed 96-well plate (Nunc, Roskilde, Denmark) was fed with 100 µl/well of HGM supplemented with 10% J774-CM and HT. A thousand hybridoma cells were diluted, by serial 2-fold, down row 1 to row 8 starting from the top left well, and then subjected to a further 2-fold dilution from columns 1-12 using a multi pipette. After 8-12 days incubation at 37°C, the supernatants from the well that contained single clone were tested as described in the screening. Positive clones were then subjected to two further rounds of limiting dilution in order to generate a monoclonal population. After obtaining single-cell lones, these hybridomas were expanded and subjected to further characterisation such as MoAb isotyping, functional testing, and also for ascites production.

2.4.5 Isotyping of MoAb

The isotyping of MoAb was carried out by using an IsoStrip mouse monoclonal antibody isotyping kit (Boehringer Mannheim, Germany) as described by the manufacturer.

2.4.6 Ascites production

Antibody containing ascites was induced by injecting 5-10x10^6 hybridoma cells into the peritonea of pristane-treated BALB/c mice. After 10-14 days, ascites from the mice were collected and ascitic supernatants were fractionated by spinning down the ascitic fluids at 3000g for 10 minutes.

2.4.7 Purifying and storing MoAbs

MoAb were purified from ascites or hybridoma supernatants using an Immobilised Protein A IgG Purification kit (Pierce, Rockford, IL). After purification, the MoAbs were buffer-exchanged to PBS and stored at 4°C for up to 6 months, or frozen at -70°C until application. The antibody containing ascites is normally stored at -70°C until use.

2.4.8 Freezing and thawing of hybridoma cells

Hybridoma cells were centrifuged at 400g for 5 min at 4 °C, and resuspended gently in hybridoma freezing medium (FCS supplemented with 10% DMSO, freshly made) at 5 x 10^{6-7} cells/ml. Cell suspensions (1ml) were then aliquoted and transfered to a freezing rack at -70 °C overnight before storing at -185 °C in the Liquid Nitrogen. When the cells were thawed, the vial was quickly warmed to 37 °C and the cell suspension washed twice with warm medium. The hybridoma cells were resuspended and cultured in the preferred growth medium.

2.5 Immunofluorescent Analysis and Cell Sorting

Indirect single-colour labelling was performed by incubating target cells ($5x10^5$) with 50 µl hybridoma supernatant or 0.25 µg purified MoAb for 45 minutes at 4°C. Cells were washed twice with washing buffer RPMI-1640 + 1% FCS + 0.1% Sodium Azide), followed by a further 45 minutes incubation with a 1:50 dilution of FITC-conjugated rabbit anti-mouse Ig (Silenus, Hawthorn, Victoria, Australia). The cells were washed as above and resuspended in 250 µl FACS-FIX (DPBS + 1% Formalin + 2% glucose + 0.02% Sodium Azide).

Dual-colour fluorescence staining was performed by incubating cells first with MoAb directly conjugated with PE. After that the cells were subjected to a further indirect single colour staining with another MoAb as described above.

To investigate the surface expression of cytokine receptor subunits on the target cells, fluorescent intensity of the sample was analysed with EPICS-Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL). Cell sorting was performed on a FACStar Plus (Becton Dickinson) containing a 250 mW argon laser that emitted at a wavelength of 488 nm. In experiment with truncated IL-3Rα-chain and IL-3Rα/GM-CSFRα chimeras, transfected COS cells were examined under a fluorescence microscope.

2.6. Immunoprecipitation

Cells (4×10^7) were surface labelled using Na ^{125}I (New England Nuclear, Boston, MA) as described (Krissansen *et al.*,1989). Cells were washed three times with PBS and lysed in 1 ml of RIPA buffer with protease inhibitors [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 0.5% Deoxycholate, 0.05% SDS, 2mM PMSF, 10 mM Soybean Trypsin Inhibitor, 20 mM Leupeptin and 5% Aprotonin (Sigma, St Louis, MI)]. The cell extracts were centrifuged at 10,000g for 15 min and the cell lysates were precleared twice with protein A sepharose

before incubating 250µl of lysates with MoAbs (2mg/ml) overnight at 4°C. Protein-A Sepharose was then added and bound proteins were washed with RIPA buffer and eluted with SDS loading buffer with 2-mercaptoethanol and analysed by SDS-PAGE. Radiolabelled proteins were visualised using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2. 7 Western Blotting Analysis

Cells (3 x 10^7) were solubilized in reducing SDS loading buffer, and the proteins separated by SDS-PAGE before transferring electrophoretically onto nitrocellulose filters. Filters were then blocked with TNT buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 3% gelatin. The test MoAbs (2 mg/ml) were diluted in TNT buffer containing 1% gelatin and incubated with the filters for 90 min. The filters were then incubated with ^{125}I-Protein A (New England Nuclear, Boston, MA) for 45 min and washed thoroughly with TNT buffer. The radio-labelled proteins were detected as described above.

2.8 Radioiodination of cytokines and binding experiments

^{125}I-IL-5 was purchased from Dupont NEN (North Sydney, NSW, Australia). Recombinant GM-CSF and IL-3 were radiolabelled by the iodine monochloride method (Contreras *et al.,* 1983) to a specific activity of about 10 to 36 mCi/mg respectively. Iodinated proteins were separated from the reaction mixture by chromatography on Sephadex G-25 PD1O Column (Pharmacia, Uppsala, Sweden), eluted with PBS containing 0.02% Tween 20 and stored in siliconised glass tubes at 4°C for up to 4 weeks. Prior to use, the radioligands were purified from Tween and non-protein-associated radioactivity by cation-exchange chromatography on a Carboxymethyl-Sepharose CL-6B column (Pharmacia,

Uppsala, Sweden) which had been pre-equilibrated with 10 mM citrate-phosphate buffer (pH 2.6). The column was washed and the radioligand was eluted with binding medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 0.5% BSA and 0.1% sodium azide.

Binding experiments were performed as described (Lopez *et al.*, 1989). Equilibrium binding assays were performed by incubating 0.5 1 x 10^6 cells in 0.15 of binding medium with radioligands or MoAbs at 24°C for 2-3 hours on a rotating table. Cell suspensions were overlayed on 0.2 ml FCS or phthalate oil (Plaetinck *et al.*, 1990) and centrifuged for 30 seconds at maximum speed in a micro-centrifuge. The visible cell pellet was removed by cutting and the radioactivity of the cell pellet was counted in a Cobra 5010 γ-counter (Packard, Meriden, CT). Nonspecific binding was determined in the presence of a 100-fold excess of unlabelled cytokines. The receptor number and affinity were determined by Scatchard analysis by using the EBDA and LIGAND programs (Munson and Rodbard, 1980; Biosoft, Cambridge, UK). Specific binding was calculated by subtracting nonspecific from total binding.

2.9 Radioiodination of MoAb and MoAb Binding Assays.

MoAb (10μg in 100 μl of PBS) was iodinated with 0.5 mCi of $Na^{125}I$ by the Chloramine T method as described (McConahey and Dixon, 1949). MoAb binding studies were performed using ^{125}I-MoAbs with the same method as cytokine binding assays.

2. 10 Proliferation Assay

GM-CSF dependent TF-1 cells or TF-1.8 cells were starved of GM-CSF for 24 to 48 hours before setting up proliferation assays. Briefly, $1x10^4$ cells were incubated in wells with preferred cytokines in the presence of a range of

concentrations of MoAbs for 48 hours at 37 °C. Wells were pulsed with 0.5 mCi/well ^{3}H-thymidine for 4 hours and then harvested onto a glass filter. Radioactivities of each well were determined by liquid scintillation. The results were expressed as disintegrations per minute (DPM).

2. 11 Histamine Release Assay

The histamine release assay was performed as previously described (Brizzi *et al.*, 1993). Briefly, low density leukocytes were separated from peripheral blood by dextran sedimentation and centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Cell suspensions (containing 1-2% of basophils) were preincubated with purified human IgE for 45 min before incubating $1x10^{6}$ cells with IL-3, a goat IgG anti-human IgE (0.8mg/ml) and a range of concentrations of MoAbs for a further 60 min. The released histamine was quantified subsequently using a radioenzymatic method (Shaff *et al.*, 1979).

2. 12 Superoxide Anion Production

Neutrophils were preincubated with medium or MoAbs for 1 hour before the test was set up with the method described by Lopez *et al.*(1986).

2. 13 Cytokine Release from Endothelial Cells.

HUVECs were obtained and cultured as previously described (Gamble *et al.*, 1989). For IL-6 measurements, HUVECs ($5x10^{5}$ per well) were treated with IFN-γ (100U/ml) for 48 hours, IL-3 (30 ng/ml) for 24 hours, or IFN-γ for 48 hours with IL-3 added for the last 24 hours with or without MoAbs 7G3 or 6H6 (100 mg/ml).

After treatment the medium was changed and supernatants were collected for 24 hours and analysed for the presence of immuno-reactive IL-6 using an ELISA method (Quantikine, R & D Systems, Minneapolis, MN).

IL-8 production from HUVEC was measured as previously described (Korpelainen *et al.,* 1993). Briefly, HUVECs ($5x10^5$ per well) were incubated with TNF-α (100U/ml) for 24 hours and or, IL-3 (30ng/ml) for 6 hours, with or without MoAb 7G3 (50mg/ml). After incubation the medium was changed and the IL-8 secreted in the following hour was quantified by ELISA.

2.14 Epitope Mapping

2.14.1 Construction and expression of chimeric and truncated IL-3Rα chains. IL-3Rα/GM-CSFRα chimera: this fusion cDNA encodes a chimeric receptor composed of the first 104 amino acids of IL-3Rα including the signal sequence fused to amino acids 118-400 of the GM-CSFRα-chain. It was generated by PCR using a sense primer 5' to the IL-3Rα coding sequence and an antisense primer corresponding to codons 104-99 and including a *Kpn* 1 site. The sequence of the resulting PCR product was checked and it was then ligated in-frame to the *Kpn* 1 site at codon 118 of GM-CSFRα.

GM-CSFRα/IL-3Rα chimera: this fusion cDNA is the converse of the IL-3Rα/GM-CSFRα chimera and encodes the first 118 amino acids of GM-CSFRα-chain including the signal sequence fused to amino acids 104-378 of IL-3Rα. It was generated by PCR using a sense primer corresponding to codons 104-110 of IL-3Rα and includes a *Kpn* I site. A downstream antisense primer was also used. The resulting PCR product was sequenced and ligated in-frame to the *Kpn* I site at codon 118 of GM-CSFRα.

IL3Rα (-31) flag: this cDNA encodes an *N*-terminally truncated form of the IL3Rα that lacks the first 31 amino acids of the mature protein but includes an 8 amino acid "flag" peptide sequence between the putative signal sequence and residue 50 of IL-3Rα. This cDNA was generated by digesting wild-type IL-3Rα with the restriction endonuclease *Eco*R V (Boehringer Mannheim GmbH, Germany), which cleaves between codons 49 and 50 and ligating it to a PCR-generated fragment encoding the 18 amino acid signal sequence of the IL-3Rα, the "flag" sequence and a short multicloning sequence which results in Val-Asp-Asp separating the flag peptide and IL-3Rα. PCR-generated sequences were verified by DNA sequence analysis.

IL-3Rα flag: this cDNA encodes an IL-3Rα in which the putative signal sequence of IL-3Rα (first 18 amino acids) is fused to the flag peptide. It was generated by PCR using an upstream sense primer corresponding to codons 19-26 and carrying an *Xba* I site at the 5' terminus of the primer. The downstream antisense primer corresponded to codons 104-99. The resultant PCR product was ligated at the 3' end to IL-3Rα (-31) flag using a common *Bam*H 1 site to restore the coding sequence for the *N*-terminal 31 amino acids missing from IL-3Rα (-31) flag. The 5' end of the PCR product was ligated via the XbaI site to the 3' end of a PCR generated fragment encoding the IL-3Rα signal peptide followed by the "flag" sequence plus the extra amino acids Val-Asp-Asp-Ile-Ser-Arg. The fidelity of the PCR generated portion was verified by DNA sequence analysis.

All chimera and truncation constructs were cloned into the expression PMX139 prior to transfection into COS cells by DEAE-dextran. Cells were grown to approximately 50-70% confluence, washed free of medium and then incubated with 3μg of cDNA (per 10 cm plate) or 6μg of cDNA (per 15 cm plate) with 0.25 mg/ml DEAE-dextran. After approximately 30 min the DEAE-dextran solution was aspirated off and cells washed and incubated in IMDM supplemented with

10% FCS and 100 μM chloroquine for 3-5 hours. Finally the cells were washed 3 times with serum free medium and incubated with IMDM supplemented with 10% FCS for 40-60 hours at 37°C.

2.14.2 β_c mutants The βc point mutants in the B'-C' and F'-G' loops have been described previously (Woodcock, *et al.*, 1994; 1996). The cDNA for wild type and all the point mutants in the B'-C' and F'-G' loops were introduced into COS cells by the electroporation method (Hercus *et al.*, 1994). Cell transfectents were then harvested for experiments 48 hours after transfection. The mutants on the B' and C' strains such as L356N, W358N, I374N and Y376N were expressed on FDCP1 cell stably as reported (Jenkins *et al.*, 1995; 1996).

2.14.3 Epitope mapping mothods

Analysis of anti-IL-3Rα MoAb 7G3 for its staining to the truncated and chimeric IL-3Rα chains was carried out by Western blot. Briefly, gels were electroeluted onto PVDF membrane and filters blocked in 5% BSA in Tris-buffered saline with 0.05% Tween-20 (TTBS). Filters were incubated with MoAbs (1-10mg/ml) in TTBS with 5% BSA for 2 hours. Flag tagged proteins were detected with 3mg/ml anti-flag MoAb M2 (IBI, New Haven, CT). The secondary antibody, alkaline phosphatase tagged goat anti-mouse (Pierce, Rockford, IL) was then added at a dilution of 1:2500 in TTBS. The MoAb bound proteins were visualised using the BCIP/NBT Western Blue stabilised substrate (Promega) as described by the manufacturer.

Epitope-mapping of anti-β_c antibodies was analysed by Immunofluorescent study. The anti-β_c MoAbs were tested for their abilities to recognise wild type β_c and the βc mutants analysed by flow cytometer using standard immunofluorescence method as just described. For each mutant, the experiment was repeated at least twice.

CHAPTER III

MoAbs TO α AND β CHAINS OF RECEPTORS FOR IL-3, GM-CSF AND IL-5

INTRODUCTION

The last two decades have witnessed the birth and development of the technique for monoclonal antibody. MoAbs are powerful reagents for analysis of their antigens, because of their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. Antibodies have been using increasingly in the field of medical diagnosis as stardard tests. In recent years, the application of MoAb has also been extended to therapy with functional MoAbs. The functional MoAbs , when bound to their antigen, may activate their antigens and producing a biological activity. Others may stabilise their antigen and prohibite the antigen from further interaction with other active molecules, and thus produce negative regulating activities. In addition, some MoAbs to specific tissue or organ have been applied as carriers by coupling them with therapeutic reagents in order to increase the specificities of the reagents.

MoAbs are also valuable tools in the study of cytokine receptors. They serve as an alternative approach over ligand binding experiments in investigating the receptor expression and distribution. As most of functional cytokine receptors are composed of multi-subunits receptors (reviewed by Miyajima *et al.*, 1992), the availabilities of MoAb against each of the receptor subunits provide a convenient way to detect the receptor subunits expressions and regulations. Up to now, MoAbs to EPOR (D'Andrea *et al.*, 1993), IL-2Rα,β (Olive *et al.*, 1986; Takeshita *et al.*, 1989; Kamio *et al.*, 1990), TNFR (Brockhaus *et al.*, 1990), and the gp130 protein (Wijdenes *et al.*, 1995; Chevalier *et al.*, 1996) have been reported.

MoAb to cytokine receptor also provide useful tools in the structural and functional studies of cytokine receptors. When MoAb binds to its epitope, which lies within the ligand and receptor interacting site, it may produce ligand-like activities, or it may also deform the ligand binding site and prevent the ligand from binding to its receptors, thus producing antagonistic effects. In addition, as most of the functional domains are predicted to occupy a small portion of the

whole receptor molecule, mapping the epitopes of the functional MoAbs may provide useful information to define these functional domains. Some neutralising MoAbs to the EPO receptor, TNF receptor and the gp130 of IL-6, IL-11, CNTF, LIF and OSM receptors are been used to study cytokine-receptor interactions(D'Andrea *et al.,* 1993; Brockhaus *et al.,* 1990; Wijdenes *et al.,* 1995; Chevalier *et al.,* 1996).

Monoclonal antibodies have played a significant role in medical diagnosis and treatment. For example, MoAbs to specific markers of human T and B cells have become routine diagnostic reagents for the cellular differentiation of acute leukarmias and lymphomas. Anti-IL-2R MoAb and chimeric IL-2 Diphtheeria-toxin have been applied for the treatment of rheumatoid arthritis (RA) (Strom *et al.,* 1993) and neutralising anti-IL-5 MoAb has been used for the control of Asthma (Mauser *et al.*, 1993; 1995). More significantly, the neutralising anti-TNF receptor MoAb has been applied successfully for the treatment of RA (Elliott *et al.,* 1993; 1994).

In this chapter, I commence this project by developing four groups of MoAbs directed to the human IL-3Rα, GM-CSFRα, IL-5Rα and the common β_c respectively.

RESULTS

3.1 Development of MoAbs to IL-3R α-chain

3.1.1 Hybridoma production

Balb/c mice were immunized 4 times with COS cell transfected with IL-3Rα cDNA and the splenocytes of one mouse were fused with NS-1 myeloma cell as described in chapter 2. Supernatants from antibody-producing hybridoma were screened on CHO F6 cells expressing high levels of IL-3Rα by the Rose-Bengal and/or FACS method, using CHO untransfected cell as a control. Three supernatants, named 6H6, 7G3 and 9F5, out of 1000 wells were found with antibodies that bound strongly to F6 cells but not CHO untransfected cells. These hybridoma cells were subcloned three times to ensure their monoclonality. These three MoAbs were then selected and used for further study.

3.1.2 Characterisation of anti-IL-3Rα MoAbs

We analysed the specificities of MoAbs 6H6, 7G3 and 9F5 on several experiments. First, I checked the MoAb staining on CHO F6 cells, COS transfectants and also on human primary cells such as neutrophils, monocytes, eosinophils and HUVEC. All the MoAb stained the above cells except fresh neutrophils. These results were in conformity with the IL-3 receptor distribution as measured by ^{125}I-IL-3 binding experiments. Figure 3-1 showed a example of cells stained with MoAb 7G3 by FACS analysis. Identical staining was also seen with 6H6 and 9F5 (data not shown). In order to confirm biochemically the identity of the antigen recognised by MoAb 6H6, 7G3 and 9F5 as the IL-3Rα-chain, immunoprecipitation and Western blotting analysis were performed. All MoAbs specifically immunoprecipitated a protein of molecular weight of 70,000 Da from ^{125}I surfaced-labelled F6 cells (figure 3-2A), COS transfectants (data not shown) and TF-1 cells (data not shown). In Western blotting analysis, a similar size protein was detected from F6 cell extracts by MoAb 6H6, 7G3 and 9F5 (figure 3-2B). An irrelevant MoAb failed to recognise any specific proteins in

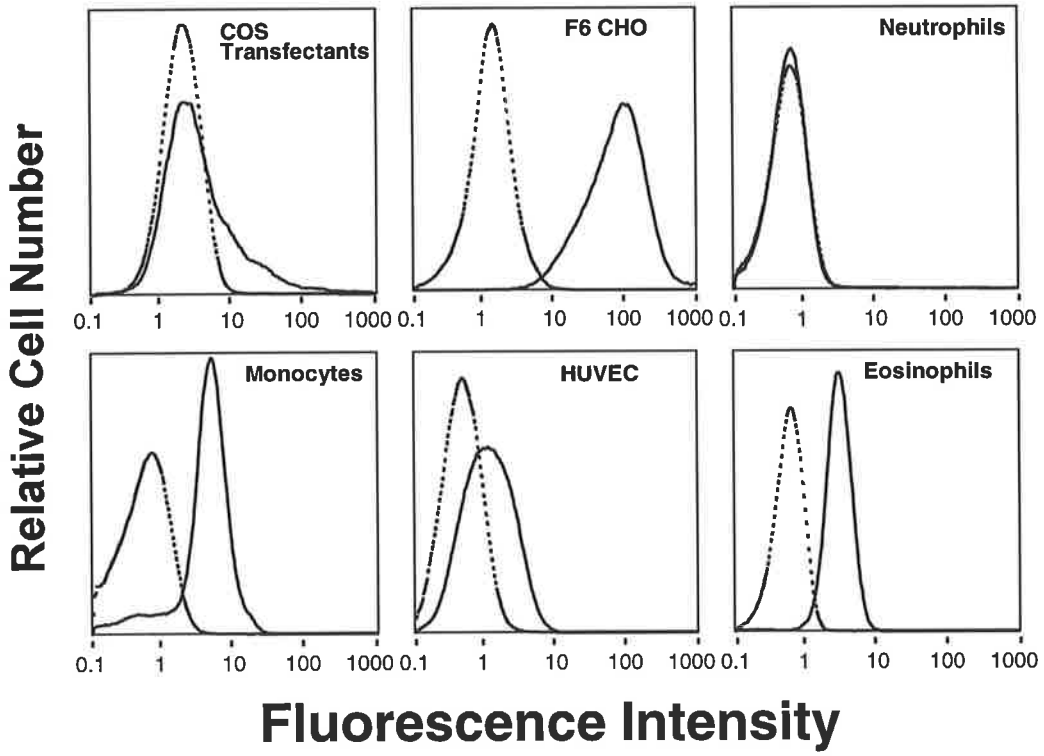


Figure 3-1 FACS analysis of staining of MoAb 7G3 (—) and the control MoAb (---) to COS cells transfected with IL-3R α-chain, CHO F6 cells, neutrophils, monocytes, HUVEC, and eosinophils.

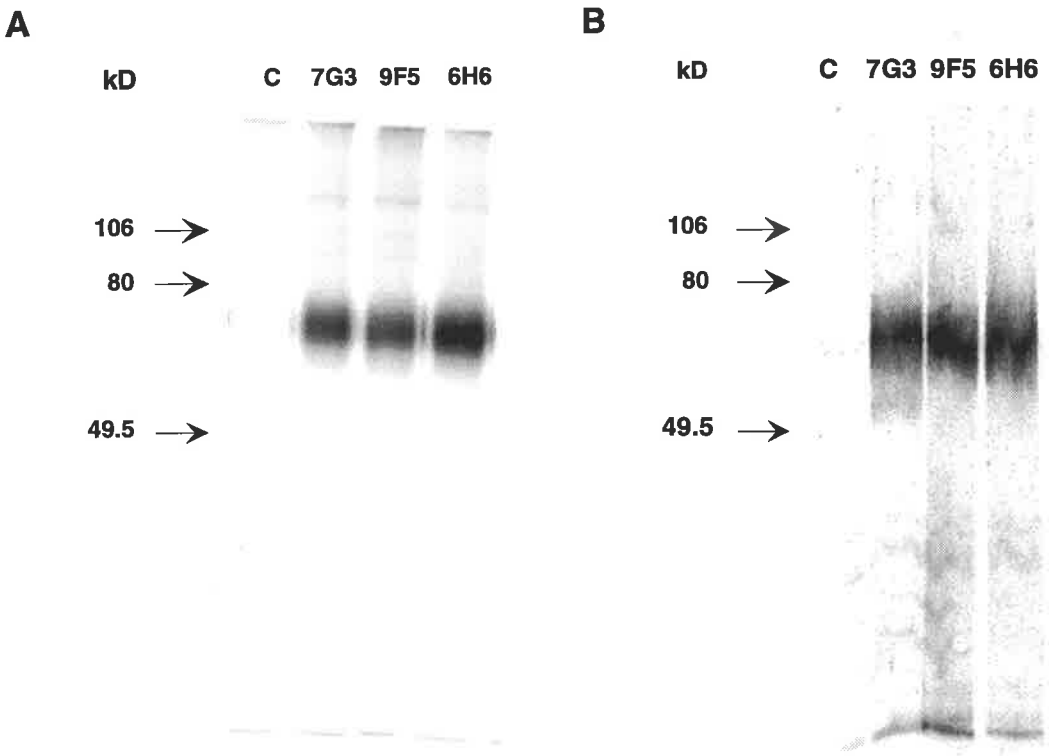


Figure 3-2 MoAbs 7G3, 6H6 and 9F5 recognise the human IL-3R α-chain. (A) immunoprecipitation of IL-3R α-chain from ^{125}I-surface-labelled F6 cells and (B) Western blot of F6 cell lysate. Both analyses were performed on 10% SDS-PAGE under reducing conditions.

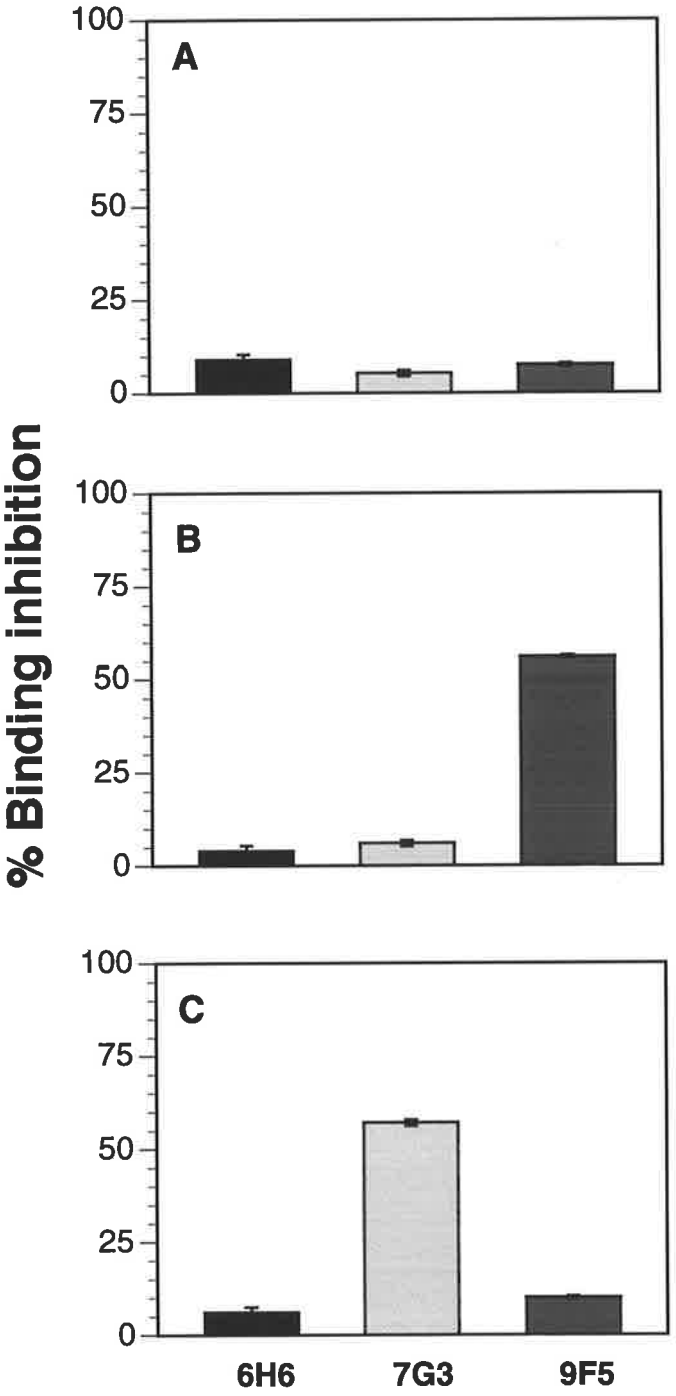


Figure 3-3 Inhibition of the binding of MoAb ^{125}I-6H6 (A), ^{125}I-7G3 (B) and ^{125}I-9F5 (C) to CHO F6 cells expressing IL-3R α-chain by unlabelled anti-IL-3Rα MoAbs.

both immunoprecipitation and Western blotting. The above data suggested that the three MoAbs could specifically recognise the native and the denatured forms of the IL-3 R α-chain. MoAb 7G3 was typed as mouse IgG2a while 6H6 and 9F5 were both IgG1.

I also examined whether these MoAbs recognised unique or overlapping epitopes. This was done by experiments using radioiodinated MoAb. The specific binding of antibody to F6 cells was determined in the presence of 200 fold excess of the same unlabelled antibody. Antibodies detecting the same or overlapping epitopes on IL-3 R α-chain were shown by their capacity to inhibit radioiodinated MoAb binding to F6 cell in the presence of the same excess concentration as that of its own unlabelled forms. As shown in figure 3-3, MoAb 6H6 could completely inhibit the binding of 7G3 and 9F5, while 7G3 and 9F5 could only inhibit half of each other's binding. These data suggested that these three MoAbs bind to three different epitopes on the IL-3 R α-chain. The epitope of 6H6 is so closed to that of 7G3 and 9F5 that it could deplete the binding of 7G3 and 9F5. But the epitopes of 7G3 and 9F5 are distinctive but still closed, so they could only inhibit part of each other's binding.

I have also tested this group of MoAbs on their abilities to interfere with IL-3 binding and IL-3 mediated functions. These data are shown in Chapter V.

3.2 Development of MoAbs to GM-CSFR α-chain

3.2.1 Hybridoma production

To produce MoAbs against human GM-CSFR α-chain, a BALB/c mouse was immunized four times with COS cell transfected with GM-CSFR α-chain cDNA and a GM-CSFR α-chain fusion protein. The mouse spleenocytes were then fused with NS-1 cells as described in Chapter II. Supernatants from hybridomas were screened on A9/C7 cells by Rose-Bengal, by FACS, or by ELISA using the GM-

CSFR α-chain fusion protein as the coating layer. In one fusion, seven MoAbs were derived. These seven MoAbs were selected for further characterisation.

3.2.2 Characterisation of anti-GM-CSF R α-chain MoAbs

I investigated the specificity of the anti-GM-CSFRα MoAbs on a series of experiments. All the MoAbs were classified as mouse IgG MoAbs. In immunofluorescence assays analysed with flow cytometer using cell lines and human primary cells, all the MoAbs except 4C11 and 8D10 could stain with COS cells transfected with GM-CSFRα cDNA, A9/C7 CHO cells, HUVECs, neutrophils, monocytes and eosinophils. These cells have been confirmed by GM-CSF binding experiments to express GM-CSF receptors. Figure 3-4 gives an example of staining of MoAb 8G6 on the cells just described above. None of them could stain with untransfected COS or CHO cells (data not shown). In order to find out the molecular species identified by this group of anti-GM-CSF R α-chain MoAbs, immunoprecipitation and Western blotting analysis were also performed using A9/C7 CHO cells expressing GM-CSFR α-chain. As shown in figure3-5, MoAb 4A5, 4H1, 6E10, 8E3, 8G6 could specifically recognise a protein of 80 kDa in immunoprecipitation but 4C11 and 8D10 save a negative or very weak reaction. All MoAb except 4A5 could recognise GM-CSFRα in immunoblotting analysis. No specific band was found with CHO parent cells (data not shown). These data suggested that this group of MoAb specifically recognised the human GM-CSFR α-chain. Some of them, 4A5, 4H1, 6E10, 8E3 and 8G6, are able to bind both the native and denatured forms of GM-CSF R α-chain, while 4C11 and 8D10 recognise predominantly the denatured form of GM-CSF R α-chain.

These MoAbs were also tested on ^{125}I-GM-CSF binding assay and GM-CSF stimulated TF-1 cell proliferation. None of them exhibited any effect on GM-CSF binding and TF-I cell proliferation (data not shown), suggesting that the epitopes of these MoAbs were not at the binding sites of GM-CSF on their GM-CSF R α-chain.

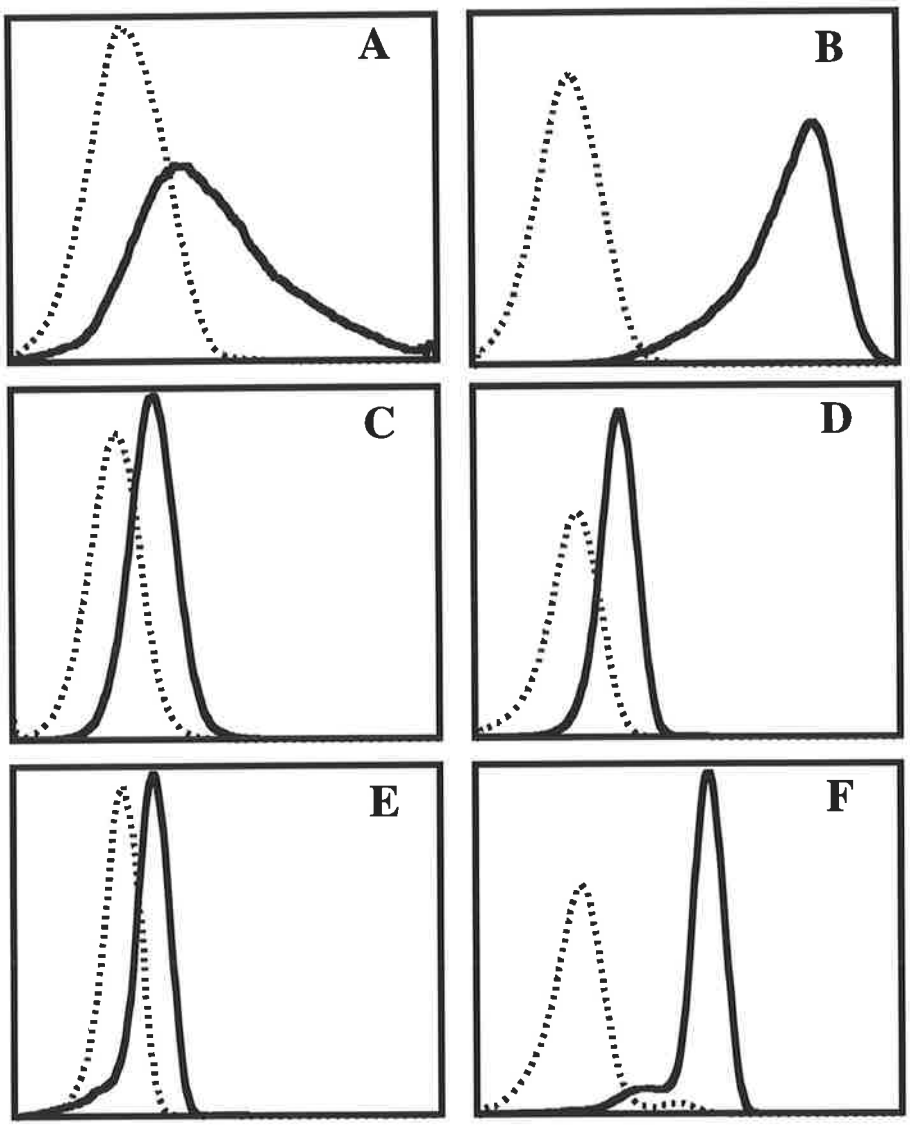
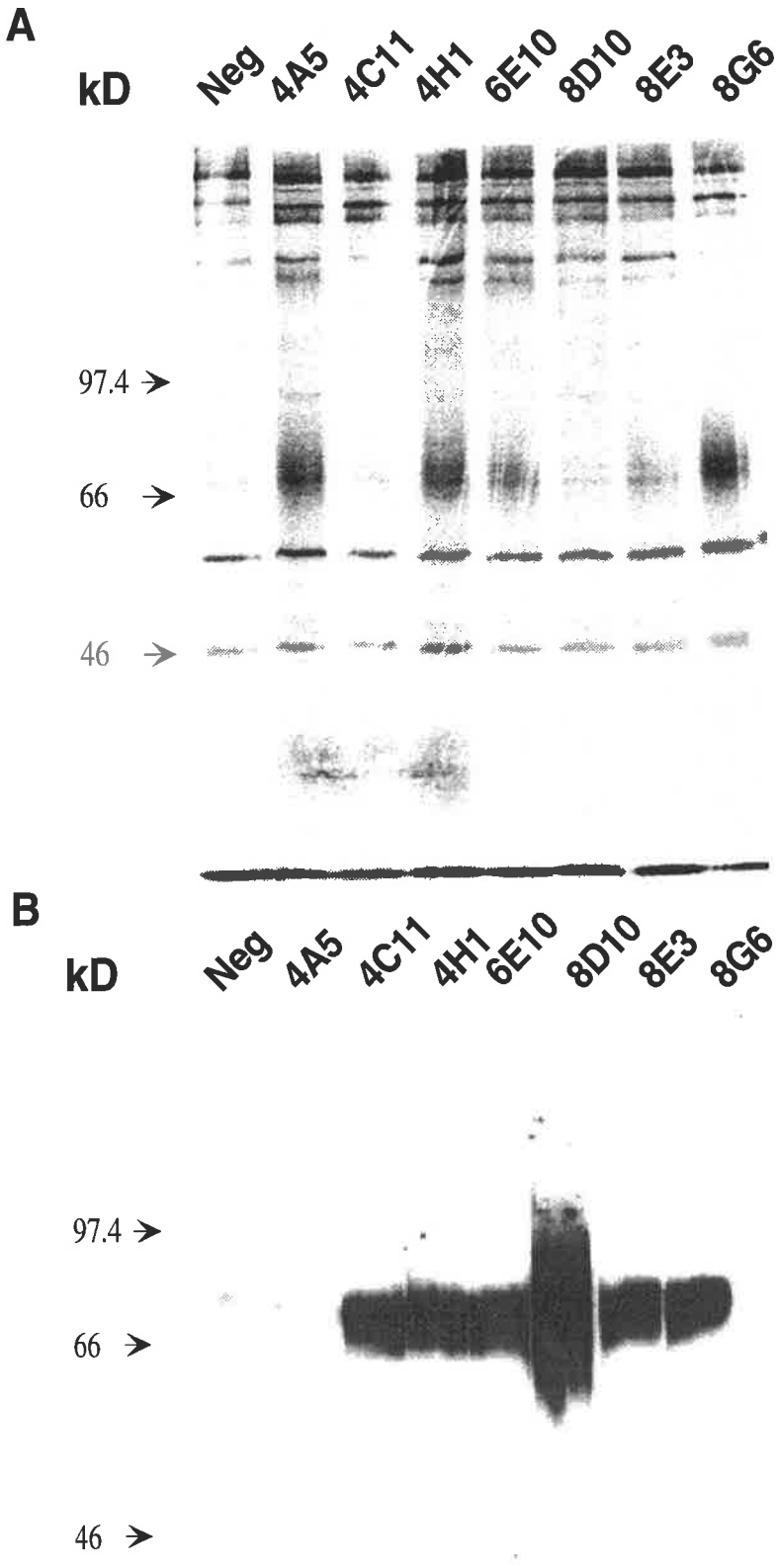


Figure 3-4 FACS analysis of the staining of MoAb 8G6(—) and a negative control MoAb (---) to (A) COS transfected with GM-CSFR α-chain, (B) CHO A9/C7 cells, (C) TF-1 cells, (D) Neutrophils, (E) Eosinophils and (F) Monocytes

Figure 3-5 MoAb 4A5, 4C11, 4H1, 6E10, 8D10, 8E3 and 8G6 recognise the human GM-CSFR α-chain. (A) immunoprecipitation of GM-CSFR α-chain from ^{125}I-surface labelled CHO A9/C7 cells and (B) Western blot of A9/C7 cell lysate. Both experiments were performed on 10% SDS-PAGE under reducing conditions.


A
kD
Neg
4A5
4C11
4H1
6E10
8D10
8E3
8G6
97.4
66
46
B
kD
Neg
4A5
4C11
4H1
6E10
8D10
8E3
8G6
97.4
66
46

3.3 Development of MoAb to IL-5R α-chain

3.3.1 Hybridoma Production

To produce MoAb against human IL-5 R α-chain, we chose the A8 CHO cell line which expresses high levels of IL-5R α-chain as an immunogen to immunise Balb/c mice. Spleenocytes from mice that had been immunized four times were then fused with NS-1 cell as described in chapter II. Supernatants from hybridoma cells in 96-well plates were screened by flow cytometry for their staining on FDCP1 cells that express IL-5 receptor. In three fusions in which a total of more than 3000 wells were analysed, only one supernatant, named A14, was found to have antibody that recognised the FDCP1 cells. This hybridoma was subcloned and MoAb purified from the supernatant as well as antibody-containing ascites.

3.3.2 Characterisation of anti-IL-5R α-chain MoAb

A14 is a mouse IgG_1 MoAb. It stained with COS cells transfected with IL-5 R α-chain cDNA, A8 CHO cells, FDCP1 cells, TF1.8 cells, eosinophils and monocytes detected by immunofluorescence analysis by flow cytometry (figure 3-6). It does not stain neutrophils (figure 3-6) nor untransfected COS, CHO, CTLL cells (data not shown). In immunoprecipitation experiment, I analysed the cell surface molecules identified by A14 on A8 CHO cells. As shown in figure 3-7, A14 could specifically immunoprecipitate a 50-55 kDa protein from ^{125}I-surface-labelled A8 CHO cell lysates, but not the CHO cell lysates (data not shown). But in Western blotting analysis, A14 failed to detect any specific protein from A8 CHO cell extracts (data not shown). These results show that MoAb A14 is specifically against human IL-5 R α-chain, and it recognises only the native form of IL-5R α-chain.

We also performed IL-5 binding assays and IL-5 stimulated proliferation assay in the presence of MoAb A14 using COS cell tranfected with IL-5 high affinity

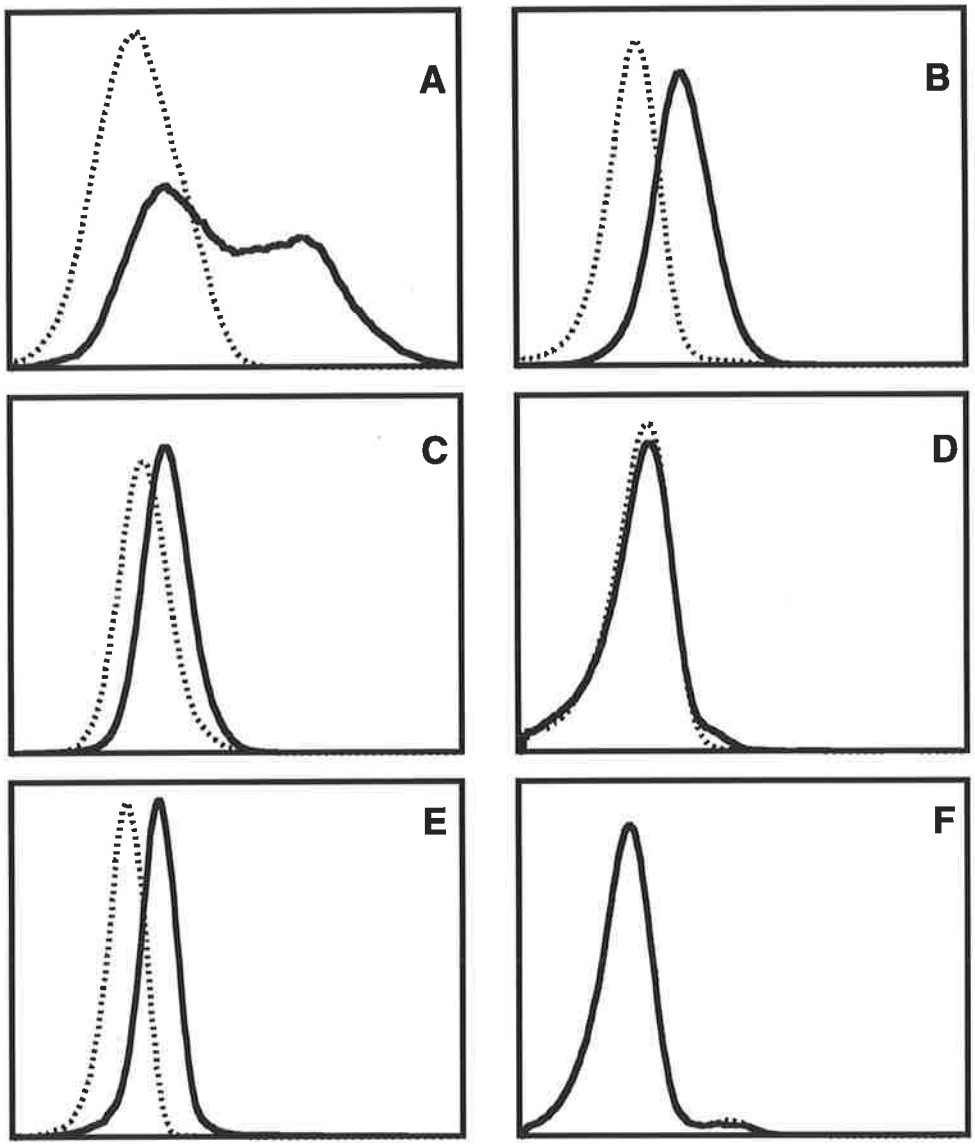
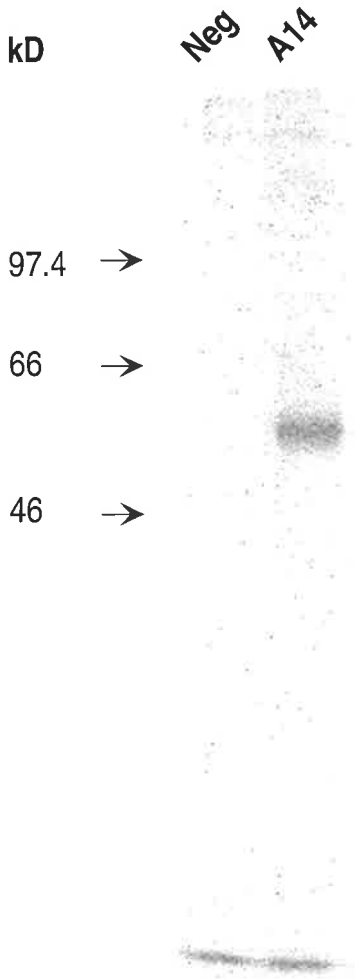


Figure 3-6 FACS analysis of the staining of MoAb A14(—) and a negative control MoAb (---) to (A) COS cells transfected with IL-5R α-chain, (B) FDCP1 cells, (C) TF-1-8 cells, (D) Neutrophils, (E) Eosinophils and (F) Monocytes

Figure 3-7 MoAb A14 recognises the human IL-5R α-chain in immunoprecipitation using ^{125}I-surface labelled CHO A8 cells expressing IL-5R α-chain. This experiment was performed on 10% SDS-PAGE under reducing condition.


kD
Neg
A14
97.4
66
46

receptor and FDCP1 cells respectively. In both experiments, MoAb A14 failed to show any inhibitory effect on IL-5 binding and function (data not shown).

3.4 Development of MoAbs to Common β_c

3.4.1 Hybridoma Production

To generate MoAbs to the β-chain, we immunized Balb/c mice using two types of cell lines. Some mice were immunized with COS β cells expressing wild type β_c; others with COS ΔQP cells expressing domain 4 of β_c. All the mice were immunized at least 4 times before fusion was conducted by fusing the spleenocytes with NS-1 cells. Four fusions were performed with spleenocytes from mice that were immunized with COS cell expressing wild type β_c. These hybridomas were screened for staining of CHOβ cells by means of the Rose-Bengal method and/or by immunofluorescence analysed by flow cytometry. More than 20 MoAb to the β_c were generated, 2 of them, 1C1 and 8B8, were chosen for this study. Another fusion was carried out using spleenocytes from a mouse immunized with ΔQP COS cell expressing domain 4 of β_c. These hybridoma supernatants were screened on ΔQP CHO cells expressing the domain 4 of β_c. Two MoAbs, named QP1 and QP2 were found to stain with ΔQP CHO cells but not the CHO parent cells. MoAb QP1 was chosen for further study along with 1C1 and 8B8.

3.4.2 Characterisation of anti-β_c MoAbs

We analysed the specificities of the MoAbs on a series of experiments. QP1, 1C1 and 8B8 are mouse IgG1 MoAbs. In immunofluorescence experiment, all three MoAbs could stain COS cells transfected with β_c cDNA, CHOβ_c cells, TF-1 cells, TF-1.8 cells, neutrophils, monocytes and eosinophils as analysed by flow cytometry. These cells have been confirmed by ligand binding assay to express the β_c as they all demonstrated high affinity ligand binding. Figure 3-8 gives the examples of flow cytometry analysis of cells stained with MoAb QP1. All the other anti-β_c MoAbs stain cells with very similar fluorescence intensities (data

not shown). None of the MoAbs could stain with COS nor CHO parent cells (data not shown). To further confirm the specificity of the MoAbs, an immunoprecipitating and immuno-blotting analysis were also performed. All three MoAbs could specifically precipitate a protein of around 120 kDa from the cell extracts of CHOβ cells (figure 3-9A) and TF-1 cells (data not shown), but not from CHO parent cells (data not shown). All three MoAbs could also recognise a protein of similar size to that seen in the immunoprecipitation and Western blot analysis using CHOβ_c cells (figure 3-9B). The molecular weight of the proteins recognised by the MoAb is consistent with the size of β_c as previously described (Kitamura *et al.*, 1991).

In addition, MoAb QP1 but not other anti-β_c MoAbs, can also recognise the ΔQP β_c mutant which only expresses the domain 4 of β_c in the extracellular region. It can detect ΔQP mutant expressed on the cell surface by immunofluorescence (data not shown) and also recognise ΔQP mutant from cell lysates by immunoprecipitation (figure 3-10). This indicates that the epitope of MoAb QP1 is conserved both in WT and ΔQP mutant β_c and the epitope is in domain 4 of β_c.

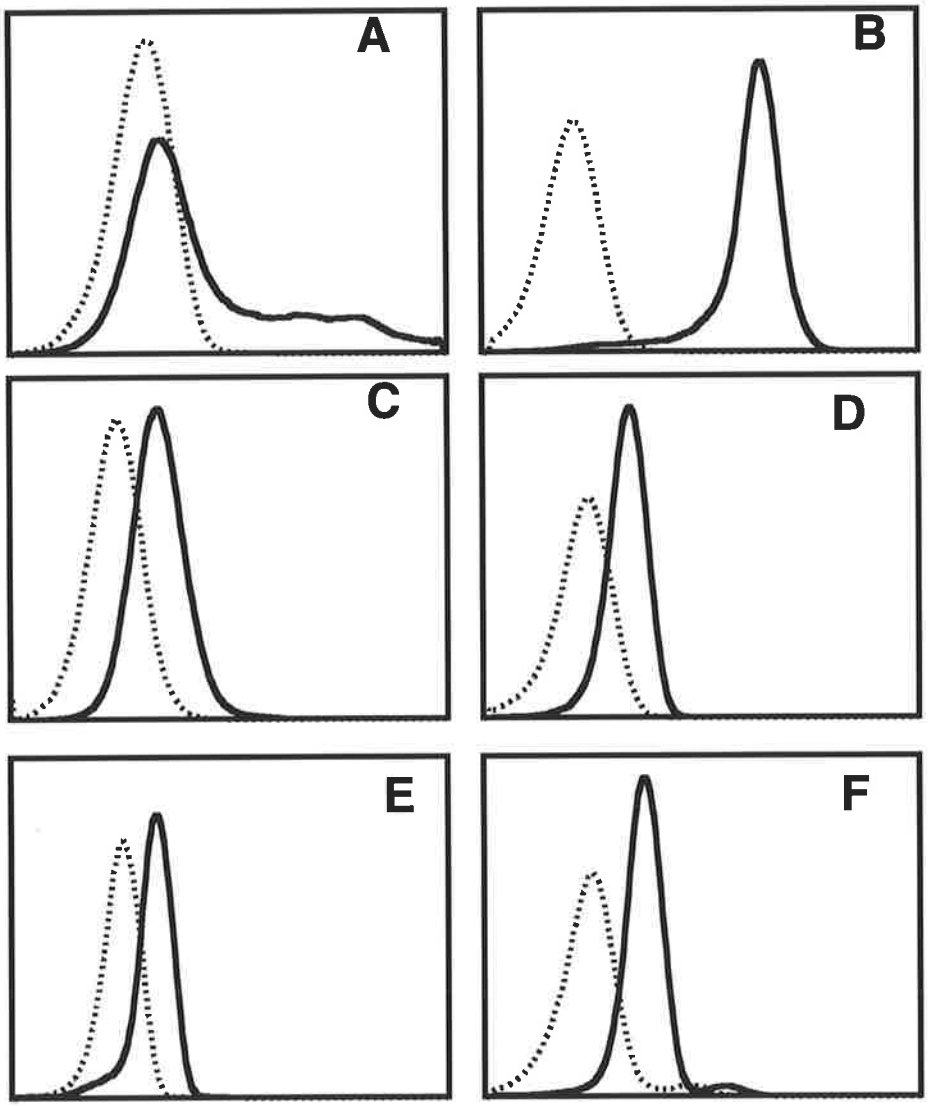


Figure 3-8 FACS analysis of the staining of MoAb QP1(—) and the control MoAb (---) to (A) COS cells transfected with β-chain, (B) CHO β–chain cells, (C) TF-1 cells, (D) Neutrophils, (E) Eosinophils and (F) Monocytes.

Figure 3-9 MoAb QP1, 1C1 and 8B8 recognise the human β-chain. (A) immunoprecipitation of β-chain from ^{125}I-surface labelled CHO β cells and (B) Western blot of CHO β cell lysate. Both experiments were performed on 7.5% SDS-PAGE under reducing conditions.

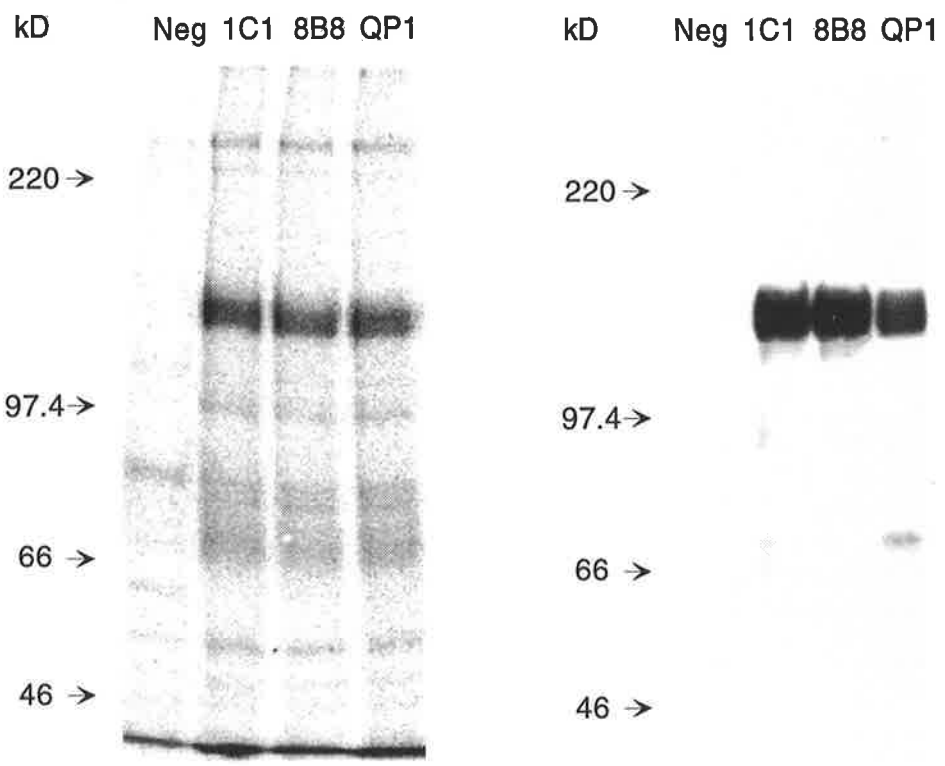
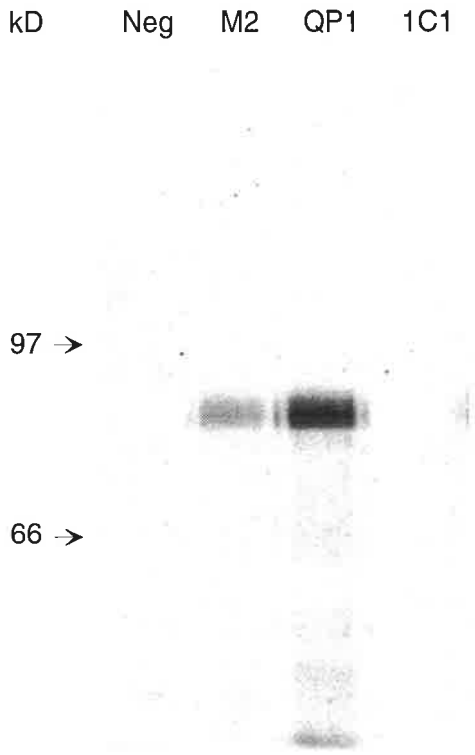

kD
Neg 1C1 8B8 QP1
220
97.4
66
46
kD
Neg 1C1 8B8 QP1
220
97.4
66
46

Figure 3-10. MoAb QP1, but not another anti-βc MoAb 1C1 , recognises β-chains mutants (βc-ΔQP) that contains only domain 4 in the extracellular regions by immunoprecipitation of CHO ΔQP cells This mutant has a flag attached so it can also be seen by anti-flag MoAb M2. The experiment was performed on 10% SDS-PAGE under reducing conditions.


kD
Neg
M2
QP1
1C1
97
66

DISCUSSION

In this chapter, I have prepared four groups of MoAbs against the human IL-3R, the GM-CSFR and the IL-5R α-chain and the common β_c respectively. These MoAbs provide very useful tools for the study of the receptors for IL-3, GM-CSF and IL-5.

Traditional investigations on cytokine receptors were mainly carried out by radio-labelled ligand binding studies. However, the limitations of ligand binding assays are several. First, they need large quantity of highly purified cells, thus many cells that are hard to be purified or to be obtained in large quantity cannot be used in these binding tests. Second, they are quite difficult to analyse the relative expression of the subunits of each receptor. Third, they cannot be used to examine in isolation the expression of subunits which do not bind ligand on their own such as β_c. Fourth, they only detect the cell surface receptor while the expression of cytokine receptor in the cytoplasm cannot be tested. Fifth, the binding experiment is a complicated one that involves radioisotopes and special equipment.

The development of monoclonal antibodies to the cytokine receptor chains provides not so much an alternative way to analyse receptor expression and distribution, but it complements binding studies using radiolabelled cytokines. With the availability of MoAb we have investigated receptor subunit expression (Sun *et al.*, 1996), regulation (Smith *et al.*, 1995) and dimerization (Stomski *et al.*, 1996). Receptor expression within the cytoplasm may also be studied if the tissue is properly fixed before applying the MoAbs (Iversen PO, personal communication).

I first described three MoAbs that specifically recognise the low-affinity α-chain of the human IL-3 receptor. All three MoAbs could specifically recognise the IL-3Rα both in its native and denatured forms. We also confirmed that the three

MoAbs define three different but adjacent epitopes on the IL-3R α-chain. It seems that all the three epitopes lie on the *N*-terminal domain. The epitope mapping and function of MoAb 7G3 are discussed in detail in chapter IV. Compared with a single MoAb to IL-3 R α-chain reported previously (Sato *et al.*, 1993), our MoAbs demonstrate similar reactivity on immunofluorescence and immunoprecipitation, but have novel properties as addressed in chapter V..

The second group of MoAb comprised 7 MoAbs to the low affinity α-chain of the human GM-CSFR. These MoAb may be divided into three groups on the basis of their reactivities. Group I MoAbs (4A5, 4C11, 6E10 and 8E3) recognise the GM-CSFR α-chain expressed on transfected cells as well as on primary cells. They could immunoprecipitate GM-CSFR α-chain from cell extracts. Most of them (except 4A5) also recognise GM-CSFR α-chain in immunoblotting. Group II MoAbs (4H1, 8G6) are more versatile in detecting the GM-CSFRα. They could recognise GM-CSFR on immunofluorescence, immunoprecipitation and Western blotting analysis. They can also stain GM-CSFR α-chain fusion protein. This indicates that the epitopes of 4H1 and 8G6 are biologically simillar. On the other hand, the third group of MoAb (4C11 and 8D10) demonstrated major reactivity only with denatured form of GM-CSFR α-chain on the immunoblotting analysis. It may suggest that these MoAb recognise a linear epitope. When the protein is folded in its native form, the epitope is not preserved. Unfortunately, none of the epitopes to which the anti-GM-CSFRα MoAb bound appear to represent GM-CSF binding sites, as the MoAbs failed to show any inhibition on GM-CSF binding and function. Anti-GM-CSFRα MoAbs have previously been reported (Nicola *et al.*, 1993), one of which has neutralising activties.

Despite extensive work on the IL-5R α-chain I have been able to develop only a single MoAb to the IL-5R α-chain. This MoAb specifically recognises IL-5R α-chain in immunofluorescence and immunoprecipitation assays. It cannot bind to the IL-5R α-chain on Western blotting analysis, suggesting that its epitope is damaged when the protein is denatured. Using this MoAb, we investigated the

distribution and expression of IL-5R α-chain on different cells. Among them, eosinophils were found to have relatively high level of IL-5R α-chain expression. This MoAb has proven to a very useful tool in detecting the IL-5R α-chain.

The β_c, which is shared by the IL-3, GM-CSF and IL-5 receptors, is critical for signal transduction. Co-expression of both the α-chain and β_c is essential for receptor high affinity binding and activation. I also created a group of MoAbs to the common β-chain. This group of MoAbs specifically recognised the wild type β_c in all the experiments tested such as the immunofluorescence, immunoprecipitation and Western blott analysis. In addition, one of them, MoAbs QP1 can also recognise a mutant β_c which only has the domain 4 in the extracellular region. I investigated these anti-β_c MoAbs on their abilities to interfere with ligand binding and ligand mediated functions and these data will be discussed in detail in Chapters VI. Although three rat anti-β_c MoAbs had been reported previously (Watanabe *et al.*, 1992), they are not very good for immunoprecipitation and Western blotting analysis. This group of MoAb represent virtually a unique set of tools with which to study the expression, regulation, structure and function of β_c. In particular, MoAb QP1 represent the first blocking antibody to β_c with important implication for identifying the binding site in β_c and perhaps for therapeutic use.

CHAPTER IV

EXPRESSION AND REGULATION OF GM-CSF, IL-3 AND IL-5 RECEPTORS

GM-CSF, IL-3 and IL-5 function through interaction and activation of their specific membrane bound receptors: GM-CSFR, IL-3R and IL-5R. Each of these receptors consists of a ligand-specific α-chain and a common β chain. Obviously, the expression of these receptors on the cell surface is a prerequisite for the biological response to these cytokines. So the understanding of the profile of receptor expression, distribution and regulation would give much indication of the biological roles of these cytokines *in vivo*.

GM-CSF, IL-3 and IL-5 are important regulators for both haemopoiesis and inflammation. Their receptors have been shown to express on many cell types of the hemopoietic system. For example, human neutrophils express GM-CSFR (Lopez *et al.,* 1988), while monocytes have both GM-CSFR and IL-3R (Elliott *et al.,* 1989) and eosinophils have all three receptors for GM-CSF, IL-3 and IL-5 (Lopez *et al.*, 1989; reviewed by Lopez *et al.,* 1992). On human hemopoietic progenitors, Testa et al. (1993) reported that high affinity IL-3R and GM-CSFR were detected on purified CD34+ CD33- cells by ligand binding assays. In addition, these cytokine receptors were also extensively expressed during abnormal hemopoiesis. For instance, GM-CSF or IL-3 have been shown to bind some leukaemic cells and stimulate their growth *in vitro* (Brizzi *et al.,* 1990).

Although the expression of cytokine receptors has been studied extensively over the past few years, the overall pattern of expression of GM-CSF, IL-3 and IL-5 receptor in the human system still needs further investigation. The examination of expressions of certain haemopoietic cells has been hindered because of the lack of purification techniques for these cells and of specific antibodies. Haemopoietic progenitors such as CD34+ cells were shown recently to express IL-3 and GM-CSF receptor α-chain, but very limited β_c (Sato *et al*, 1993; Sun *et al*, 1996, our unpublished observation). Moreover, the expression of IL-3

receptor has been confirmed on cells other than those of hemopoietic origins, such as HUVECs (Korpelainen *et al.,* 1993; 1995).

The expression of cytokine receptors on the cell surface seems to be a dynamic process rather than a static one (Korpelainen *et al.,* 1993; 1995; Smith *et al.,* 1995). In haemopoiesis, cell differentiation *in vivo* are usually regulated by a network of cytokines. The interaction among these cytokines results in the sequential expression of cytokine receptors. That means a cascade transactivation of cytokine receptors during differentiation (Testa *et al.*, 1992) in which the activation of one cytokine receptor by certain cytokine favours the expression of receptors of other cytokines. This process amplifys signal of cytokines by initiating cell response to several other cytokines. In inflammation, it was found that IL-3R lost its expression during differentiation (Lopez *et al.*, 1988), but IL-3R can be re-expressed if neutraphil were stimulated (Smith *et al.*, 1995). The investigation of receptor regulation may help to understand the functional complexity of cytokines in the regulation of haemopoiesis and inflammation.

In this chapter, I examine the expression patterns of the receptor for GM-CSF, IL-3 and IL-5 on both hemopoietic and non-hemopoietic cells using the antibodies developed in chapter III. More importantly, the regulation of these cytokine receptors on human neutrophils and HUVECs was also documented.

4.1 Expression of receptor subunits on CD34+ cells

CD34+ cells were purified from bone marrow mononuclear cells with an anti-CD34 antibody coupled to the magnetic beads and their purity was more than 95%. These cells were then stained with anti-receptor MoAb to the α and β-chains. The receptor expression was analysed with a flow cytometer. The profile obtained from the flow cytometry analysis on receptor expression was summarised in figures 4-1 and 4-2. In these figures, all CD34+ cells expressed IL-3R α-chain and GM-CSF α-chains, but the expression of IL-3R α-chain is much higher than GM-CSFR α-chain. In contrast, the expression of β_c on CD34 is very poor as immunofluorescence only increased slightly when cells were stained with anti-β_c MoAbs. The anti-IL-5Rα MoAb stain was at the same level of the control MoAb indicating that there is no detectable IL-5R expression on these CD34+ cells (data not shown).

Interestingly, although all the CD34+ cell have IL-3R and GM-CSFR α-chains, the distribution of these α-chains was not followed the role of a Normal Distribution. A portion of the CD34+ cells (1/10) express relatively high levels of IL-3R α-chain (figure 4-3) and GM-CSFR α-chain (figure 4-3) compared with the majority of CD34 cells with lower expression. These experiments were repeated at least three times and the profiles are very consistent. I have not analysed the phenotypes of these high expressing cells yet, but from clone-forming studies the IL-3R and GM-CSFR α-chains high CD34+ population seems more mature (data not shown in this thesis). These data correlate very well with the finding of Kurate *et al.* (1995) about the cytokine receptor distribution on CD34+ cells.

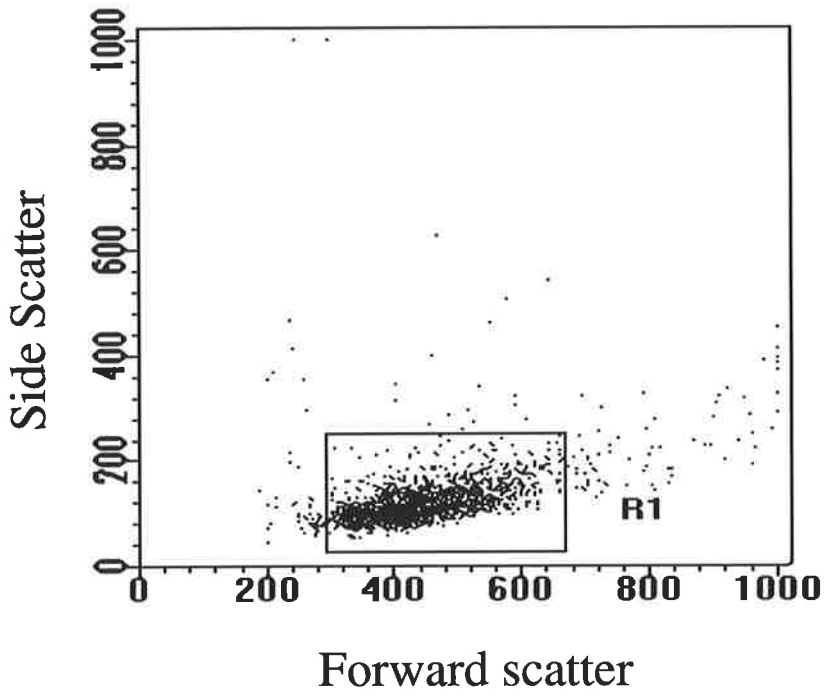


Figure 4-1. The analysis window of CD34+ cells on flow cytometer

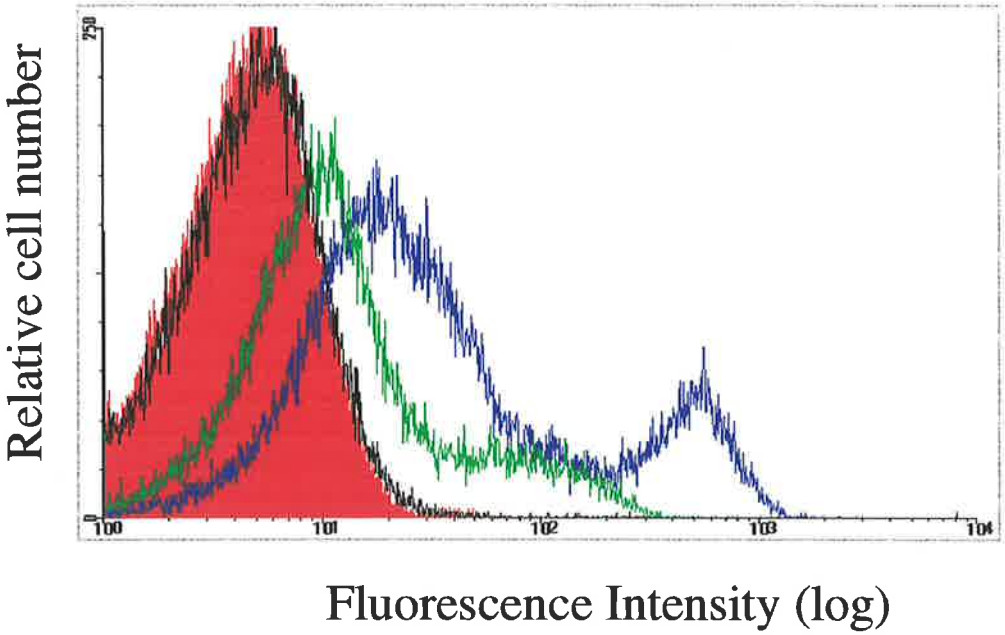


Figure 4-2. Expression of IL-3R and GM-CSFR α-chains and the common β-chain on CD34+ cells. Flow cytometry analysis of CD34+ cells stained with anti-IL-3Rα MoAb 7G3 (blue), anti-GM-CSFRα 8G6(green), anti-βc MoAb 1C1(black) and a negative control MoAb 3D3(red).

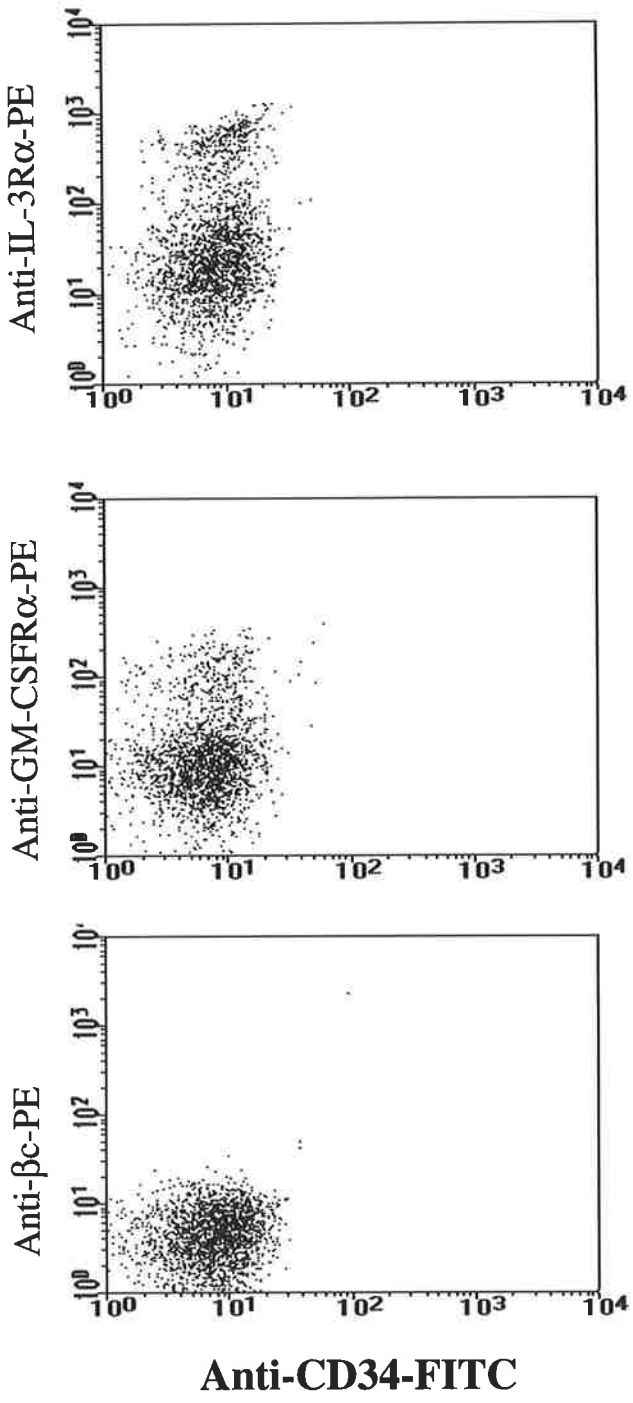


Figure 4-3. Two-color folw cytometry analysis of CD34+ cells by anti-IL-3Rα (7G3), anti-GM-CSFRα (8G6) and anti-β chains (1C1) MoAbs, in the presence of anti-CD34-FITC.

4.2 Receptor Subunits Expression on Monocytes, Eosinophils and Basophils

Using these MoAbs developed in chapter III, the expression of α and β chain on the haemopoietic mature cell population was analysed by flow cytometer. On monocytes (figure 4-4A), GM-CSFR and IL-3R α-chains and the β-chain were expressed but not the IL-5R α-chain. Surprisingly, the GM-CSFR α-chain have very high level of expression on monocytes compared with that of IL-3R α-chain and the β-chain. This finding may help to explain the data obtained from the GM-CSF and IL-3 cross-competition binding assays on monocytes (Elliott *et al.*, 1992). In that study they found that GM-CSF could compete very well for the binding of IL-3 but IL-3 competed very poorly to the binding of GM-CSF. That phenomenon was only detected on monocytes but not on eosinophils. On purified eosinophils (figure 4-4B), all three α- and the β- chains were expressed on the cell surface and level of β-chain expression was very similar to that of the α-chains. This feature of expression may explain the direct cross-binding competition by GM-CSF, IL-3 and IL-5 on eosinophils (reviewed by Lopez *et al.*, 1992). In another experiment, when human bone marrow or peripheral blood mononuclear cells were stained with anti-IL-3Rα MoAb, but not the other MoAbs, there is a very distinctive small portion of cells that expressed very high level of IL-3R α-chain but not the other receptor chains. When this samll cell population was purified by cell-sorting, it was found to consist of 98% basophils as judged by their morphological characteristics. Figure 4-5 showed the flow cytometer profiles of bone marrow mononuclear cells analysed by normal window and figure 4-6 illustrated the dual-colour analysis with an anti-CD34 MoAb and anti-IL-3Rα MoAb 6H6 or anti-GM-CSFRα MoAb 8G6. More interestingly, the basophils also express a lower level of CD34 antigen (figure 4-6).

4.3 Expression and Regulation of Receptor Subunits on Neutrophils

Human neutrophils were analysed by the MoAbs to IL-3R, GM-CSFR and IL-5R α-chains and the β-chain. Freshly purified neutrophils were found to express only GM-CSFR α-chain and β-chain but not IL-3R and IL-5R α-chain, as

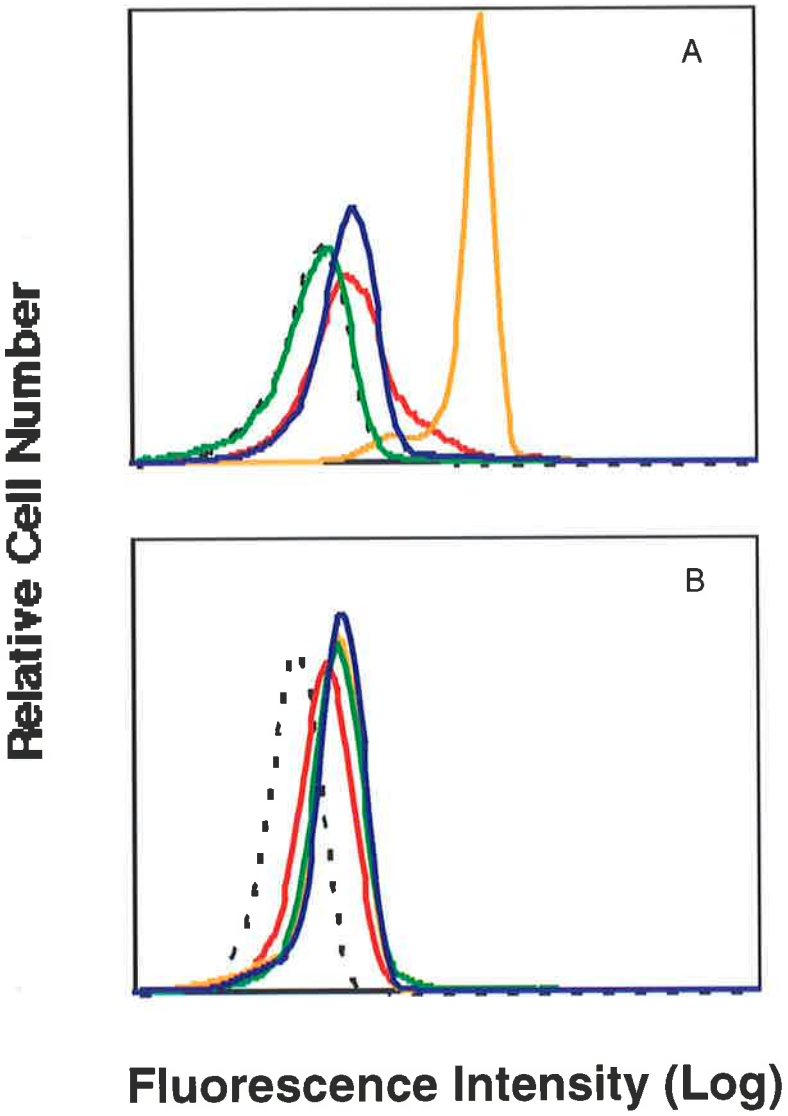


Figure 4-4 FACS analysis cytokine receptor expressions on human monocytes (A) and eosinophils (B) detected by anti-IL-3Rα (—), anti-GM-CSFRα (—), anti-IL-5Rα (—), anti-β (—) chain MoAbs and the control MoAb (---).

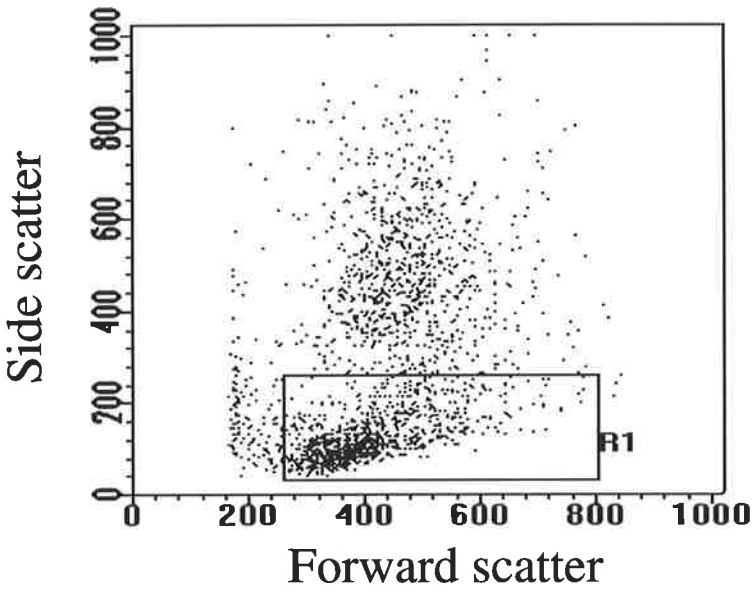


Figure 4-5. The analysis window of bone marrow mononuclear cells on flow cytometer

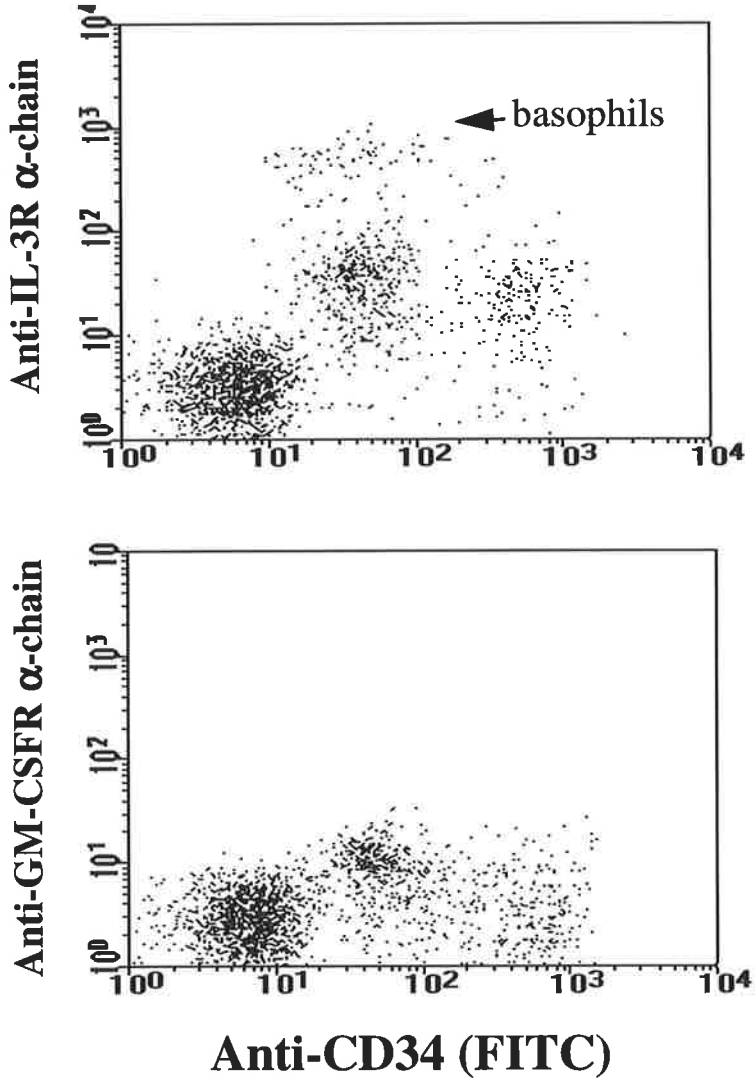


Figure 4-6. Two-color flow cytometry analysis of bone marrow mononuclear cells stained with anti-IL-3Rα MoAb 7G3 or anti-GM-CSFRα MoAb 8G6, in the presence of anti-CD34-FITC

previously predicted by binding assays (Lopez *et al.*, 1988). However, when neutrophils were stimulated by GM-CSF, but not by medium alone for 2 hours or overnight, they began to express relatively high levels of IL-3R α chain on the cell surface (figure 4-7). This GM-CSF-induced IL-3R α-chain was seen in 5 samples from different donors (figure 4-8). Binding studies with radio-labelled IL-3 confirmed that the GM-CSF stimulated neutrophils demonstrated both high and low affinity IL-3 binding sites (figure 4-9). These data indicated that the up-regulation of IL-3R α-chain in neutrophils also can lead to its association with β_c thus forming high affinity ligand binding sites. In other experiments we also found that IL-3 could induce function on GM-CSF stimulated neutrophils such as the up-regulation of HLA class II expression. IL-3 failed to show any class II antigen up-regulation on the freshly purified neutrophils or these stimulated with medium alone (Smith *et al.*, 1995). The ability of GM-CSF to induce expression of IL-3R α-chain on the neutrophil appears to be unique as other neutrophil-activating cytokines such IFN-γ, TNF-α and IL-1 did not induce any detectable expression of this molecule (data not shown).

4.4 Expression and regulation of receptor subunit on HUVEC

To investigate the receptor expression on HUVEC, cells were stained with anti-IL-3Rα MoAb 7G3, anti-GM-CSFRα MoAb 8G6, anti-β_c MoAb 1C1 and a control MoAb, and analysed by flow cytometry. The FACS profiles shown in figure 4-10 represent the fluorescence intensity of HUVEC stained with anti-receptor MoAbs. In that figure MoAb 7G3 and MoAb 1C1 give a strong staining. But MoAb 8G6 gives the same staining as the control MoAb. These data indicate that HUVEC expresses relatively high levels of IL-3R α-chain and the β-chain, but not the GM-CSFR α-chain.

In addition, inflammatory (TNF-α, IFN-γ) and preinflammatory (IL-4) cytokines were examined for their potential to regulate receptor expression in HUVEC. The cells were preincubated with TNF-α (100 U/ml), IFN-γ (100 U/ml) or IL-4 (10

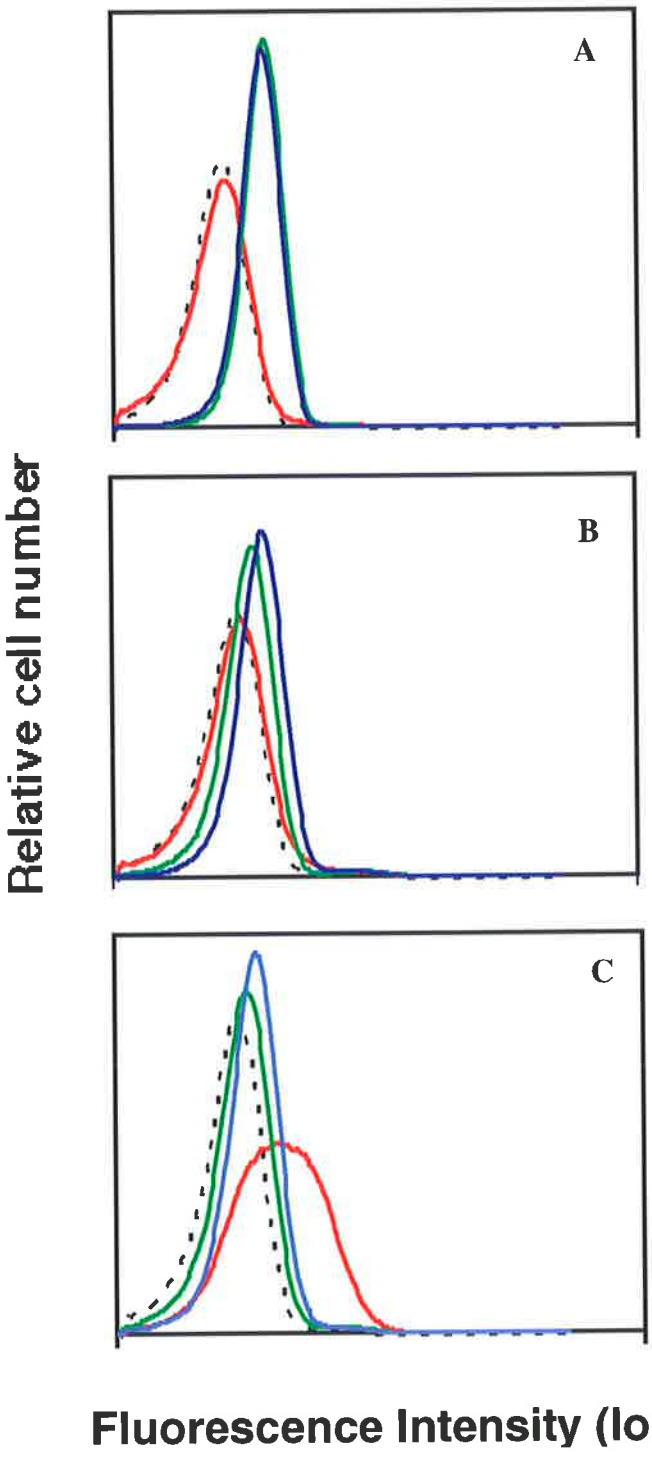


Figure 4-7 FACS analysis of cytokine receptor expression detected by anti-IL3Rα (—) , GM-CSFRα (—) and β (—) chains MoAbs and the control MoAb (---) on human neutrophils, (A) when neutrophils were freshly purified, (B) cultured overnight with medium, or (C) with GM-CSF at 2ng/ml.

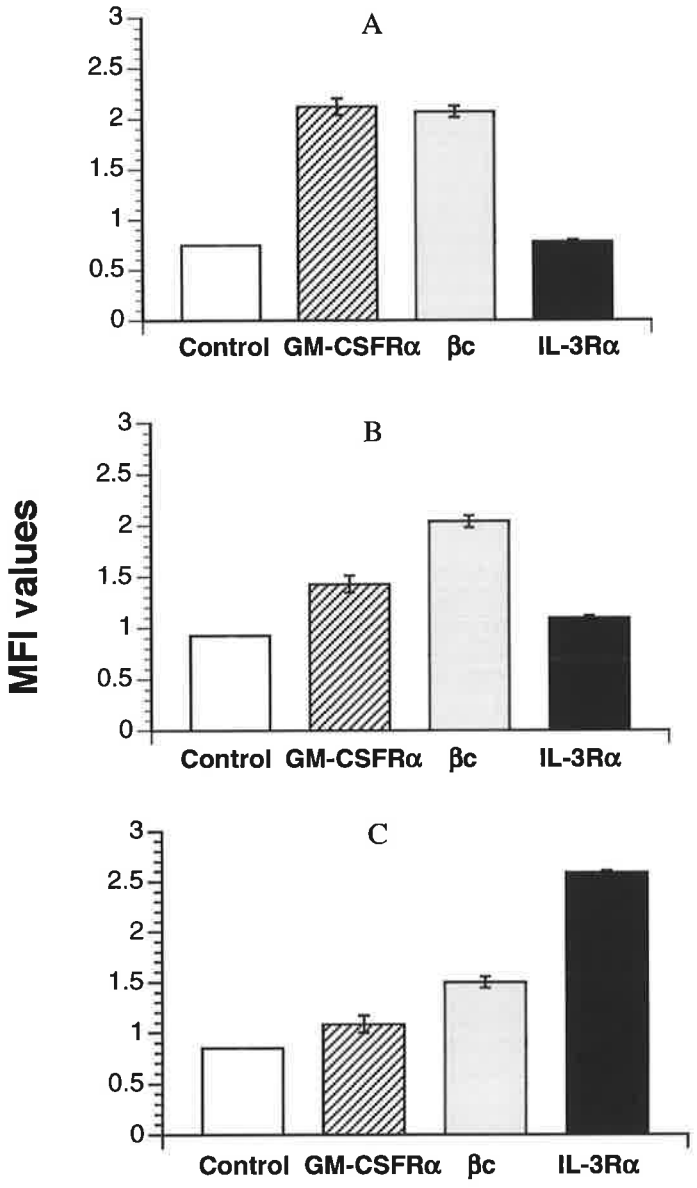


Figure 4-8 Pooled mean fluorescence intensity (MFI) values from 5 donors confirm expression of the subunits of receptor for GM-CSF and IL-3 on neutrophils. Neutrophils either fresh (A), cultured overnight in medium alone (B), or in 1ng/ml GM-CSF(C) were stained with control MoAb (□), MoAb 8G6 (▨), 1C1 (□) and 9F5 (■).

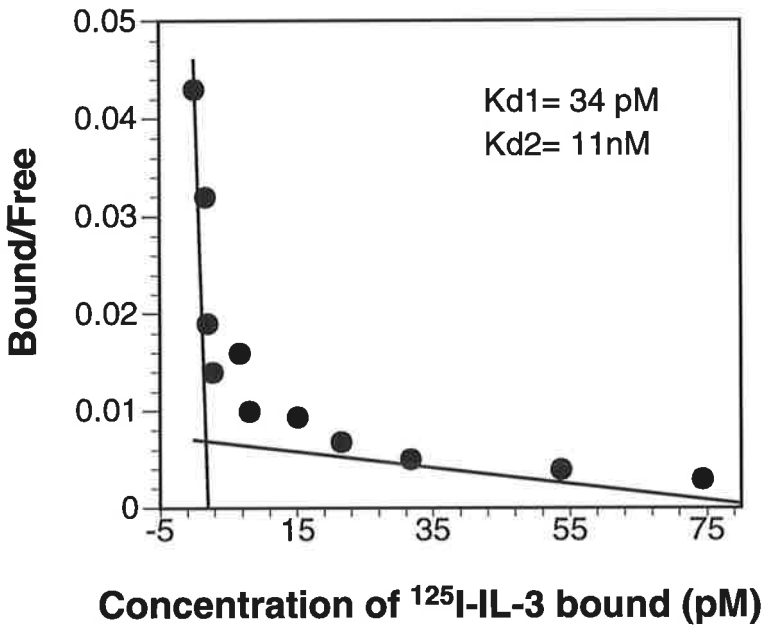


Figure 4-9 Scatchard transformation of ^{125}I-IL-3 binding to GM-CSF stimulated neutrophils. Binding curve was generated by incubating 3×10^6 neutrophils (>98% pure) with varying concentration of ^{125}I-IL-3 in triplicate for each point. This analysis showed 50 high-affinity and 2,900 low-affinity binding sites per cell.

ng/ml) for 24 hours before analysing their surface expression of IL-3R α-chain and β-chain by MoAbs on a flow cytometer. HUVEC from three donors were analysed and the data are summarised on figure 4-11. Both TNF-α and IFN-γ increased expression of IL-3R α-chain by ~3 fold and the β-chain by ~2 fold. The combination of TNF-α and IFN-γ had a synergistic effect, they up-regulated IL-3R α-chain by ~14 fold and the β-chain by 8 fold. Interestingly, although IL-4 strongly up-regulated the β chain expression, it consistently down-regulated the expression of IL-3R α-chains.

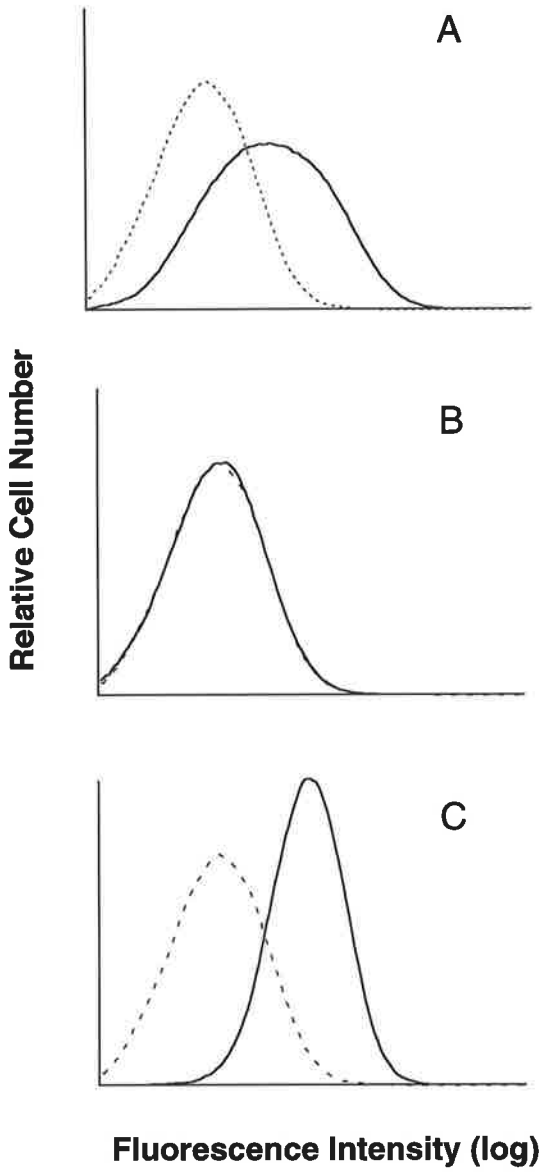


Figure 4-10 IL-3R, GM-CSFR α-chains and β-chain expression on HUVEC stained with anti-IL-3Rα MoAb 7G3 (A), anti-GM-CSFRα MoAb 8G6 (B) and anti-βc MoAb 1C1 (C) with a control MoAb (dashed line), and analysed by flow cytometer.

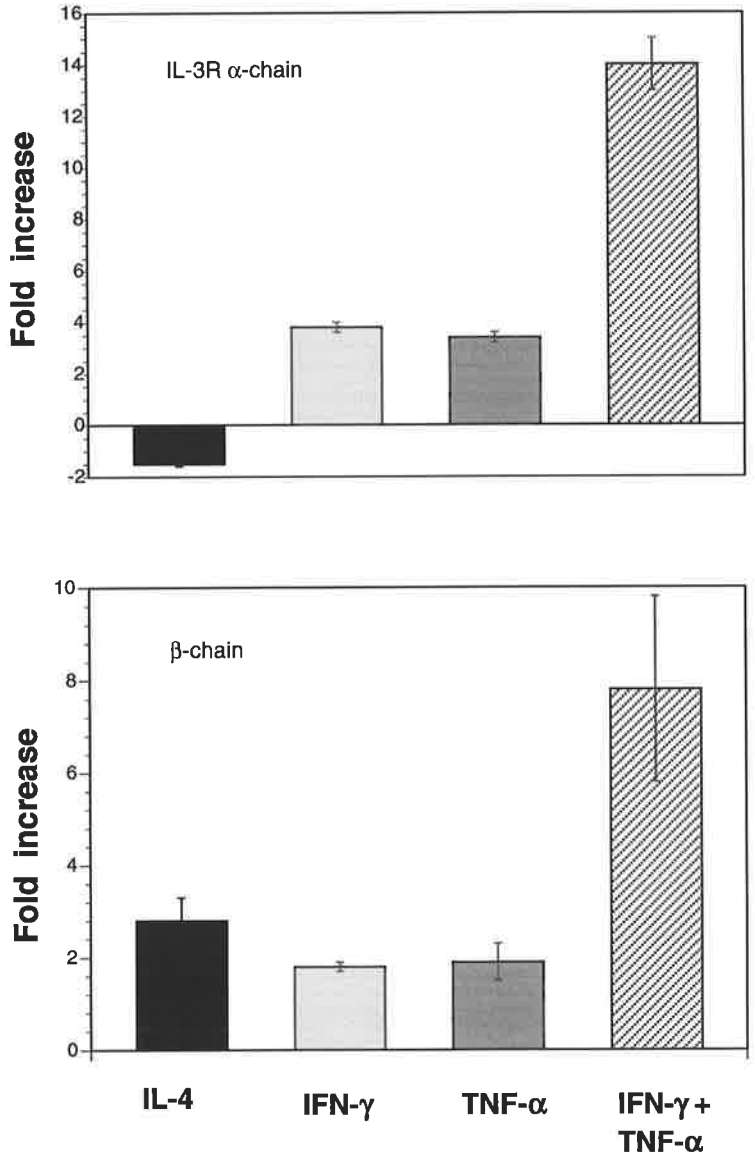


Figure 4-11 Quantitation of cytokine-induced IL-3R α- and β- chain expression in HUVEC. Cell were stimulated with medium (basal line), or IL-4 (10ng/ml), or TNF-α (100U/ml), or IFN-γ (100U/ml), or both TNF-α and IFN-γ for 24 h. HUVEC were then stained with anti-IL-3Rα MoAb 7G3, or anti-βc MoAb 1C1, and analysed by flow cytometer. The increase of IL-3Rα and β chain expression over basal level was calculated using the mean fluorescence intensity values (MFI). The mean MFI and SEM were obtained from four experiments with cells from different donors.

Discussion

This chapter shows the expression pattern of receptors for GM-CSF, IL-3 and IL-5 on human hemopoietic cells such as CD34+ cells, neutrophils, eosinophils, monocytes and basophils. The non-hemopoietic HUVEC are also shown to have IL-3 receptors. The receptor expression on human neutrophils and HUVEC, however, is not a static process, and can be regulated by several other cytokines. The wide distribution of GM-CSF, IL-3 receptors, and to a lesser extent IL-5 receptor, confirms the broad spectrum of biological functions of these cytokines. The pattern of receptor regulation also suggests the complexity of cytokine interaction on their target cells.

The pattern of GM-CSF, IL-3 and IL-5 receptors expression on human hemopoietic progenitors has long been a puzzle. CD34 antigen is an important marker of early hemopoietic stem/progenitor cells. CD34+ bone marrow cells comprise 1.5% of marrow mononuclear cells, but contain precursors for all lymphohaemopoietic lineages (reviewed by Kraus *et al*, 1996). CD34+ cells have been shown by *in vitro* studies to respond to IL-3 and GM-CSF. I show here that CD34+ cells express relatively high levels of IL-3R α-chains, and to a lesser degree, GM-CSFR α-chain. The expression of βc is very much limited compared with that of α-chains for IL-3 and GM-CSF receptors. This finding coincides with the results of receptor expression on CD34+ cell detected using another anti-IL-3R α-chain MoAbs by Sato *et al.* (1993). As the β-chain is a key component to form functional receptors and responsible for signalling, the very poor expression of β_c to IL-3R and GM-CSFR α-chain raises the issue that the pattern of receptor activation in CD34+ cells may be different from other β_c rich hemopoietic cells. The higher expression of IL-3 and GM-CSFR α-chains may also have some unknown biological roles besides forming cytokine receptors. In addition, we have also performed clone-forming studies using the CD34+ cells with different cytokine receptor expressions. It seems that the CD34+, IL-3R α-chain low cells are more immature than the IL-3R α-chain high cells (data not

shown in this thesis). Similar results also found GM-CSFR α-chain low and high cells. This finding suggests that IL-3R and GM-CSFR α-chains may also be served as differentiation markers.

The distribution of GM-CSF, IL-3 and IL-5 receptor expression on the haemopoietic sub-populations such as neutrophil, monocytes and eosinophil is consistent with the findings from ligand binding assays. Although it is very difficult to predict the exact receptor number with the FACS data shown here, in general, the expression of receptor subunits was much higher on mature cells than on progenitors. The β_c is always have limited expression comparing with the α-chain expressions in all the cells tested and the β_c expression on haemopoietic stem cells such as CD34+ cell is much more limited. As for the receptor subunits distribution among haemopoietic mature cells, it seems that the β_c expression is consistently at lower level while the α-chain expression is in a higher level but rather variable. For example, IL-3R α-chain has the highest expression on basophils, while GM-CSFR α-chain was highest on monocytes. The limited expression of β_c help to explain the cross-competions of ligand among GM-CSF, IL-3 or IL-5 on human monocytes (Elliott *et al.*, 1989) and eosinophils (Lopez *et al.,* 1989). In addition, I have successfully applied these anti-IL3Rα MoAbs in purifying human basophils from bone marrow or peripheral bloods mononuclear cells (documented elsewhere). This finding expands the application of these anti-IL-3Rα MoAbs.

The profiles of cytokine receptor expression on haemopoeitc cells were also correlated well with their biological responses to these cytokines. In many *iv vitro* studies, IL-3, GM-CSF and many other cytokines in combination were shown to induce strong multiplication of CD34 + cells (Egeland *et al.*, 1991; Saeland *et al*, 1988; 1989; Caux *et al.,* 1990). However, neither stem cell populations responses to single cytokines *in vitro* and each requires the synergistic actions of two or more cytokines for proliferation (McKinstry *et al.*, 1997). These phenomena may be explained by the very limited β_c expression on

progenitor cells such as CD34+ cells. That means there may be a critical receptor number and the occupancy of that number of receptors is necessary to reach the threshold for receptor activation. But there is also another possibility that different cytokines may transduce their signals in different ways and the signalling built up together to facilitate the receptor activation or cell response. On the other hand, receptor rich cell such as mature haemopoeitic cells can be stimulated by single cytokine. For example eosinophils and monocytes can be activated by either IL-3 or GM-CSF. It is still not concluded that the very high levels of GM-CSF receptor α-chain expression on monocytes compared with the other receptor chains indicate that monocytes are more sensitive to GM-CSF than to other cytokines. However, the fact that basophils express very high level of IL-3R α-chains and, to a lesser degree, the β_c (data not documented in this thesis) may explain why IL-3 is the most important stimulator in basophil activation (Lopez *et al.,* 1990).

The expression of cytokine receptors on the cell surface seems to be a dynamic process and is regulated by other cytokines. One of the striking findings in this chapter is that neutrophils, when simulated by GM-CSF, can express relatively high level of IL-3 receptor α-chain. The α-chain expression also leads to IL-3 functions as judged by the fact that IL-3 can up-regulate HLA-DR class II expression in GM-CSF stimulated neutrophils but not the fresh ones (Smith *et al.,* 1995). This finding provides further evidence for the role of this cytokine in inflammation. It also gives strong evidence GM-CSF serves as an important amplification factor in inflammation. Cytokine receptor up-regulation may also exist in other inflammatory effector cells such as monocytes, basophils, eosinophils and mast cells as well.

Another striking finding is that IL-3 receptor can also be expressed on non-hemopoietic cells such as endothelial cells from umbilical vein. Endothelial cells line the vessels of the vascular system and also form an important component of bone marrow stromal cell population (Tavassoli, 1992; Hasthorpe *et al*, 1992).

They play an important role in both the immune, inflammatory response and hemopoiesis. Although the actual role of IL-3R expression on HUVEC still needs further investigation, it at least indicates that the function of IL-3 is not restricted to hemopoietic cells as previously thought. In addition, I also showed here that the expression of IL-3R on HUVEC can be regulated by some cytokines such as TNF-α, IFN-γ and IL-4. From the functional point of view, regulation of IL-3R by TNF-α, IFN-γ and IL-4 is important in these endothelial cells. Assuming that a certain threshold level of IL-3 expression is essential to generate a biological response, the TNF-α and IFN-γ induced up-regulation of IL-3R would allow cells to respond to IL-3 more easily. On the other hand, the inhibition of IL-3R α-chains expression by IL-4 may reduce the responsiveness of endothelium to IL-3. As TNF-α, IFN-γ and IL-4 are all important cytokines in inflammation, the pattern of IL-3R regulation in cytokine-activated conditions of endothelium may provide clues to the functional role of IL-3 in vascular biology *in vivo*. In contrast, the expression of GM-CSFRα on endothelial cells is still not conclusive. Bussolino *et al.* (1989) reported that there are certain numbers of GM-CSF receptor on human endothelial cells, but in this thesis the endothelial cells failed to show any detectable staining by the anti-GM-CSFRα MoAbs either in fresh nor stimulated conditions. The absence GM-CSFRα expression is consistent with the finding of Yong *et al.* (1991). Human endothelial cells can produce GM-CSF themself (Broudy *et al.,* 1986), however, their responses to GM-CSF have been controversial by a few studies (Bussolino *et al.*, 1989; Yong *et al.*, 1991). So the function of GM-CSF on human endothelial cells and there receptor expression thereof still need further investigation.

CHAPTER V

MoAb 7G3 RECONGNISES THE *N*-TERMINAL DOMAIN OF IL-3R α-CHAIN AND FUNCTIONS AS AN IL-3 RECEPTOR ANTAGONIST

INTRODUCTION

Human IL-3 is a pleiotropic cytokine that stimulates the production of haemopoietic cells of multiple lineages including neutrophils, eosinophils, basophils, monocytes, megakaryocytes, erythroid cells and B lymphocytes. IL-3 has also been shown to regulate vascular endothelial cell functions by enhancing adhesion molecule expression, neutrophil transendothelial migration and cytokine production (Korpelainen *et al.,* 1993; 1995). Some of the effects of IL-3 such as the stimulation in of haemopoiesis are desirable, which have prompted its clinical application for accelerating bone marrow reconstitution and haemopoietic cell maturation after bone marrow transplantation or chemotherapy (Orazi *et al.,* 1992; Haylock *et al.*, 1992). However, it is also apparent that abnormal, excessive production of IL-3, or excessive expression of IL-3 receptor has the potential to play a pathological role in some conditions. For example, some acute myeloid leukaemic cells proliferate in response to IL-3 (Deliwel *et al.,* 1988; Park *et al.,* 1989) and follicular B cell lymphoma cells produce IL-3 and depend on IL-3 for their growth (Clayberger *et al.,* 1992). IL-3 has also been implicated in allergy not only for its ability to stimulate eosinophil and basophil production (Lopez *et al.,* 1988; 1990) but also for being a strong stimulus for histamine release from basophils *in vitro* (Haak-Frendscho *et al.*, 1988; Lopez *et al.,* 1990). The elevation of IL-3 mRNA levels in the skin and bronchi of allergic individuals (Kay *et al.,* 1991) further suggests a pathogenic role for IL-3 *in vivo*.

The biological activities of human IL-3 are initiated by the binding of IL-3 to its receptor. The IL-3 receptor consists of two subunits: an α-chain that binds IL-3 specifically with low affinity (Kitamura *et al.*, 1991) and a β-chain that is shared by IL-3, GM-CSF and IL-5 and is responsible for receptor high affinity conversion. Although both chains are required for signalling (Kitamura *et al.,* 1992), IL-3 binding to IL-3R α-chain is the initial step in receptor activation and signal transduction. The consequences of IL-3 receptor activation are not

fully understood but probably involve the activation of specific kinases associated with the receptor (Ihle *et al.,* 1994). Our observation also suggests that IL-3 induces IL-3Rα and β chain dimerization during receptor activation (Stomski *et al.,* 1996).

In the previous chapters I showed the development and cellular binding of three MoAbs to the IL-3Rα. In this chapter we analyse this group of MoAbs for effects on IL-3 binding and function. I show here that MoAb 7G3 could completely neutralise IL-3 binding and function (Sun *et al.,* 1996). The MoAb 7G3 epitope maps to amino acid 19-49 of the *N*-terminal domain of IL-3R α–chain. These results offer the potential to block IL-3 activity *in vivo*. These data also suggest that the *N*-terminal domain of IL-3Rα, and by analogy also of GM-CSF and IL-5R α-chain, may be involved in ligand binding. In addition, this MoAb may provide a useful tool in the manipulation of IL-3 functions both in *in vitro* and *in vivo* studies.

RESULTS

5.1 MoAb 7G3 inhibits both the low and high affinity binding of IL-3

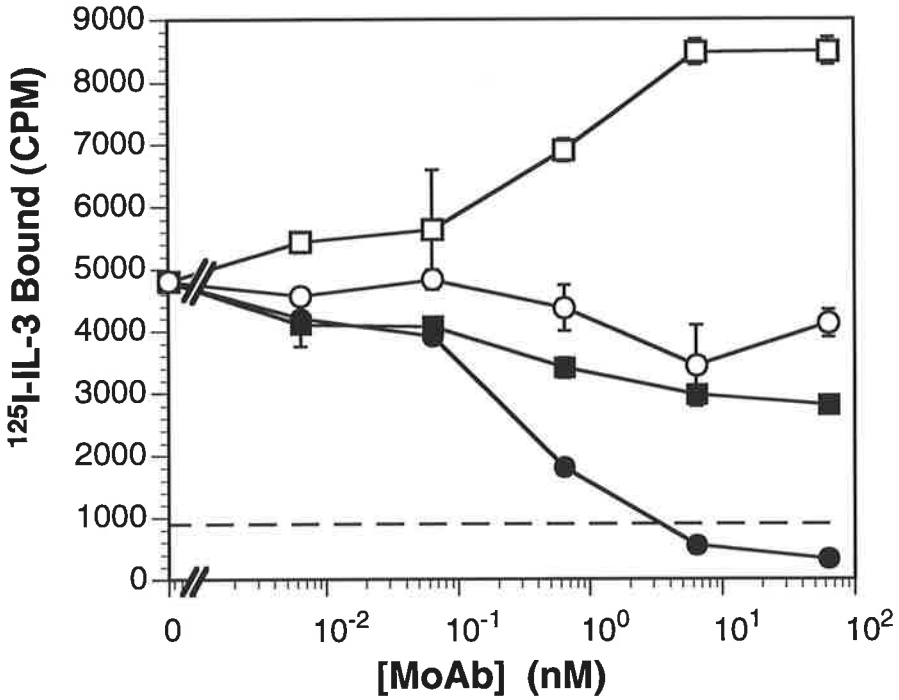
To examine whether the anti-IL-3Rα chain MoAbs could interfere with IL-3 binding we performed competition IL-3 binding experiments using a fixed concentration of ^{125}I-IL-3. In the low affinity binding assay, 4 nM of ^{125}I-IL-3 was incubated with $5x10^5$ CHO F6 cells that express IL-3Rα in the presence of anti-IL-3Rα MoAbs over a concentration range from 0.065 to 65 nM. The high affinity binding assay was carried out by incubating 150 pM ^{125}I-IL-3 with $7x10^5$ COS cells co-expressing IL-3R α and β chains in the presence of anti-IL-3Rα MoAbs over the same concentration range as in the low affinity binding. We found that MoAb 7G3 but not other MoAb inhibited the binding of ^{125}I-IL-3 to F6 cells expressing IL-3Rα in a dose-dependent manner (Figure 5-1A). Similarly MoAb 7G3 also blocked the binding of ^{125}I-IL-3 to COS cells transfected with the IL-3Rα and β chain cDNA (Figure 5-1B). In both cases, MoAb 7G3 gave 50% inhibition of ^{125}I-IL-3 binding around 0.7 nM and complete inhibition around 10 nM. MoAbs 6H6 and 9F5 did not inhibit IL-3 binding to low or high affinity IL-3 receptors, however, 6H6 enhanced ^{125}I-IL-3 binding to the IL-3Rα chain above (Figure 5-1A) in 3/3 experiments performed.

5.2 IL-3 reciprocally inhibits the binding of MoAb 7G3

In order to investigate whether IL-3 and MoAb 7G3 could directly compete for the binding of each other, I performed reciprocal competition binding experiments. F6 CHO cells expressing the IL-3Rα chain alone (Fig 5-2A) or COS cells transfected with the IL-3Rα and β chain cDNA (Fig 5-2B) were preincubated with IL-3 or GM-CSF over a range of concentration before the addition of 1 nM ^{125}I-7G3. In both cases, IL-3 but not GM-CSF inhibited the binding of ^{125}I-7G3 to the IL-3R. Interestingly IL-3 induced 50% inhibition of ^{125}I-7G3 binding on F6 cells at concentration of around 300 nM, while on COS cells co-expressing IL-R α and β chains at concentration of around 3 nM. This difference can be explained by affinity variations between low and high affinity

Figure 5-1. Dose-dependent competition for ^{125}I-IL-3 binding by anti-IL-3Rα MoAb 7G3(●), 6H6(□), 9F5(■) and a control MoAb 3D3 (O) to (A) F6 CHO cells stably expressing the IL-3R α-chain and (B) COS cells transiently transfected with IL-3R α- and β-chains. In (A) ^{125}I-IL-3 was used at 4nM and in (B) at 150pM. The dashed line represents competition by 200 fold excess unlabelled IL-3. Each point is the mean of triplicate determinations and the error bars represent standard deviations.

A


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
^{125}I-IL-3 Bound (CPM)
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
[MoAb] (nM)

B

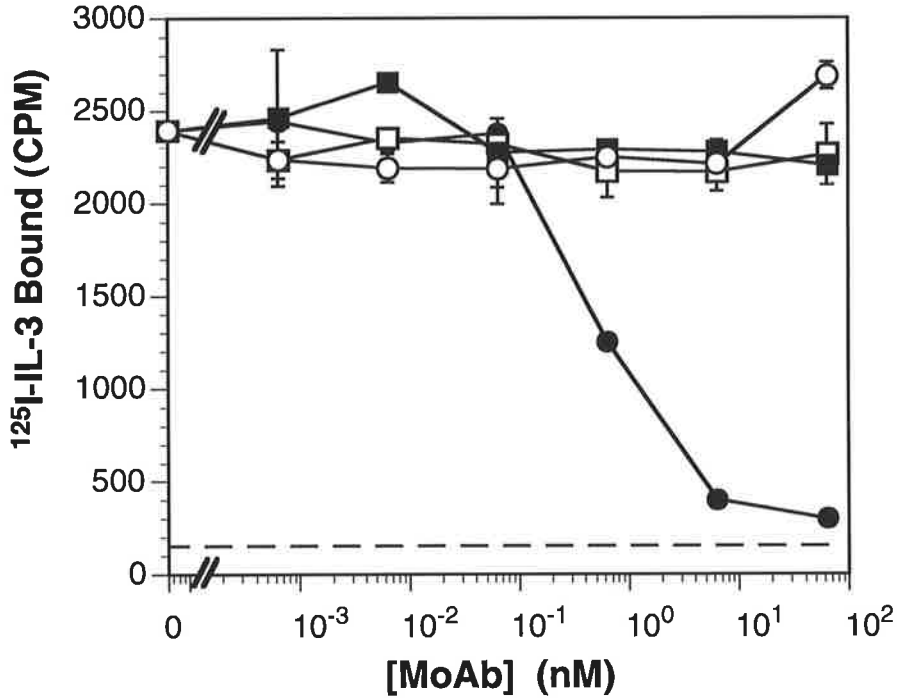
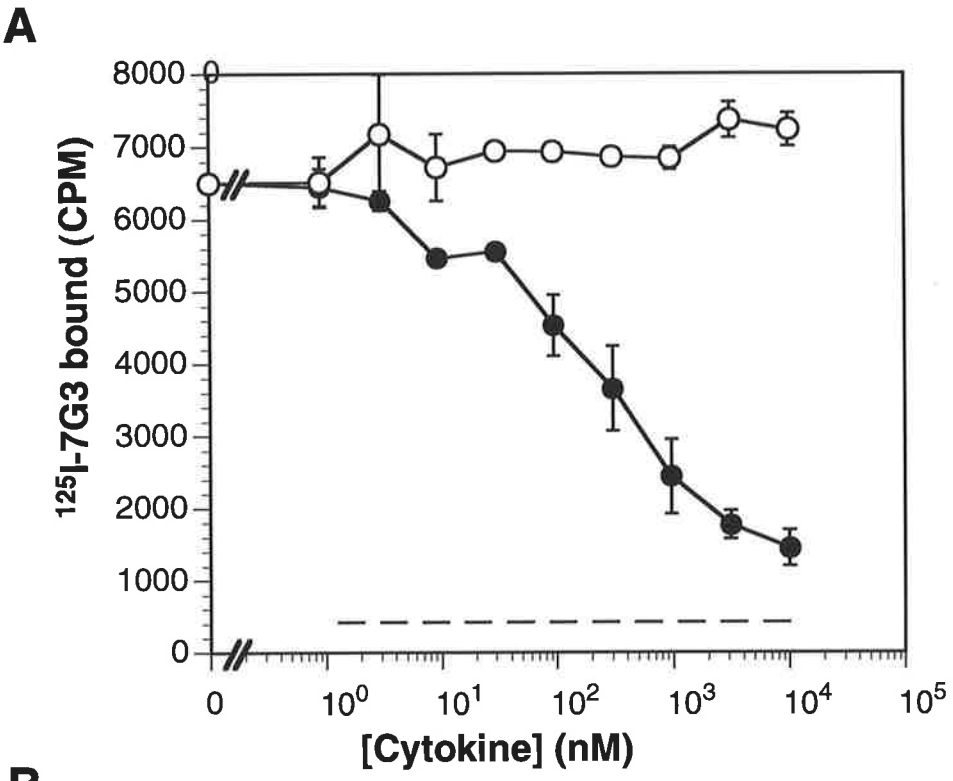
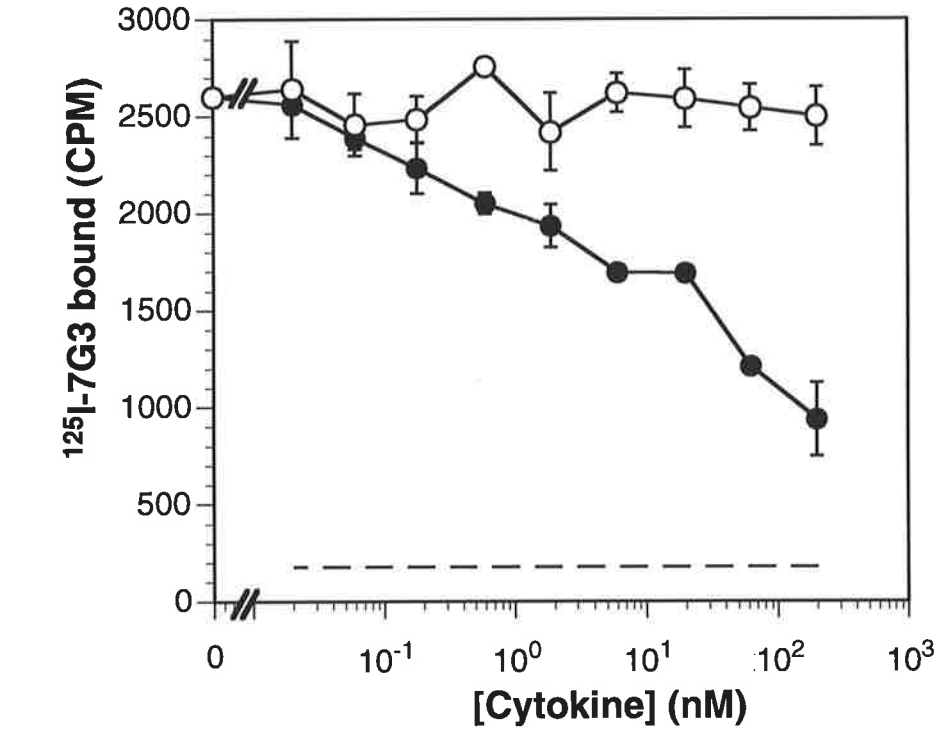

3000
2500
2000
1500
1000
500
0
^{125}I-IL-3 Bound (CPM)
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
[MoAb] (nM)

Figure 5-2. Dose-dependent competition for ^{125}I-7G3 binding to (A) F6 cells stably expressing the IL-3R α chain, and to (B) COS cells transiently transfected with the IL-3R α and β chains by human IL-3(●) or GM-CSF(O). The dashed line represents the inhibition in the presence of 100 fold excess unlabelled 7G3. Each point represents the mean of triplicate determinations and the error bars represent standard deviations.


A
8000
7000
6000
5000
4000
3000
2000
1000
0
^{125}I-7G3 bound (CPM)
0
10^0
10^1
10^2
10^3
10^4
10^5
[Cytokine] (nM)


B
3000
2500
2000
1500
1000
500
0
^{125}I-7G3 bound (CPM)
0
10^{-1}
10^0
10^1
10^2
10^3
[Cytokine] (nM)

receptor. The above data indicate that IL-3 and MoAb 7G3 bind to the same or adjacent epitopes on the IL-3R α chain.

5. 3 The affinity of MoAb 7G3 for the IL-3Rα chain

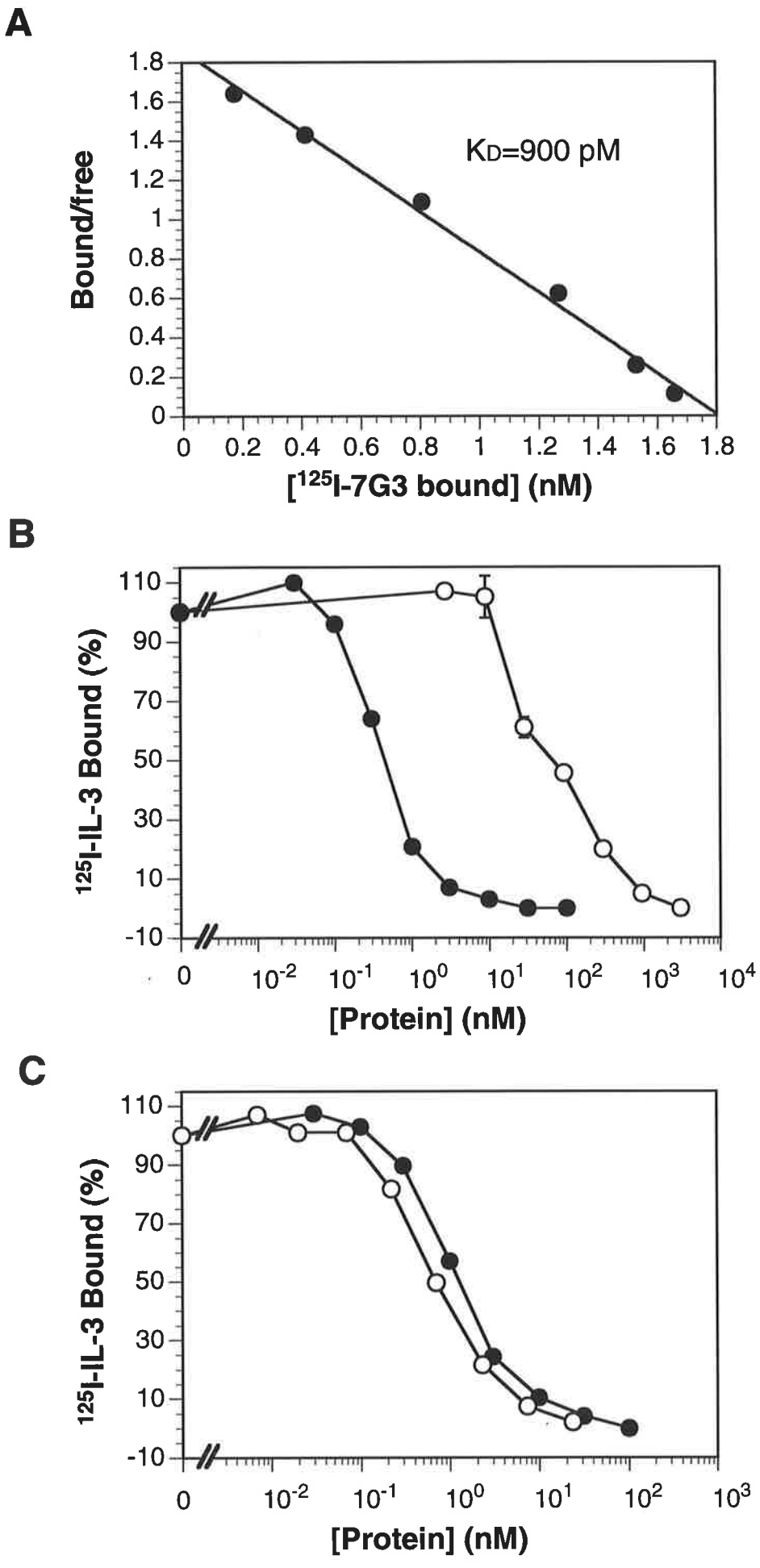
As MoAb 7G3 and IL-3 recognised the same or adjacent binding sites on IL-3Rα chain we next performed direct measurements of MoAb 7G3 binding affinity and compared them to IL-3. Scatchard transformation of a saturation binding curve of ^{125}I-7G3 on F6 CHO cells revealed a kDa of 900 pM (Figure 5-3A). This represents a hundred fold higher affinity of IL-3Rα for 7G3 than reported for IL-3 itself (Kitamura *et al.*, 1991). Consistent with these values MoAb 7G3 competed with an approximately 100 fold greater affinity than IL-3 for ^{125}I-IL-3 binding to F6 cells (Figure 5-3B). On the other hand, MoAb 7G3 competed with approximately three fold lower affinity than IL-3 on COS cells expressing the IL-3 high affinity receptor (Figure 5-3C).

5. 4 MoAb 7G3 antagonises IL-3-mediated biological functions

Having demonstrated that MoAb 7G3 could completely inhibit IL-3 binding, we then examined MoAb 7G3 for its ability to antagonise IL-3 functions in situations where IL-3 may play a pathogenic role. These experiments examined IL-3 stimulated cell proliferation, basophil histamine release and endothelial cell activation.

To study the effects of MoAb 7G3 on IL-3 stimulated cell proliferation, we used the human myeloid leukaemic cell line, TF-1 cells, which is dependent on IL-3 for growth *in vitro*. A dose-response study indicated that a concentration of approximately 0.3 ng/ml of IL-3 induced half-maximal proliferation of TF-1 cells (Figure 5-4A). I then performed experiments by incubating TF-1 cells with a constant concentration of 0.3 ng/ml of IL-3 in the presence of a range of concentration of MoAb 7G3 from 0.007 to 70 nM. We found that the addition of MoAb 7G3, but not other anti-IL-3Rα chain MoAbs, antagonised cell proliferation in a dose-dependent manner (Figure 5-4B), with 50% inhibition

Figure 5-3. Characterisation of MoAb ^{125}I-7G3 binding to the IL-3R. (A) Scatchard transformation of a saturation binding curve using ^{125}I-7G3 on F6 cells stably expressing the IL-3R α chain; (B) competition for ^{125}I -IL-3 binding to F6 cells expressing IL-3Rα chain by MoAb 7G3(●) or IL-3(O); and (C) competition for ^{125}I-IL-3 binding to COS cells expressing IL-3Rα and β chains by MoAb 7G3(●) or IL-3(O). Each point is the mean of triplicate determinations.


A
Bound/free
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KD=900 pM
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8
[125I-7G3 bound] (nM)
B
125I-IL-3 Bound (%)
110
90
70
50
30
10
-10
0 10-2 10-1 100 101 102 103 104
[Protein] (nM)
C
125I-IL-3 Bound (%)
110
90
70
50
30
10
-10
0 10-2 10-1 100 101 102 103
[Protein] (nM)

occurs at the concentration of 0.7 nM. The dose required for half-maximal inhibition correlated very well with that observed in binding assay.

IL-3 has been shown to be one of the strongest enhancer of histamine release from human basophils, suggesting an effector role in allergy (Haak-Frendscho *et al.,* 1988; Lopez *et al.,* 1990). I also tested MoAb 7G3 for its ability to antagonise histamine release. From a dose-response assay (Figure 5-5A), we selected a concentration of 1 ng/ml of IL-3, which gave a half-maximal stimulation of the histamine release, to examine the effect of MoAb 7G3. We found that MoAb 7G3, but not MoAb 9F5, was able to completely antagonise the IL-3-dependent stimulation of basophil histamine release, again in a dose-dependent manner (Figure 5-5B).

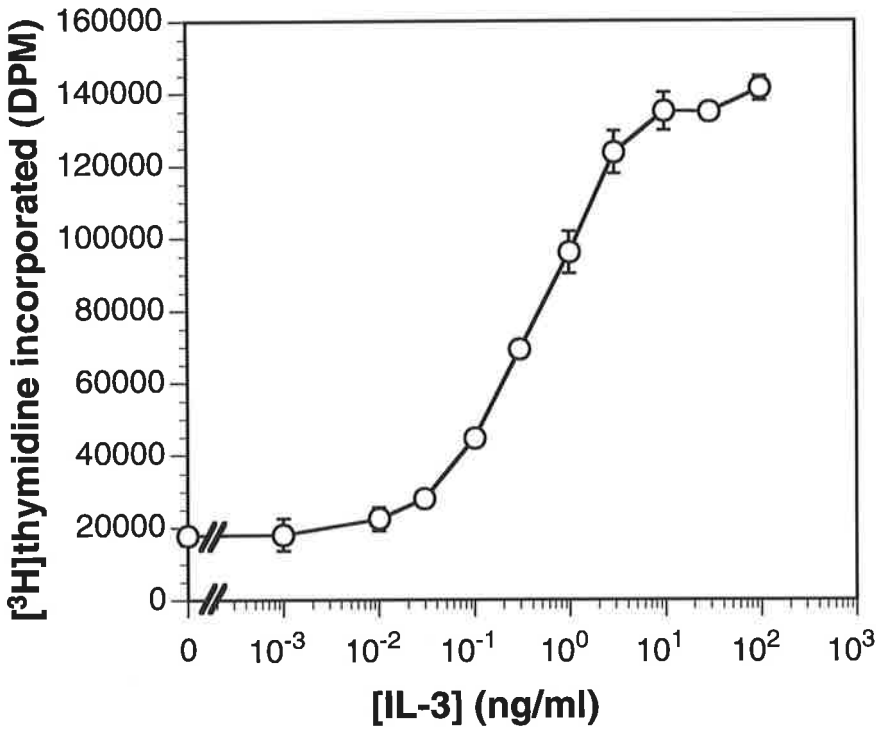
Human endothelial cells have recently been shown to express IL-3 receptor α and β chain (Korpelainen *et al.,* 1993; Brizzi *et al.,* 1993) and it has been demonstrated that IL-3 acts as an amplification factor enhancing several endothelial cell functions including cytokine secretion (Korpelainen *et al.*, 1995). MoAb 7G3 was also examined for its ability to inhibit IL-6 and IL-8 secretion from IL-3-stimulated HUVEC. We found that MoAb 7G3 was able to antagonise the synergy of IL-3 with interferon γ in the stimulation of IL-6 secretion. This effect was specific for the IL-3 amplification effect and did not affect the small stimulatory effect of interferon γ alone (Figure 5-6A). Similarly, MoAb 7G3 was able to antagonise the enhancing effect of IL-3 on IL-8 secretion by TNF-α-stimulated cells without inhibiting the effect of TNF-α (Figure 5-6B).

5.5 Epitope mapping of MoAb 7G3.

To identify the region/s in IL-3Rα recognised by MoAb 7G3 we initially tested MoAb 7G3 for binding to overlapping peptides of 14 amino acids in length encompassing the full extracellular domain of the IL-3Rα chain. However, no specific binding of MoAb 7G3 was observed (data not shown). Since these

Figure 5-4. Inhibition of IL-3-mediated proliferation of TF-1 cells by MoAb 7G3. (A) TF-1 cell proliferation in response to different concentrations of IL-3; (B) TF-1 cell proliferation stimulated by 0.3 ng/ml of IL-3 in the presence of a range of concentrations of anti-IL-Rα MoAbs 7G3(●), 6H6(□), 9F5(■) and a control MoAb(O) . Each point represents the mean of triplicate determinations and the error bars represent standard deviations.

A


160000
140000
120000
100000
80000
60000
40000
20000
0
[³H]thymidine incorporated (DPM)
0
10⁻³
10⁻²
10⁻¹
10⁰
10¹
10²
10³
[IL-3] (ng/ml)

B

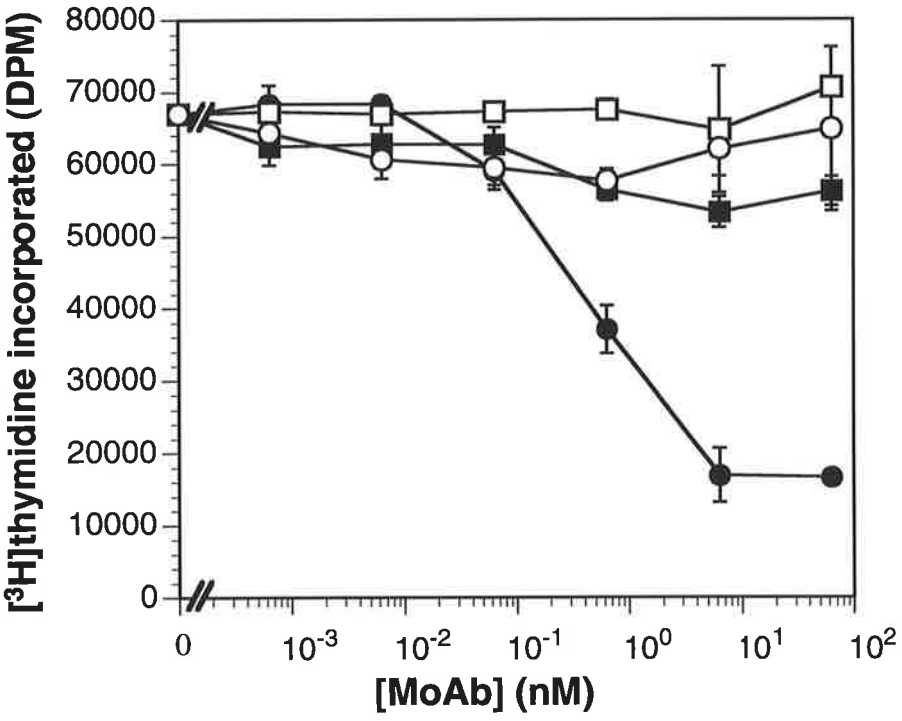

80000
70000
60000
50000
40000
30000
20000
10000
0
[³H]thymidine incorporated (DPM)
0
10⁻³
10⁻²
10⁻¹
10⁰
10¹
10²
[MoAb] (nM)

Figure 5-5. Inhibition of IL-3-mediated stimulation of human basophil histamine release by MoAb 7G3. (A) Histamine release in response to a range of concentrations of IL-3 and (B) histamine release stimulated by 1 ng/ml of IL-3 in the presence of a range of concentrations of anti-IL-Rα MoAb 7G3(●), 9F5(■) and the control MoAb(O). Each value represents the mean of quadruplicate determinations and the error bars represent standard deviations.

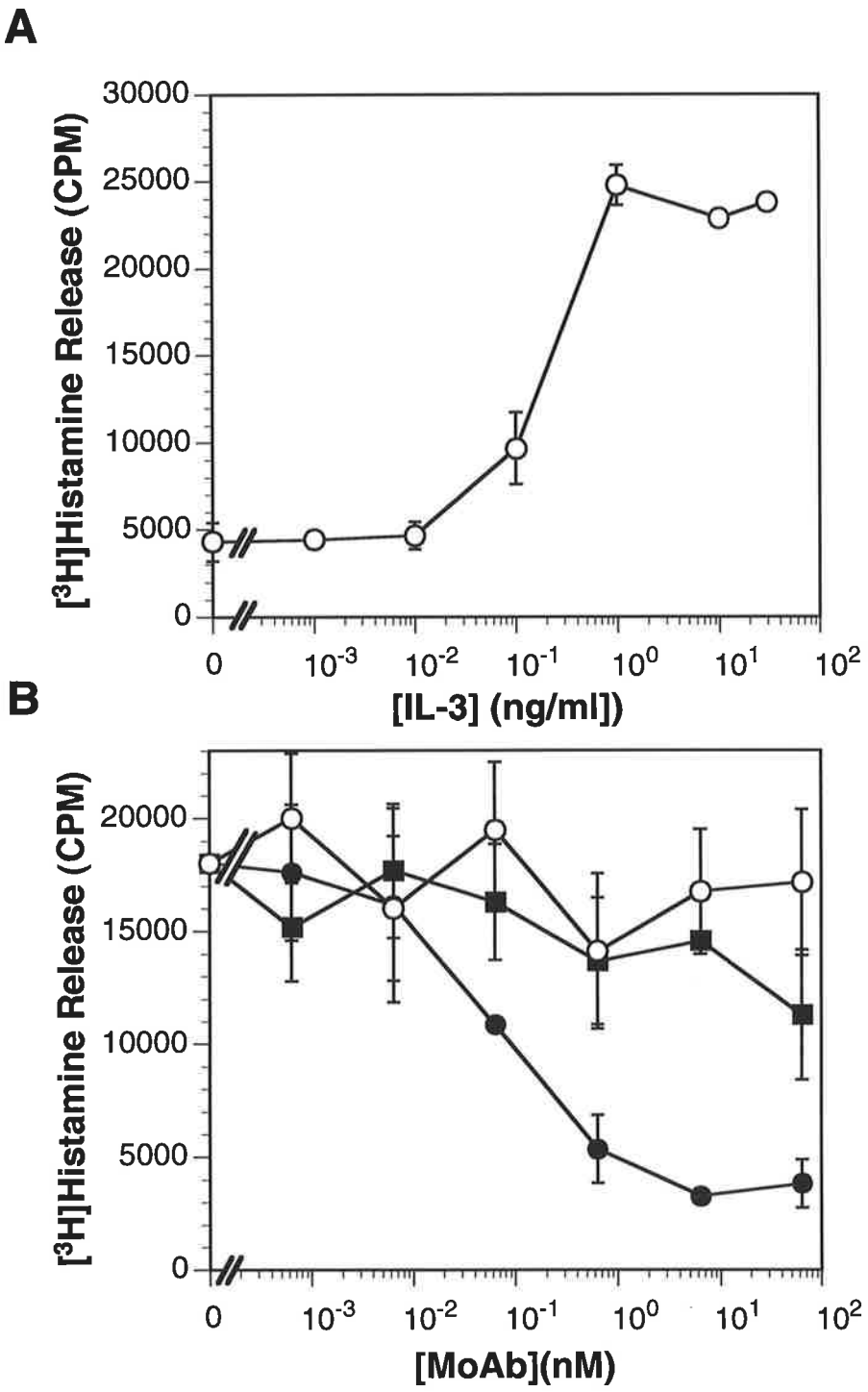
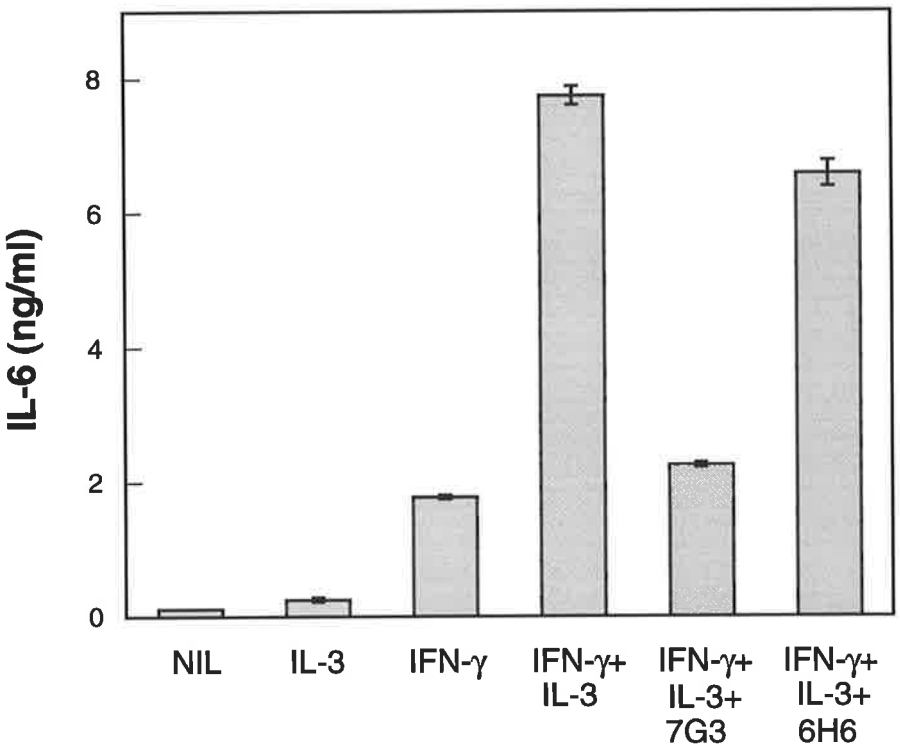
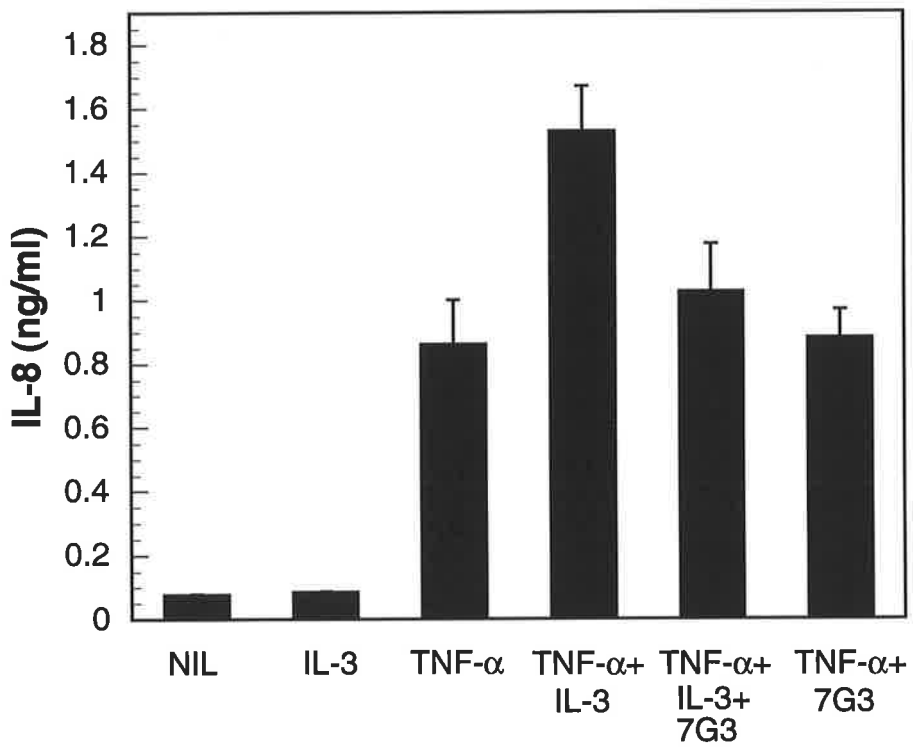

A
30000
25000
20000
15000
10000
5000
0
[3H]Histamine Release (CPM)
0
10-3
10-2
10-1
100
101
102
[IL-3] (ng/ml])
B
20000
15000
10000
5000
0
[3H]Histamine Release (CPM)
0
10-3
10-2
10-1
100
101
102
[MoAb](nM)

Figure 5-6. MoAb 7G3 selectively inhibits IL-3-mediated stimulation of (A) IL-6 release and (B) IL-8 release from HUVEC stimulated by IL-3 (30ng/ml) together with IFN-γ (100U/ml) or TNF-α (100U/ml). MoAb 7G3 was used at 30μg/ml. The values represent the means of triplicate determinations and the error bars represent standard deviations.

A


IL-6 (ng/ml)
8
6
4
2
0
NIL
IL-3
IFN-γ
IFN-γ+ IL-3
IFN-γ+ IL-3+ 7G3
IFN-γ+ IL-3+ 6H6

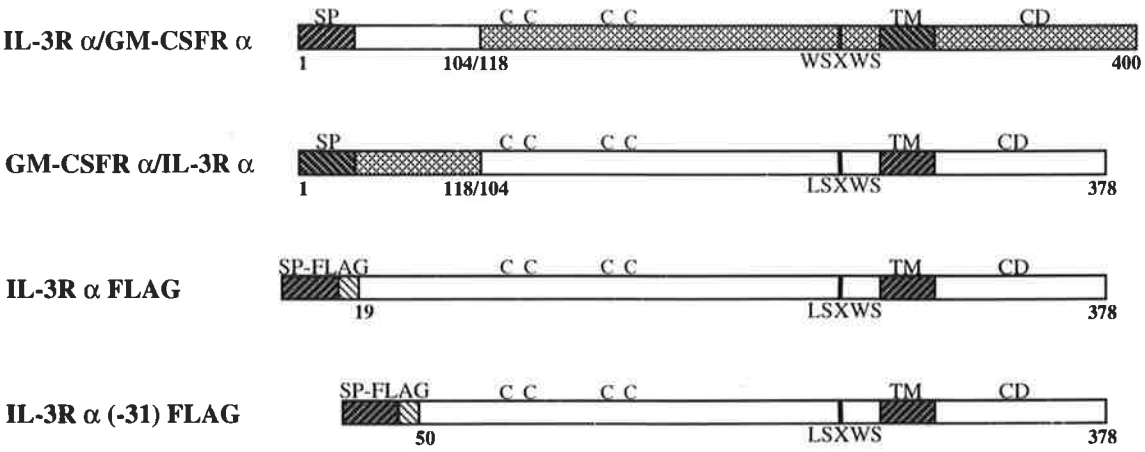
B


IL-8 (ng/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NIL
IL-3
TNF-α
TNF-α+ IL-3
TNF-α+ IL-3+ 7G3
TNF-α+ 7G3

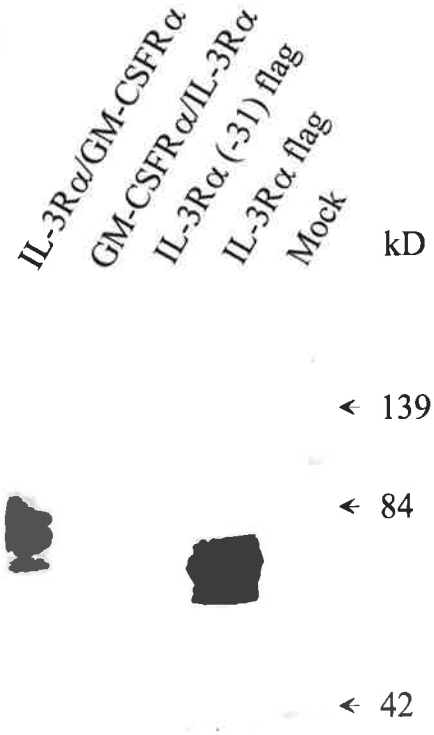
results suggest that MoAb 7G3 may recognise a conformational rather than a linear epitope, we generated cDNAs encoding IL-3Rα/GM-CSFRα chimeras and truncated IL-3Rα chains (figure 5-7A). These cDNAs were expressed in COS cells and binding of MoAb 7G3 to the mutant receptors was examined by Western blotting and immunofluorescence. Although the IL-3Rα/GM-CSFRα chimera composed of amino acids 1-104 of IL-3Rα and amino acids 118-400 of GM-CSFRα bound 7G3 by both Western blot analysis (Figure 5-7B) and immunofluorescent microscopy (data not shown), the converse chimera (GM-CSFRα/IL-3Rα) composed of amino acids 1-118 of GM-CSFRα and amino acids 105-378 of IL-3Rα failed to do so. This suggests that the epitope for 7G3 is located in the amino terminal 104 amino acids of IL-3Rα. A receptor deletion mutant, IL-3Rα (-31) flag, lacking the first 31 residues beyond the signal peptide (Thr19-Asp49 absent) but containing an 8 residue "flag" sequence also failed to bind 7G3. However another receptor mutant, IL-3Rα flag, containing Thr 19-Asp 49 along with the "flag" sequence did bind 7G3 (Figure 5-7B). Strong expression of the IL-3Rα (-31) flag and IL-3Rα flag could be demonstrated by immunofluorescent microscopy (data not shown) and Western blotting (Figure 5-7C) using an anti-flag M2 MoAb. These results suggest that the epitope of 7G3 may be located within amino acids Thr19-Asp49 of the amino terminus of IL-3Rα.

Figure 5-7. (A) Schematic representation of the IL-3Rα constructs used to epitope map MoAb 7G3. SP = signal peptide; TM = transmembrane region; CD = cytoplasmic domain. The conserved cysteines (c) and WSXWS motifs are indicated. The numbering of the primary sequence includes the signal peptide. The shaded regions represent GM-CSFRα chain and the clear regions IL-3Rα chain encoding DNA. (B) Western blot analysis of COS cells transiently transfected with various IL-3Rα mutants. Binding of 7G3 was seen with the IL-3Rα/GM-CSFRα chimera (B, lane 1) and with the wild-type IL-3Rα containing a "flag" sequence interposed between the signal peptide and residue 19 (IL-3Rα flag) (B, lane 4) but not with the GM-CSFRα/IL-3Rα chimera (B, lane 2) nor with a truncated IL-3Rα lacking Thr19-Asp49 [IL-3Rα (-31) flag] (B, lane 3). (C) Expression of IL-3Rα (-31) flag (C, lane 1) and IL-3Rα flag (C, lane2) are demonstrated by Western Blot using an anti-flag MoAb M2.

A

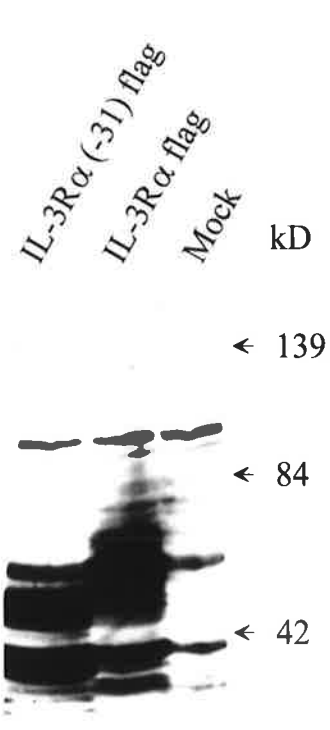


B



MoAb 7G3

C



Anti-flag MoAb M2

DISCUSSION

I describe here the generation of a specific anti-IL-3Rα MoAb, 7G3, which completely and reciprocally inhibits the binding of IL-3 to its high and low affinity receptor and also antagonises IL-3 activity in all functions tested. In addition, I show that the epitope recognised by MoAb 7G3 lies within the *N*-terminal domain of IL-3Rα, implicating this *N*-terminal domain, conserved among the IL-3Rα, GM-CSFRα and IL-5Rα superfamily, in ligand binding.

MoAb 7G3 was one of a panel of MoAbs produced against hIL-3R α-chain. Cytokine binding experiments showed that MoAb 7G3 inhibited both the high and low affinity IL-3 binding in a dose-dependent manner, with 50% inhibition occuring at a concentration of 0.5 nM. MoAb 7G3 bound IL-3Rα with an approximate Kd of 900pM. Thus the affinity of MoAb 7G3 is about 100-300 fold greater than IL-3 for IL-3Rα [100nM (Kitamura *et al.,* 1991)] and about 3-10 fold lower than IL-3 for the IL-3Rαβ high affinity receptor [100pM (Kitamura *et al.,* 1992)]. This was reflected in the inhibition of IL-3 binding (Figure 5-3) and in the ED_{50} of MoAb 7G3 in functional assays (Figure 5-4 to 5-6).

IL-3 is believed to play important roles in both haemopoiesis. Although IL-3 has been shown to stimulate several cell types *in vitro* (Metcalf, 1984), it is somewhat puzzling that this cytokine has not been detected in the bone marrow or serum of normal animals (Metcalf *et at*, 1991), and IL-3 gene knock-out mice undergo normal haemopoiesis (Mulligan RC personal communication) suggesting that it is not required for basal haemopoiesis. On the other hand, the injection of IL-3 into mice and humans stimulates haemopoiesis as well as having significant side-effects such as bone marrow fibrosis (Metcalf *et at.*, 1986; Falk *et al.,* 1991). In this respect IL-3 may be viewed as a "reactive" rather than a "steady-state" cytokine and its production may lead to desirable as well as potentially deleterious effects.

Consistent with this role, the production of IL-3 is under tight regulatory control in T cells (Ryan *et al.*, 1994). We show here that MoAb 7G3 is an effective antagonist of IL-3 activities with an ED_{50} of 0.4 to 1 nM, which correlates with its Kd value (Fig 5-3). In the proliferation assay, MoAb 7G3 completely antagonised IL-3-stimulated proliferation of the leukaemic cell line, TF-1 cells (Fig 5-4), at similar concentrations used in the IL-3 binding assay. Antagonism of IL-3-mediated cell proliferation is likely to be useful in some leukaemias where IL-3 has been shown to promote growth (Deliwel *et al.*, 1988; Park *et al.*, 1989). In particular follicular B cell lymphomas that bind IL-3 with high affinity and proliferate in an IL-3-dependent manner (Clayberger *et al.*, 1992) may be ideally suited for intervention with MoAb 7G3.

IL-3 is also an important regulator in inflammation. It functions by stimulating the production of eosinophil, basophil, monocyte and the activation of their functions. Recently IL-3 was also shown to facilitate neutrophil function when neutrophils were stimulated with GM-CSF (Smith *et al.*, 1995). The effect of MoAb 7G3 on two types of IL-3 functions was also studied here as the antagonism of IL-3 in these situations is likely to be of clinical significance. Firstly, MoAb 7G3 antagonised IL-3-mediated enhancement of histamine release from basophils (Figure 5-5). Antagonising IL-3 may be useful in allergic situations as elevated IL-3 mRNA has been noted in the skin and bronchi of atopic individuals (Kay *et al.*, 1991), and the presence of IL-3 may lead to excessive stimulation of basophils and eosinophils at allergic reaction sites. Secondly, MoAb 7G3 antagonised IL-3-mediated functions on HUVEC, namely the enhancement of TNF-α stimulation and the synergism with interferon γ (Figure 5-6). The presence of IL-3 receptors on HUVEC and their up-regulation by TNF-α and interferon γ has recently been noted (Korpelainen *et al.*, 1993;1995; Brizzi *et al.*, 1993), and their stimulation by IL-3 enhances IL-8 and IL-6 production, HLA class II expression (Korpelainen *et al.*, 1995), and neutrophil transmigration (Korpelainen *et al.*, 1993). Although the full significance of these *in vitro* findings needs to be ascertained, these effects are

likely to contribute to a systemic phase of inflammation and may be amenable to control with MoAb 7G3.

Since IL-3 is such an important cytokine in both haemopoiesis and inflammation and no recombinant IL-3 or IL-3 receptor antagonists have been reported, MoAb 7G3 will be valuable to block IL-3 biological activities in *in vitro* and possibly *in vivo* studies. It is interesting to compare the dose of the 50% inhibition produced by anti-IL-3Rα MoAb 7G3 and the neutralising anti-β_c MoAbs (Chapter VI) in both ligand binding and functional studies. The anti-βc MoAb QP1 could neutralise 50% of IL-3 binding and function at the concentration of 50 to 100 nM. While MoAb 7G3 is 100 times or more potent than MoAb QP1 in antagonising IL-3 activity. This dosage difference may represent the low to high affinity conversion of IL-3 receptor. As MoAb 7G3 directly competes with IL-3 for binding to IL-3R α-chain, it prevents IL-3 from binding and thus blocks the IL-3Rα β heterodimerization. In other competition experiments I also found that MoAb 7G3 and IL-3 reciprocally inhibited each others binding. This suggests that the IL-3 binding site may lie within or adjacent to the epitope recognised by MoAb 7G3. The fact that all three anti-IL-3Rα MoAbs bind three adjacent epitopes but only MoAb 7G3 demonstrated antagonising function suggests that the IL-3 binding site may site is in a specific area.

The *N*-terminal domain of IL-3Rα was identified as a region required for MoAb 7G3 binding based on the positive immunofluorescence and Western blotting results with a chimeric receptor comprising the *N*-terminal domain of IL-3Rα and the CRM (Goodall *et al.,* 1993) of GM-CSFRα. In contrast, MoAb 7G3 failed to bind to a chimeric receptor comprising the *N*-terminal domain of GM-CSFRα and the CRM of the extracellular region of IL-3Rα (Figure 5-7). This suggests that the *N*-terminal domain of IL-3Rα is necessary for MoAb 7G3 binding. Further truncations in the *N*-terminus with retention of MoAb 7G3 reactivity suggest that the 19-49 region of the *N*-terminal domain of IL-3Rα forms part of the epitope recognised by MoAb 7G3. In other experiments (Barry *et al.,* 1997)

we have found that truncation of the *N*-terminal domain of IL-3Rα does not abolish the binding of IL-3 although the affinity of this binding is much decreased. These results have implications for defining the binding site for IL-3 and suggest that this may be formed by two non-contiguous regions in the primary structure of IL-3Rα, one of which is in the *N*-terminal domain and is recognised by MoAb 7G3. The existence of a conformational epitope for IL-3 and MoAb 7G3 is further supported by the inability of MoAb 7G3 to bind linear sequences as represented by the overlapping 15-amino acid peptides.

It is interesting to note that the *N*-terminal domains of IL-3Rα, GM-CSFRα and IL-5Rα represent a unique feature of this sub-family of receptors. They are not classical Ig-like domains and differ from the two domains of the cytokine receptor module encompassing the rest of each α chain (Goodall *et al.*, 1993). Their function is not known, although the presence of apparently free Cys residues suggests a role in disulphide-linked heterodimerization with β_c (Stomski *et al.*, 1996). The results shown here with IL-3Rα raise the possibility that the *N*-terminal domain of this sub-family of receptors is involved in ligand binding.

CHAPTER VI

MoAb QP1 TO DOMAIN 4 OF THE COMMON β-CHAIN OF GM-CSF, IL-3 AND IL-5 RECEPTORS BINDS TO A CONFORMATIONAL EPITOPE FORMED BY B'-C' AND F'-G' LOOPS AND INHIBITS RECEPTOR DIMERASATION, BINDING AND ACTIVATION BY ALL THREE CYTOKINES

Introduction

The receptors for IL-3, GM-CSF and IL-5 are heterodimeric receptors consisting of a ligand-specific α chain (Hayashida *et al.*, 1990; Gearing *et al.*, 1989; Tavernier *et al.*, 1991; Kitamura *et al.*, 1991) and a common β chain (Kitamura *et al.*, 1991). Although the β-chain has no detectable binding to any of the three ligands, it is a key component of the receptor complex, as it is responsible for the receptor high affinity binding conversion and more importantly signal transduction.

Structurally the β-chain belongs to the cytokine receptor superfamily, and functionally it is analogous to a subset of this family such as gp130, IL-2R β-chain and γ-chain. They are shared receptor components for several cytokine receptors. The gp130 acts as an affinity converter and signal transducer for cardiotrophin-1, ciliary neurotrophic factor, IL-6, IL-11, LIF and oncostatin M (Pennica *et al.*, 1995; Davis *et al.*, 1993; Hibi *et al.*, 1990; Hilton *et al.*, 1994; Liu *et al.*, 1992). IL-2R β-chain is shared by IL-2 and IL-15 receptor, and IL-2R γ-chain by IL-2, IL-4, IL-7, IL-9 and IL-15 receptors (Giri *et al.*, 1994; Takeshita *et al.*, 1992; Russell *et al.*, 1993; Noguchi *et al.*, 1993; Kimura *et al.*, 1995).

In terms of receptor activation for GM-CSF, IL-3 and IL-5 receptors, it is accepted that signalling is mediated by a complex comprising the ligand and both the α and β chains, and the signal is induced by a series of kinase pathways as described in Chapter I. However, some fundamental steps in the signal are still unknown, such as the ligand binding site in the β_c, or the interaction site between the α and β_c. Ligand binding experiments have revealed that β_c increases the affinity of IL-3Rα 500~1000 fold (Kitamura *et al.*, 1991; 1992), GM-CSFRα 20~100 fold (Hayashida *et al.*, 1990) and IL-5Rα 2~5 fold (Tavernier *et al.*, 1991, 1992), which raises the possibility that each cytokine may bind to different region of the β_c. Mutagenesis studies of the β_c clearly indicate that

certain residues in the extracellular domain are essential for the binding of GM-CSF and IL-5 but have little effect on IL-3, while other residues are necessary for the binding of all three cytokines (Woodcock *et al.*, 1994; 1996). In addition we have also suggested that the pattern of receptor dimerization among GM-CSF, IL-3 and IL-5 may be different (Stomski *et al.*, 1996; personal communication).

It is proposed that some modification to β-chain that results in interference with the important residues in the β_c may inhibit the binding and function of all three cytokines. It also opens the potential to control the action of these cytokines by manipulating the receptor β-chain when such functional residues or domains are revealed. Recently MoAbs to gp130 have revealed blocking activities to binding and function of multi-ligands (Wijidenes *et al.*, 1995; Chevalier *et al.*, 1996). This also represents an important approach to analyse the function of gp130 related cytokines.

In this chapter, I present an anti-β_c MoAb which neutralises the binding and function of IL-3, GM-CSF and IL-5. I have mapped the epitope of this neutralising MoAb to domain 4 of the β_c, more precisely to a conformational epitope in the B'-C' and F'-G' loops. In addition I show that this MoAb prevents receptor dimerisation. These data give strong indications that domain 4 is an important functional domain of the β_c. This MoAb also provides a useful tool for the manipulation of IL-3, GM-CSF and IL-5 function *in vitro* and may form the basis of small antagonistic molecules for *in vivo* use.

6.1 Inhibition of IL-3, GM-CSF and IL-5 binding by anti-β_c MoAbs

To examine whether these anti-β_c MoAbs could interfere with ligand binding, the MoAbs were tested for their capacity to compete for the binding of ^{125}I-IL-3, ^{125}I-GM-CSF and ^{125}I-IL-5 to TF-1.8 cells which express high affinity receptors for all three ligands. It was found that MoAb QP1, but not the other anti-β_c MoAbs nor the control MoAb, inhibit the high affinity binding of IL-3, GM-CSF and IL-5 (figure 6-1). All the inhibitions were demonstrated in a dose-dependent manner. The dosages of MoAb QP1 that produced 50% inhibition of ligand binding ranged from 30 to 100 nM. In the IL-5 binding assay (figure 6-1) MoAb QP1 did not completely eliminate IL-5 binding. This may be explained by the fact that the antibody only inhibits ligand binding to the β_c and thus only blocks the high affinity binding of IL-5. As the affinity difference between low and high affity IL-5 receptor is much narrower than that of IL-3R or GM-CSFR, when IL-5 was applied at 50 pM it can still demonstrate some degree of low affinity binding to the α-chain and this portion of binding can not be neutralised by this anti-β_c MoAb.

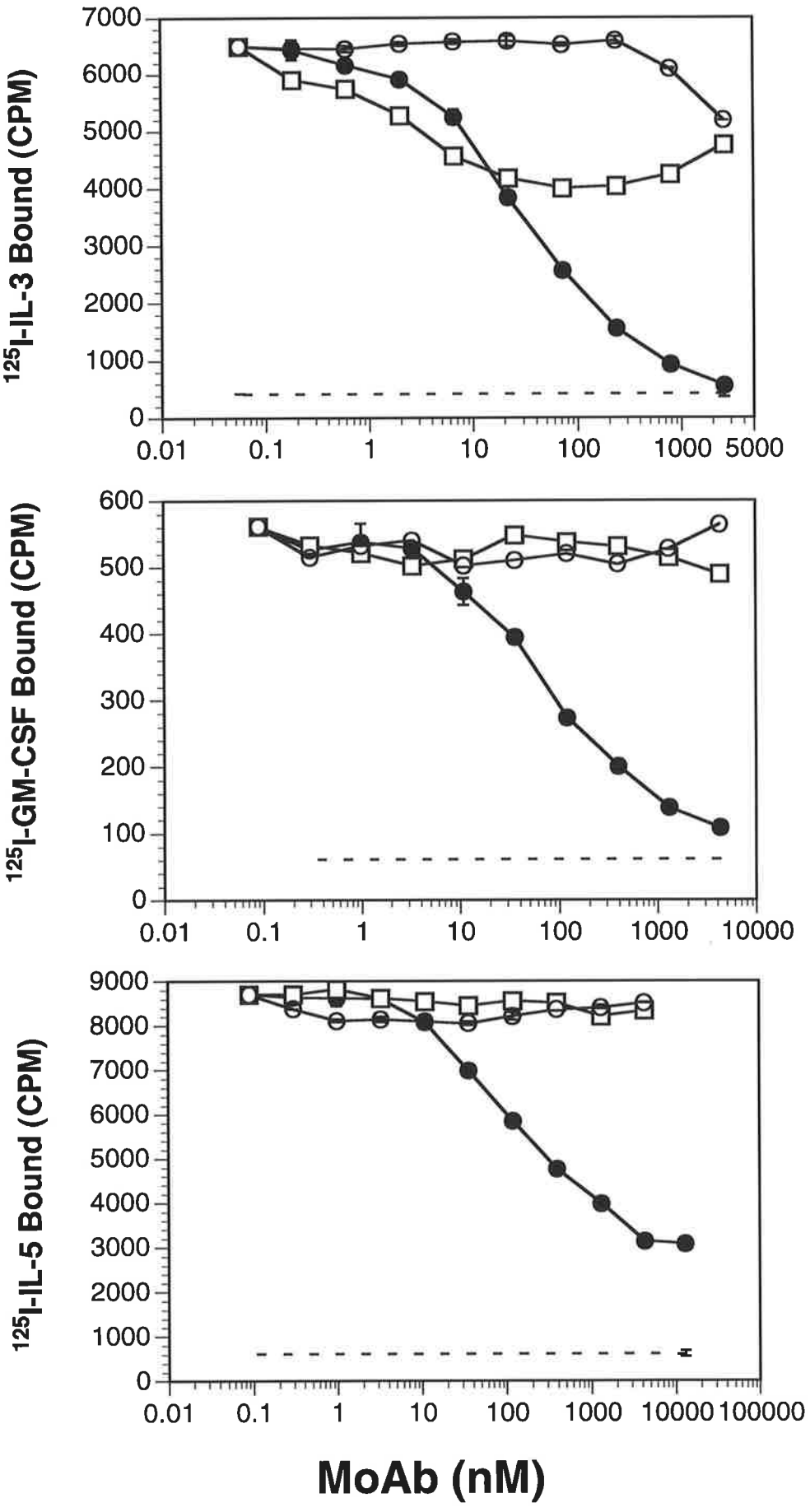
6.2 The Affinity of MoAb QP1 to β_c

To determine the affinity of QP1, the MoAb QP1 was radio-labelled and saturation binding of MoAb QP1 to TF-1.8 cells set up. Scatchard transformation analysis suggests that the affinity of MoAb QP1 is around 2 nM (figure 6-2). This affinity correlates well with the affinities of other neutralising MoAbs to anti-IL-3R α-chain (Sun *et al.,* 1996) and the anti-GM-CSFR α-chain (Nicola *et al.,* 1993).

6.3 Inhibition of anti-β_c MoAb binding by IL-3, GM-CSF and IL-5

To investigate whether the three cytokines could also reciprocally inhibit the binding of anti-β_c MoAb QP1, binding assays were set up to test the binding of radio-labelled MoAb QP1 to TF-1.8 cells in the presence of varied concentrations of IL-3, or GM-CSF, or IL-5. We found (figure 6-3) that IL-3 and GM-CSF

Figure 6-1. Dose-dependent competition for the binding of ^{125}I-IL-3 (200pM), ^{125}I-GM-CSF (50pM) and ^{125}I-IL-5 (50pM) by MoAbs QP1 (●), 1C1 (□) and a negative control MoAb 3D3 (○) to TF-1.8 cells. (---) represents ligand binding in the presence of 200-fold excess unlabelled ligand. Each point is the mean of triplicate determinations, and error bars represent standard deviations.


125I-IL-3 Bound (CPM)
7000
6000
5000
4000
3000
2000
1000
0
0.01
0.1
1
10
100
1000
5000
125I-GM-CSF Bound (CPM)
600
500
400
300
200
100
0
0.01
0.1
1
10
100
1000
10000
125I-IL-5 Bound (CPM)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0.01
0.1
1
10
100
1000
10000
100000
MoAb (nM)

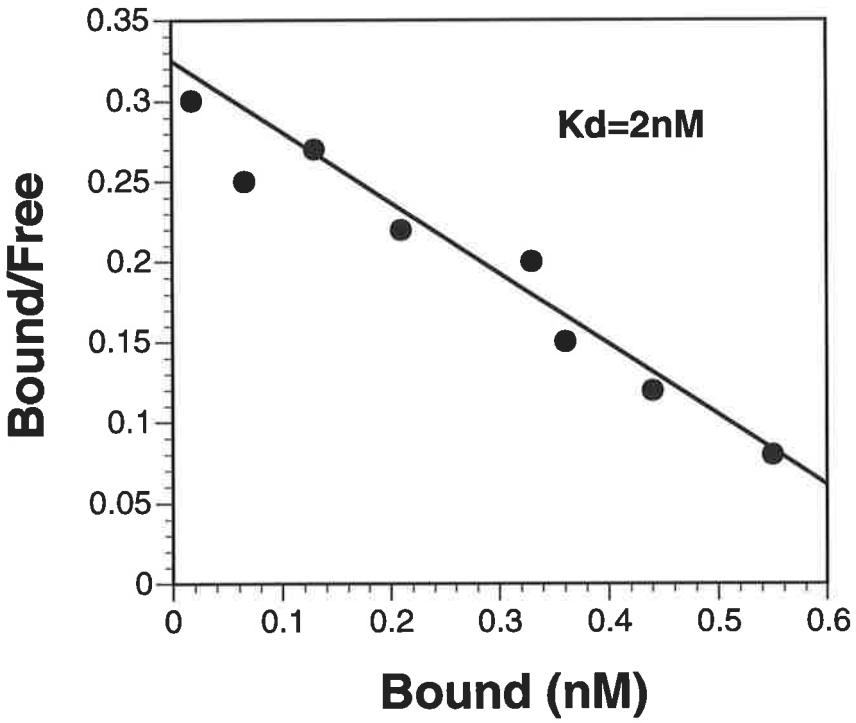


Figure 6-2. Scatchard transformation of a saturation binding curve using ^{125}I-QP1 on TF-1.8 cells

produced a dose-dependent inhibition of MoAb QP1 binding to TF1.8 cells, but IL-5 failed to show any inhibiting effect even at the concentration up to 3000 nM. Human TNF showed no inhibiting effect on MoAb QP1 binding. The half maximal inhibition by IL-3 occurred at concentration of 0.2 nM, for GM-CSF at around 200 nM. This difference may suggest that the binding site of MoAb QP1 is more related to the IL-3 binding site and to a lesser extent the GM-CSF binding site. But more likely, this difference may be due to the affinity of ligands to the β_c. Ligand binding studies have revealed that in affinity conversion from low to high affinity, β_c increases IL-3 affinity 500~1000 fold (Kitamura *et al.*, 1991; 1992), GM-CSF binding affinity 20~100 fold (Hayashida *et al.*, 1990). So IL-3 may bind more stronger than GM-CSF to the β_c.

6.4 Inhibition of IL-3-induced α and β chain dimerisation and phosphorylation

We have showed previously that IL-3 quickly induced IL-3R α and β chain dimerisation and β_c phosphorylation (Stomski et al., 1996). MoAb QP1 was also tested for its ability to inhibit such IL-3 induced phenomena. The experiment was performed by preincubating MO7e cells with or without IL-3 in the presence of MoAb QP1 or the control MoAbs before performing immunoprecipitation on ^{125}I-surface-labelled cells with anti-IL-3Rα and anti-β_c MoAbs. The same samples were also probed subsequently with anti-phosphotyrosine antibody by Western blotting. The results in figure 6-4 showed that IL-3R α and β chain dimerisation occurred in the presence of IL-3 and IL-3 plus the non-neutralising anti-β_c MoAb 1C1. However, when the MoAb QP1 or a neutralising anti-IL-3Rα MoAb 7G3 was present, both neutralising MoAbs blocked the IL-3-induced α and β chain dimerisation. In addition, no β_c phosphrylation can be detected (figure 6-4) accordingly. These data suggest that MoAb QP1 inhibits IL-3 signalling by preventing α and β chain association.

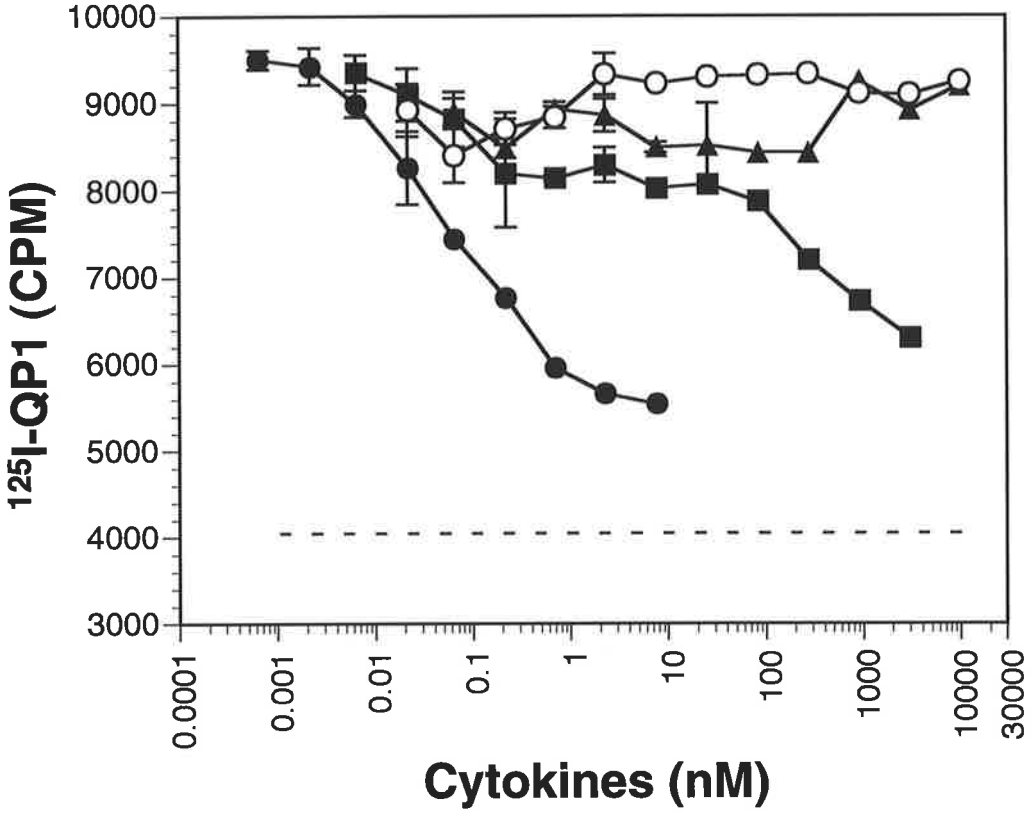
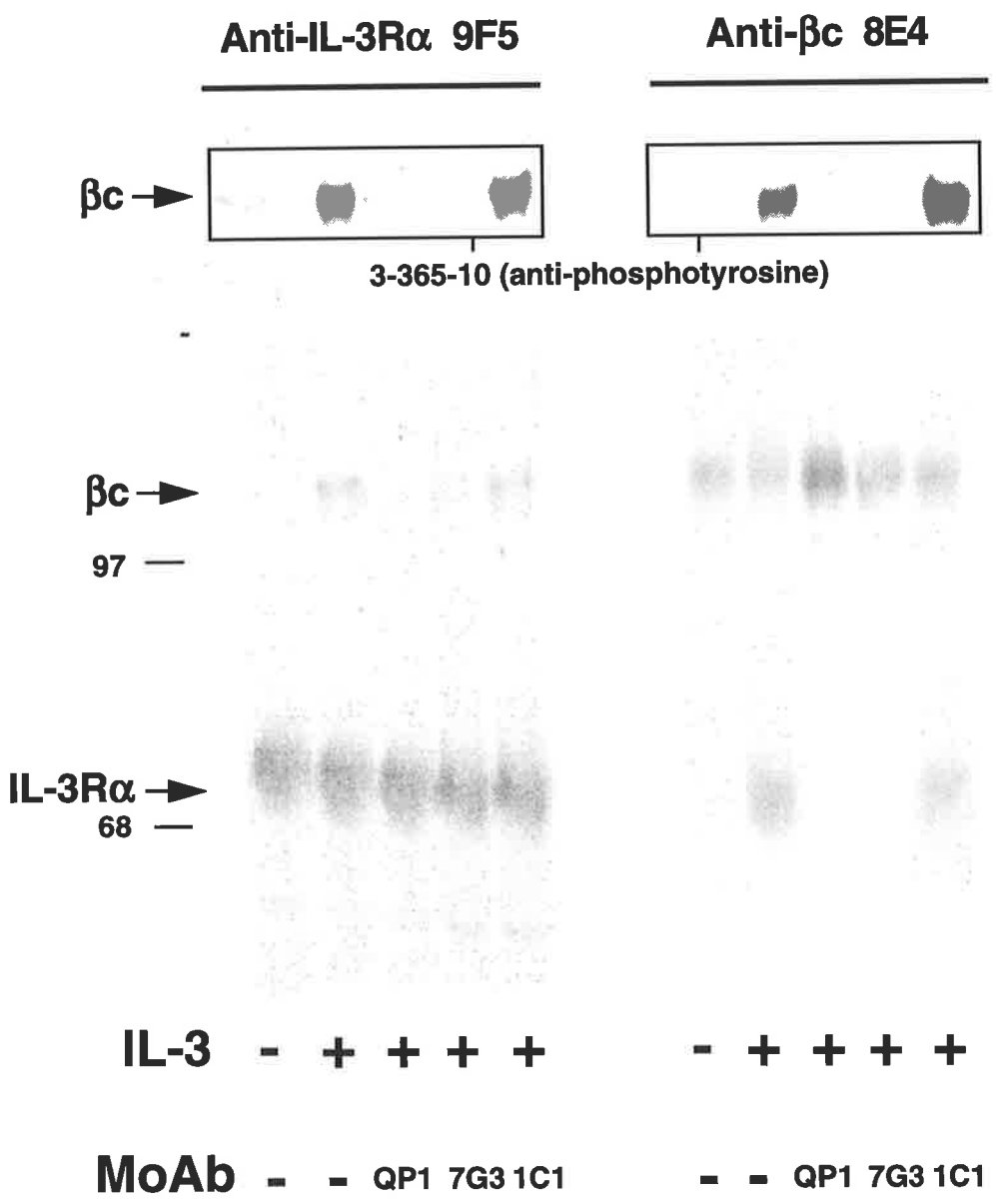


Figure 6-3. Dose-dependent competition for ^{125}I-QP1 (1nM) binding to TF-1.8 cells by IL-3 (●), GM-CSF (■), IL-5 (▲) and TNF-α (O). (---) repesents inhibition in the presence of 200-fold excess unlabelled MoAb QP1.

Figure 6-4. Inhibition of IL-3-induced α and β chain dimerisation and phosphorylation. Immunoprecipitations using anti-IL-3Rα MoAb 9F5 or anti-β_c MoAb 8E4 from MO7e cells preincubated with MoAbs QP1, 7G3 and 1C1 or medium alone (-) for 1 min, and then treated (+) or not treated (-) with IL-3 (50nM) for 5 min. The figure was visualised by PhosphorImaging and the position and molecular weight (in thousands) of marker proteins are shown to the left of the gels. The gels were reprobed by Western blotting analysis using anti-phosphotyrosine MoAb 3-365-10 and the top panels show the image of part of the gels in the β_c area.


Anti-IL-3Rα 9F5
Anti-βc 8E4
βc
3-365-10 (anti-phosphotyrosine)
βc
97
IL-3Rα
68
IL-3 - + + + + - + + + +
MoAb - - QP1 7G3 1C1 - - QP1 7G3 1C1

6.5 Neutralisation of ligand-dependent growth by the anti-β_c MoAbs

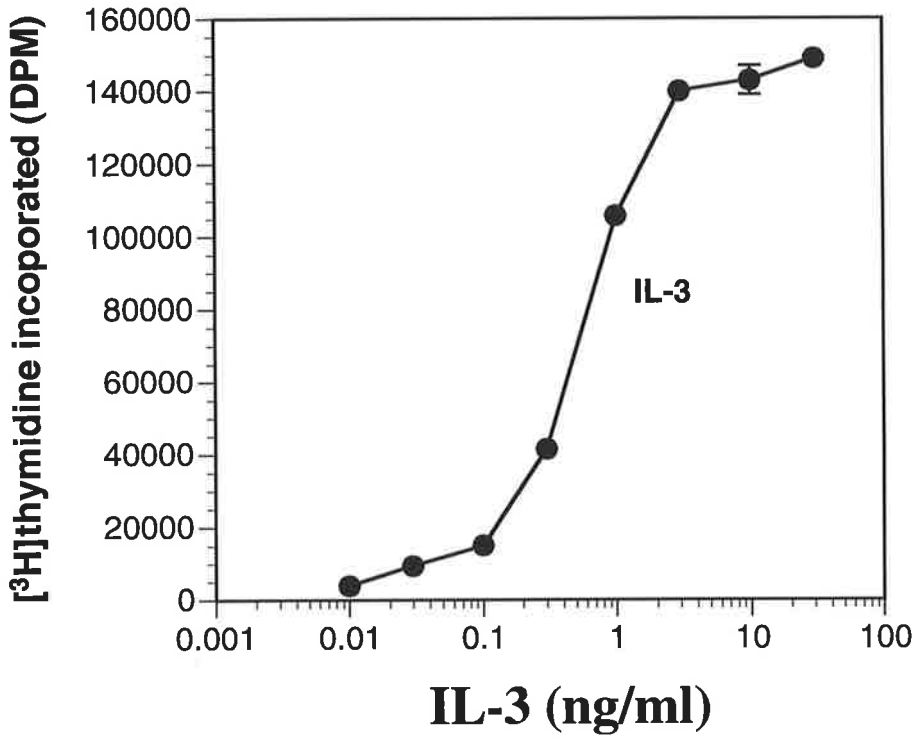
To assay the bioactivity of the anti-β_c MoAbs, we used TF1.8 cells which are dependent on IL-3, GM-CSF, IL-5, or EPO for their growth *in vitro*. The ligand dose-response studies (figure 6-5 to 6-8) indicated that the dosages for half-maximal proliferation of TF-1.8 cells by IL-3, GM-CSF, IL-5 or EPO were 0.3 ng/ml, 0.03 ng/ml, 3 ng/ml and 5ng/ml respectively. The anti-β_c MoAbs were tested for their capacities to block the proliferation of TF-1.8 cells, stimulated by each of the four ligands at the concentration supporting half-maximal proliferation. It was shown that MoAb QP1 could inhibit the ligand-dependent proliferation stimulated by IL-3 (figure 6-5), GM-CSF (Figure 6-6), or IL-5 (figure 6-7) in a dose-dependent manner, while non-neutralising anti-β_c MoAb 1C1 and 8B8, and the control MoAb failed to show any inhibiting effects. The concentration of 50% maximal inhibition produced by the neutralising MoAbs QP1 was consistent with that demonstrated by ligand binding assays. In contrast, MoAb QP1 did not show any inhibition of EPO (figure 6-8) stimulated proliferation with the same batch of TF-1.8 cell, which indicates that the effect of this antibody was specific to IL-3, GM-CSF and IL-5, which share the receptor β_c.

6.6 Antagonising of neutrophil superoxide production.

As GM-CSF is a strong enhancer of the superoxide production from human neutrophils, the inhibiting activity of QP1 was tested. MoAb QP1 could specifically inhibit the superoxide production (figure 6-9), with a half-maximal inhibition dose corresponding to that in the proliferation assays. In this test QP1 was used in the form of a whole MoAb. It would have been preferable to use Fab fragment instead of whole antibody in order to prevent non-specific Ig interaction with the neutrophil Fc receptor. However, the data here seem very reliable as the control MoAbs (one irrelevant, another non-neutralising anti-β_c) with the same isotype as QP1 failed to produce any inhibiting activity of neutrophil superoxide production.

Figure 6-5. Inhibition of IL-3-mediated proliferation of TF-1.8 cells. (A) TF-1.8 cell proliferation in response to different concentration of IL-3. (B) TF-1.8 cell proliferation stimulated by 0.3ng/ml IL-3 in the presence of MoAbs QP1 (●), 1C1 (■) and a negative control MoAb 3D3 (O). Each point is the mean of triplicate determinations, and error bars represent standard deviations.

A


[3H]thymidine incoporated (DPM)
160000
140000
120000
100000
80000
60000
40000
20000
0
0.001
0.01
0.1
1
10
100
IL-3
IL-3 (ng/ml)

B

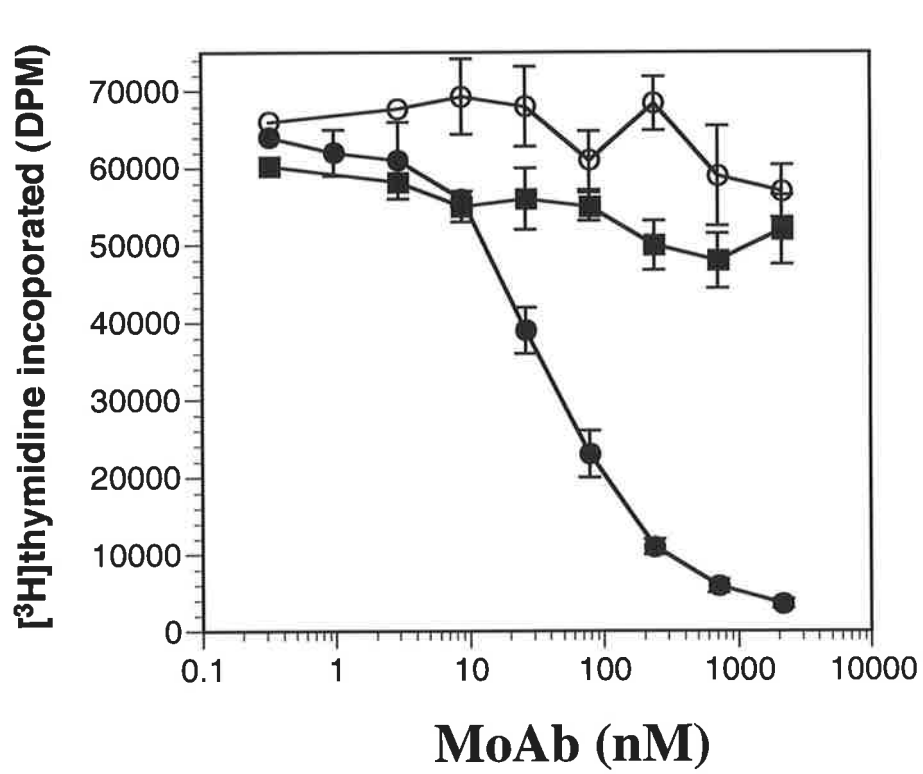
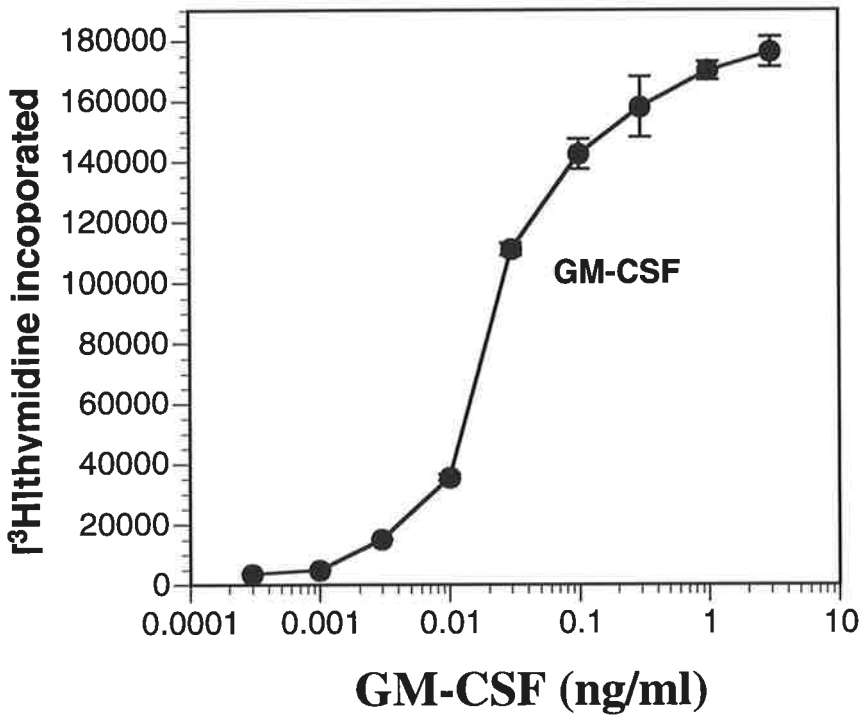

[3H]thymidine incoporated (DPM)
70000
60000
50000
40000
30000
20000
10000
0
0.1
1
10
100
1000
10000
MoAb (nM)

Figure 6-6. Inhibition of GM-CSF-mediated proliferation of TF-1.8 cells. (A) TF-1.8 cell proliferation in response to different concentration of GM-CSF. (B) TF-1.8 cell proliferation stimulated by 0.03ng/ml GM-CSF in the presence of MoAbs QP1 (●), 1C1 (■) and a negative control MoAb 3D3 (○). Each point is the mean of triplicate determinations, and error bars represent standard deviations.

A


180000
160000
140000
120000
100000
80000
60000
40000
20000
0
[3H]thymidine incoporated
GM-CSF
0.0001
0.001
0.01
0.1
1
10
GM-CSF (ng/ml)

B

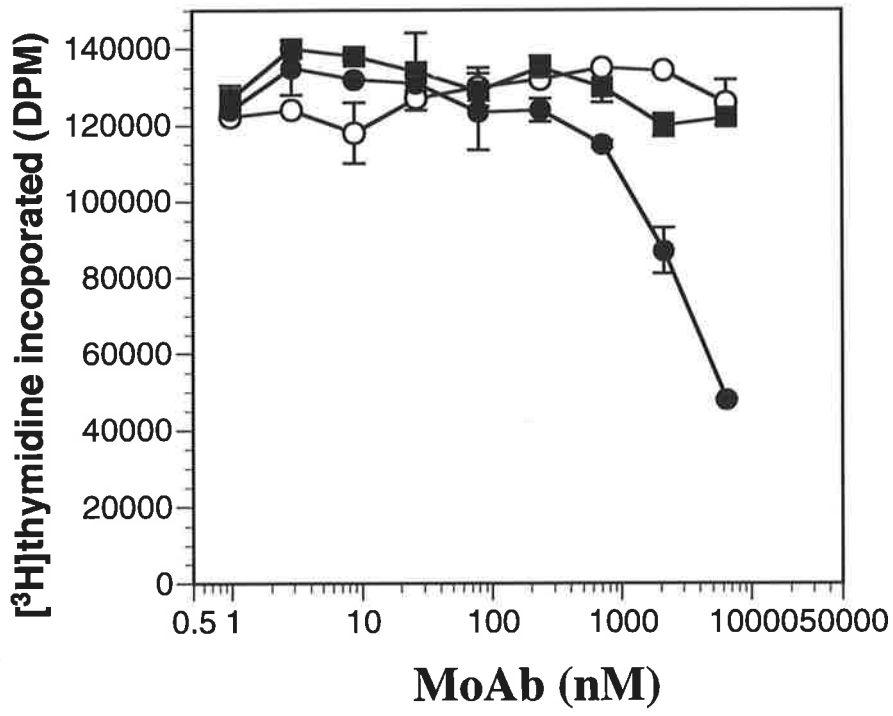
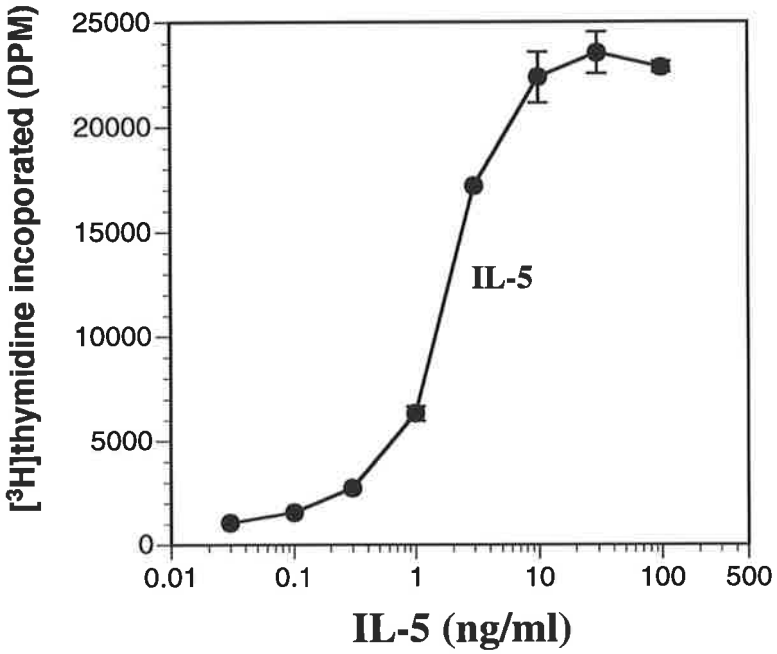

140000
120000
100000
80000
60000
40000
20000
0
[3H]thymidine incoporated (DPM)
0.5 1
10
100
1000
1000050000
MoAb (nM)

Figure 6-7. Inhibition of IL-5-mediated proliferation of TF-1.8 cells. (A) TF-1.8 cell proliferation in response to different concentration of IL-5. (B) TF-1.8 cell proliferation stimulated by 3ng/ml IL-5 in the presence of MoAbs QP1 (●), 1C1 (■) and a negative control MoAb 3D3 (○). Each point is the mean of triplicate determinations, and error bars represent standard deviations.

A


[3H]thymidine incoporated (DPM)
25000
20000
15000
10000
5000
0
0.01
0.1
1
10
100
500
IL-5
IL-5 (ng/ml)

B

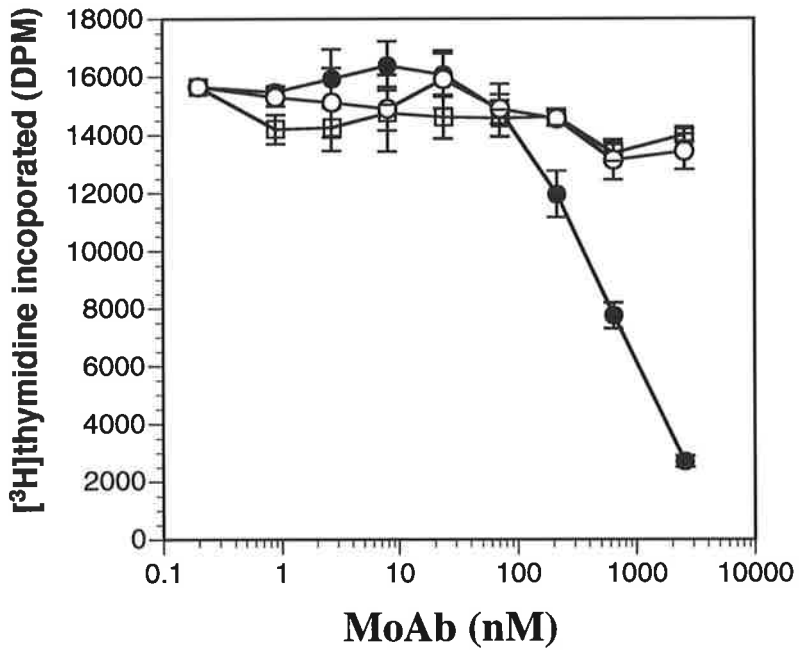
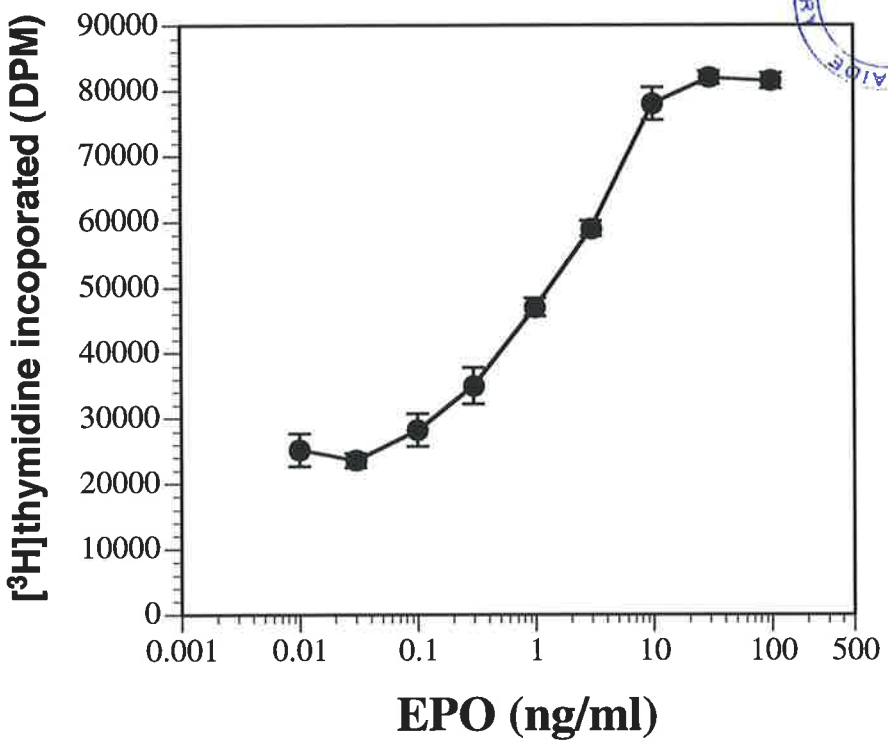

[3H]thymidine incoporated (DPM)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.1
1
10
100
1000
10000
MoAb (nM)

Figure 6-8. Inhibition of EPO-mediated proliferation of TF-1.8 cells. (A) TF-1.8 cell proliferation in response to different concentration of EPO. (B) TF-1.8 cell proliferation stimulated by 5ng/ml EPO in the presence of MoAbs QP1 (●), 1C1 (■) and a negative control MoAb 3D3 (O). Each point is the mean of triplicate determinations, and error bars represent standard deviations.

A



B

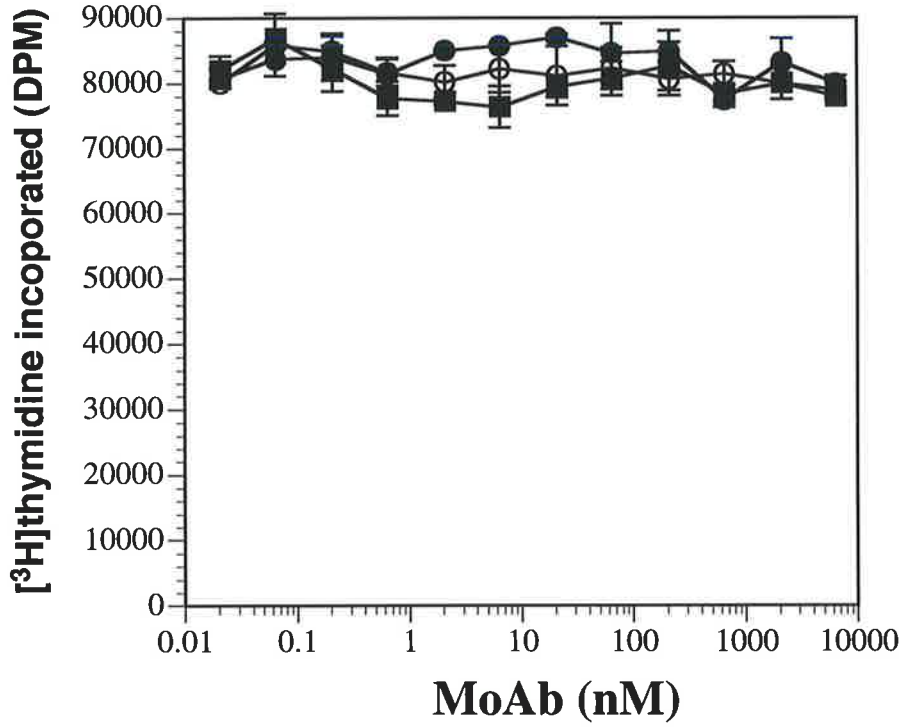
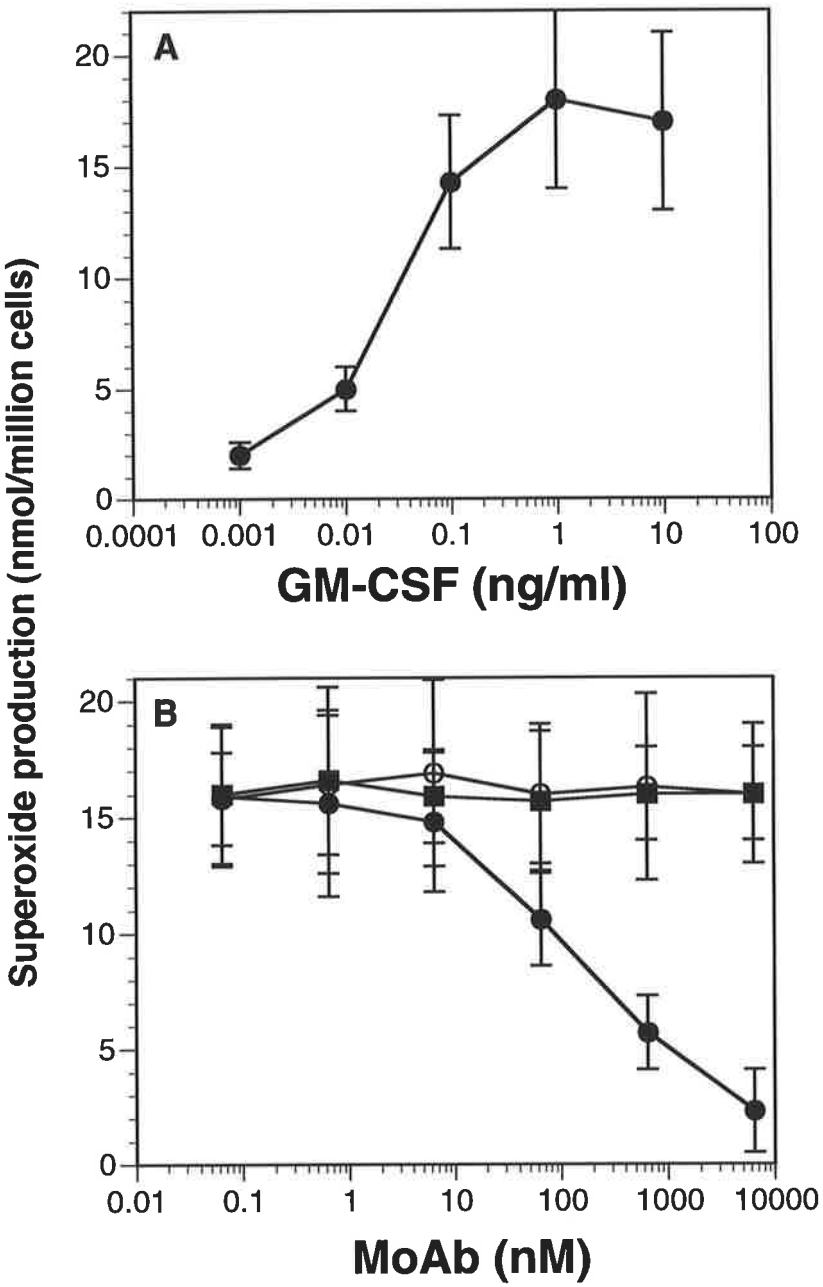


Figure 6-9. Inhibition of GM-CSF-stimulated neutrophil superoxide production. (A) neutrophil superoxide production in response to different concentration of GM-CSF. (B) neutrophil superoxide production stimulated by 1ng/ml GM-CSF in the presence of anti-βc MoAbs QP1 (●), 1C1 (■) and a negative control MoAb 3D3 (O). Each point is the mean of triplicate determinations, and error bars represent standard deviations.


A
20
15
10
5
0
0.0001
0.001
0.01
0.1
1
10
100
GM-CSF (ng/ml)
B
20
15
10
5
0
0.01
0.1
1
10
100
1000
10000
MoAb (nM)
Superoxide production (nmol/million cells)

6.7 Inhibition of IL-5-stimulated CD69 up-regulation on human eosinophils

CD69 is an early activation marker for human lymphocytes (Hara *et al.*, 1986). However, CD69 expression on eosinophils can be induced dramatically by GM-CSF, IL-3 and IL-5 although the significance of that up-regulation has not been fully understood (Hartnell *et al.*, 1993). In this study, IL-5 stimulated CD69 expression was tested by means of FACS analysis. Eosinophils from three donors were preincubated with MoAb QP1 or non-neutralising MoAb 1C1 before staining with PE-conjugated anti-CD69 MoAb. The mean fluorescence intensities of anti-CD69 MoAb on those eosinophils were shown in figure 6-10. I found that MoAb QP1 could specifically inhibit the IL-5-stimulated CD69 up-regulation to the basal line level while the control MoAb 1C1 failed to show any inhibiting effects. The inhibiting dose of MoAb QP1 also correlates well with that in the binding studies.

6.8 Epitope mapping of MoAb QP1

I have confirmed that MoAb QP1 recognises domain 4 of the β_c (chapter III), while the other anti-β_c MoAbs such as 1C1 recognise domain 2 of β_c (our unpublished observation). In order to identify more precisely the binding epitope of MoAb QP1, we first tested its binding to a series of overlapping peptides of 14 amino acids in length spanning the full extracellular region of β_c. However, no specific binding was obtained (data not shown). This suggests that MoAb QP1 may recognise a conformational rather than a linear epitope.

I then investigated the epitope of the antibody using a series of existing β_c mutants with one or more residues substituted in domain 4. MoAb QP1 was tested for its ability to stain wild type β_c as well as β_c mutants expressed on COS cells by means of immunofluorescence, with an irrelevant MoAb as a negative control and a non-neutralising anti-β_c MoAb (recognises regions other than the mutated domain of β_c) as a positive control. On wild type β_c transfectants, both

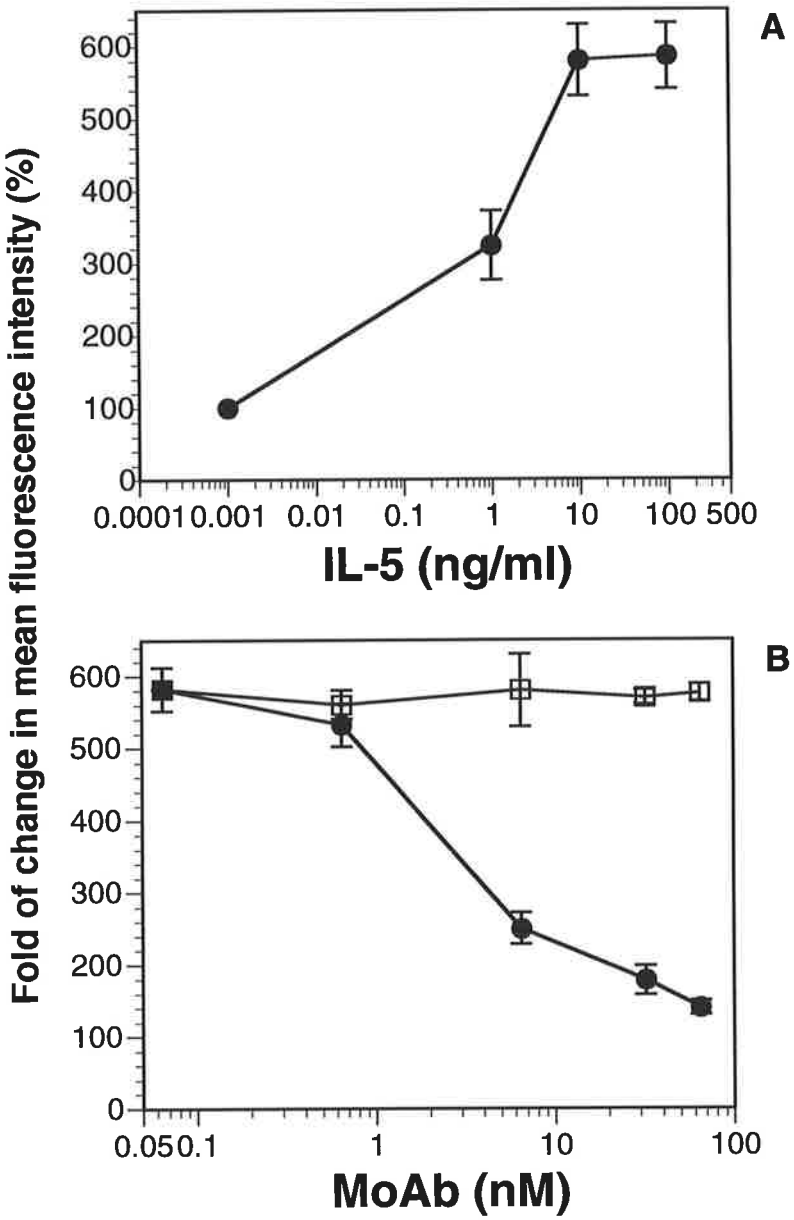


Figure 6-10. MoAb QP1 inhibits IL-5-stimulated CD69 up-regulation on human eosinophils. (A) CD69 up-regulation in response to IL-5. (B) CD69 up-regulation stimulated by 10ng/ml of IL-3 in the presence of anti-βc MoAbs QP1 (●) and 1C1 (O). Each point is the mean value of three samples from different donors, and error bars reprensent standard deviations.

MoAb QP1 and the non-neutralising anti-β_c MoAb showed the same profile(figure 6-11). On the β_c mutants with mutations in the B'-C' loop (figure 6-12) from residue M361 to H370 (Woodcock *et al.,* 1994), MoAb QP1 was unable to bind to the β_c mutant with point mutations of M363A/R364A, E366A and H367A. It also showed significantly decreased binding to β_c mutant D369A/H370A. But it maintained its identical staining to the rest of β_c mutants such as M361A/K362A, Y365A and I368A. In experiments on β_c mutants in the F'-G' loop (figure 6-13) from R418 to N422, MoAb QP1 failed to recognise mutant R418A and showed dramatically decreased binding to N422A mutant. Normal binding to the rest of the mutants such as T419A, G420A and Y421A was observed with MoAb QP1. In addition, I also tested MoAb QP1 on some β_c mutants in the B' and C' strands. Interestingly MoAb QP1 recognises all four mutants tested the same way as the non-neutralising anti-β_c MoAb. These mutants include L356N, W358N, I374N and Y377N (data not shown). In these experiments, the non-neutralising anti-β_c MoAb 1C1 which recognises domain 2 of β_c can stain all the β_c mutants tested. The differences in FACS profiles among the mutants in figure 6-12 and 6-13 by MoAb 1C1 are due to the alterations of mutant β_c expression as a result of different batches of COS cells transfected. The overall data here suggests that MoAb QP1 may bind to a conformational epitope comprised of residues in the B'-C' and F'-G' loops. The epitope of QP1 may include at least residues M363/R364, E366, H367, D369/H370, R418 and N422.

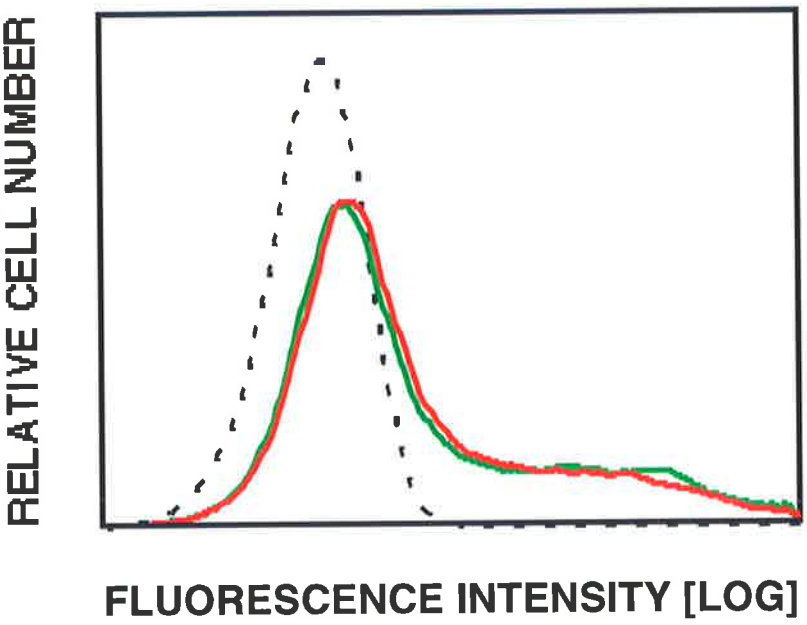
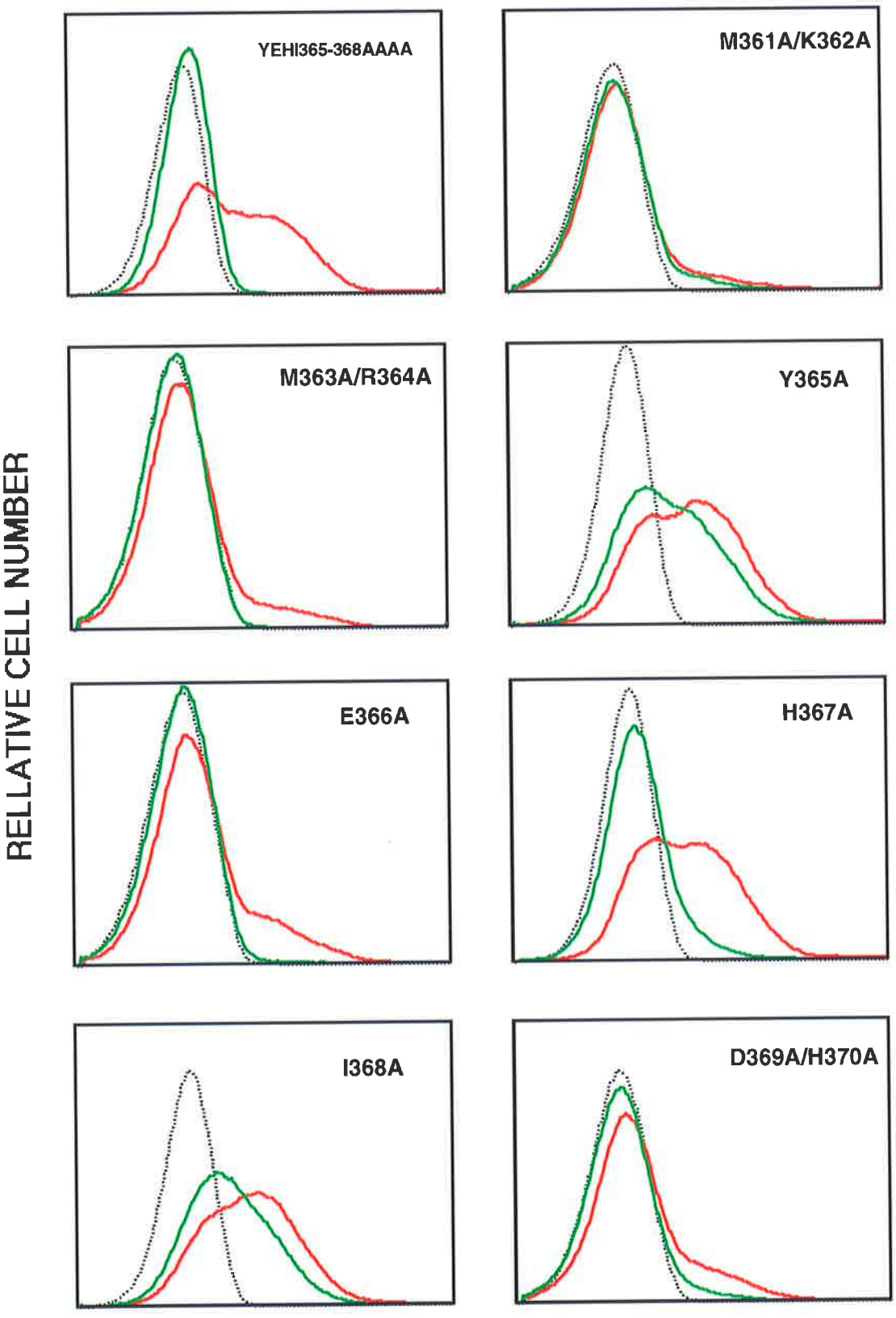


Figure 6-11. Flow cytometry analysis of COS cells expressing wild type β_c stained by anti-β_c MoAb QP1 (green), 1C1 (red) and a negative control MoAb 3D3 (black).

Figure 6-12. Flow cytometry analysis of COS cells expressing a variety of β_c mutants in the B'-C' loop stained by anti-β_c MoAbs QP1 (green), 1C1(red) and a negative control MoAb 3D3 (black).


YEHI365-368AAAA
M361A/K362A
M363A/R364A
Y365A
E366A
H367A
I368A
D369A/H370A
RELLATIVE CELL NUMBER
FLUORESCENCE INTENSITY [LOG]

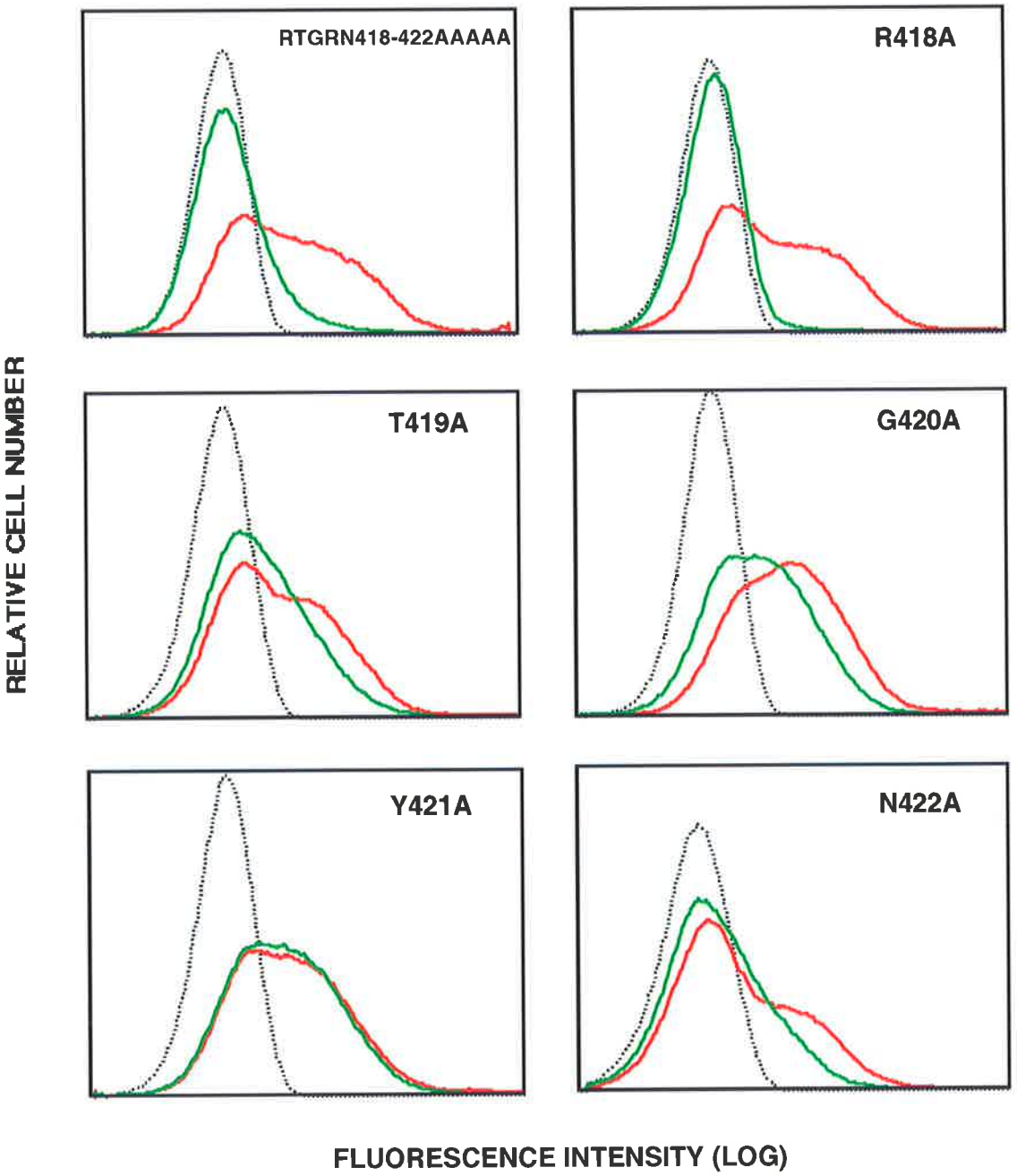


Figure 6-13. Flow cytometry analysis of COS cells expressing a variety of β_c mutants in the F'-G' loop stained by MoAbs QP-1 (green), 1C1 (red) and a negative control MoAb 3D3 (black).

Discussion

I have developed a panel of MoAbs to the common β_c of the human IL-3, GM-CSF and IL-5 receptors in chapter III. In this chapter a neutralising anti-β_c MoAb, QP1 that binds to the functional domain of the β_c, is analysed in detail. Importantly, I show here that the epitope of MoAb QP1 was in domain 4 of the β-chain, and more precisely may be on the B'-C' and F'-G' loops in domain 4. These data imply that the ligand binding sites may lie on a conformational site consisting of B'-C' and F'-G' loops.

MoAb QP1 is the first reported MoAb that exhibits antagonising activity to the β_c. It blocks the binding of IL-3, GM-CSF and IL-5 to their high affinity receptors and, accordingly, inhibits ligand-induced receptor phosphorylation. It can also inhibit the cytokine stimulated proliferative response *in vitro*. Importantly MoAb QP1 can also specifically block the appropriate cytokine activation on some human primary cells. It completely inhibit GM-CSF-stimulated superoxide production from neutrophils and IL-5-stimulated CD69 up-regulation on eosinophils. MoAb QP1 produced 50% maximal inhibition of binding and function of the appropriate ligand at the concentration of 30 ~ 100 nM, with complete inhibition at 2000 nM. This inhibitive dose range is about 30 ~ 100 times higher than that of neutralising MoAb 7G3 to IL-3R α-chain (Sun *et al.*, 1996), although the affinities of MoAb QP1 to the β_c and MoAb 7G3 to IL-3Rα are very similar.

Although MoAb QP1 is less potent than the neutralising anti-α-chain MoAb in terms of its inhibiting effects, it may be more useful over the anti-α-chain antibody in terms of the antagonistic significance. In many physiological and pathological conditions, all three cytokines function simultaneously and have overlapping or synergistic activities. For example, in inflammation, effector cells such as eosinophils (Lopez *et al.*, 1989), monocytes (Elliott *et al.*, 1989; 1992) and neutrophils (Lopez *et al.*, 1988; Smith *et al.*, 1995) are all activated by

combinations of cytokines such as IL-3, GM-CSF and IL-5. As MoAb QP1 can block the function of all three cytokines, it may be more powerful in controlling the excessive stimulation of these effector cells in conditions like allergy and some other inflammatory reactions. In addition, many studies have suggested that IL-3 and GM-CSF can promote leukaemic cell growth *in vitro* (reviewed by Lowenberg and Touw, 1992). MoAb QP1 may have a potential application in antagonising the IL-3, GM-CSF or IL-5 stimulated growth of leukaemia cells both *in vitro* and possibly *in vivo*.

I have mapped the epitope of MoAb QP1 to the domain 4 of β_c. From the study of ligand and receptor modelling it was predicted that domain 4 may the interacting site between ligand and the β_c. Experiments with β_c truncation mutants have also suggested that the domain 4 may be essential to maintain the ligand-independent proliferation of the transfected cells (D'Andrea *et al.*, 1995). So it is not surprising that when MoAb binds to domain 4 it may modify the structure of domain 4 and thus produce antagonising activity.

In competition assays, I found that MoAb QP1, IL-3 and GM-CSF reciprocally inhibited each other's binding to the β_c. This suggests a direct two-way competition between MoAb and IL-3, or GM-CSF for a common or adjacent binding sites. Therefore, molecular definition of the epitope of MoAb QP1 should help in defining the ligand interacting sites on β_c.

Since MoAb QP1 did not recognise any linear epitope in the β_c, I mapped the epitope of MoAb QP1 using substitution mutant β_c expressed on the surface of COS cells. It was demonstrated that MoAb QP1 failed to recognise some β_c mutants such as M363A/R364A, E366A and H367A in the B'-C' loop, and R418A in the F'-G' loop, while these mutants were definitely expressed on the cell surface. MoAb QP1 also dramatically lost its binding affinity to some other mutants such as D369A/H370A in B'-C' loop and N442A in the F'-G' loop. In contrast, it maintained binding to several other mutants tested with mutations

in the same loops and in the B' and C' strands. These data give evidence that this MoAb may recognise a conformational epitope covering the B'-C' and F'-G' loops. Since the FACS analysis does not alter the structure of β_c, it may also imply that the residues Met^{363}/Arg^{364}, Glu^{366}, His^{367}, Asp^{369}/His^{370}, Arg^{418} and Asn^{422} may be close to each other in the three dimensional structure of β_c and this region may involve ligand interactions.

This finding correlates very well with the binding studies of β_c residue substitution mutants (Woodcock *et al.*, 1994, 1996) in which we showed that Tyr^{365}, His^{367} and Ile^{368} in the B'-C' loop were important for the high affinity binding of GM-CSF and IL-5 while Tyr^{421} in the F'-G' loop was essential for the binding of all three ligands. More interestingly, from the modelling studies using the GH-GHR complex as a template, the B'-C' and F'-G' loops of β_c lie close together and form a cleft which faces the ligand (Woodcock *et al.*, 1996; Bagley *et al.*, in press).

The data shown in this chapter provide more evidence that the B'-C' and F'-G' loops are the functional domains in the β_c. Support for this notion also stems from the EPO study (Liunak *et al.*, 1996; Wrighton *et al.*, 1996) which showed that a 20-residue cyclic peptide unrelated to the native EPO targeted to the B'-C' and F'-G' loops of the EPO receptor produced functional mimicry. In addition, structural and functional studies of the GH and its receptor complex also revealed that the B'-C' and F'-G' loops in the GHR were part of the interactive determinants and responsible to for GH binding and function (De Vos *et al.*, 1992; Rowlinson *et al.*, 1994). By analogy, this may also imply that the B'-C' and F'-G' loops may also be the functional domains for other receptor chains in the cytokine receptor superfamily, especially those that are analogous to the β_c for IL-3, GM-CSF and IL-5 receptor.

CHAPTER VII

GENERAL DISCUSSION

GM-CSF, IL-3 and IL-5 are a group of well known glycoprotein cytokines. Since their discovery two decades ago, and especially since the cloning of these cytokines, their physiological roles in both human and mice have been studied extensively. All three cytokines are important regulators of haemopoiesis and inflammation. GM-CSF and IL-3 are more broad spectrum cytokines, while IL-5 mainly functions on the eosinophil and basophil lineages. The properties of these cytokines have led to many clinical applications. For example, GM-CSF and IL-3 have been shown to have beneficial effects in accelerating the haemopoietic recovery from the agranulocytosis following bone marrow transplantation (Nemunaitis *et al.*, 1990) or chemotherapy (Tylor *et al.*, 1989; Gulati and Bennett, 1990). Both cytokines have also been applied to stimulate the differentiation of haemopoietic stem cells for the *in vitro* expansion of bone marrow cells in order to shorten the agranulocytosis period after stem cell transplantation (Haylock *et al.*, 1992). In addition, GM-CSF and IL-3 have been used for the stem cell mobilisation technique which is widely applied as an important therapeutic approach for haemopoietic malignancies as well as some solid tumours (Gianni *et al.*, 1990; Brugger *et al.*, 1992). More recently, many attempts have been made to use GM-CSF and IL-3 to help rescue the haemopoietic and immune systems in the immuno-compromised individuals such as AIDS patients (Hermans, 1995; Scadden *et al.*, 1994; Matsuda *et al.*, 1995).

Although these cytokines are being used in many clinical situations, their roles in the real physiological setting is still somewhat controversial. For instance, no IL-3 is found either in the bone marrow or in the circulation (Garland *et al.*, 1983; Crapper *et al.*, 1984; Cheers *et al.*, 1988; Cluitmans *et al.*, 1995). Transgenic mice, with IL-3, GM-CSF or IL-5 genes knocked out respectively, still undergo nearly normal haemopoiesis but with impaired immune responses. Some clinical studies from the patients using IL-3 suggest that IL-3 may lead to some significant side-effects such as bone marrow fibrosis (Falk *et al.*, 1991). From this respect, GM-CSF, IL-3 and IL-5 may be regarded as "reactive" rather than

"steady" state cytokines. The production of these cytokines may lead to desirable as well as potentially deleterious effects; while overproduction may be associated with the pathogenesis of certain diseases such as some leukaemias and lymphomas, allergy, and possibly arthritis. From this point of view, reagents that can neutralise the function of these cytokines may have beneficial effects in these disease conditions. A GM-CSF antagonist has been reported (Hercus et al., 1994) and its application in many disease conditions is under investigation.

GM-CSF, IL-3 and IL-5 function through their specific cell surface receptors. Since the cloning of genes for GM-CSFRα (Gearing *et al.,* 1989), IL-3Rα (Kitamura *et al.,* 1991), IL-5Rα (Tavernier *et al.,* 1991) and the β_c (Hayashida *et al.*, 1990), the research on these receptors has advanced very rapidly. The progress of this work is mainly focussed on three areas:

(I). **Receptor expression and regulation**: Pioneering investigators on GM-CSF, IL-3 and IL-5 receptors typically applied binding assays with radio-labelled cytokines. As binding assays need a large quantity of cells, most of the investigations were limited to cell lines and purified human primary cells such as neutrophils, monocytes and eosinophils. With the availability of MoAbs to cytokine receptors (MoAbs described in this thesis and MoAbs described by Sato *et al.*, 1993 and Nicola *et al.*, 1993), the investigations have extended to other cells that can be purified only with difficulty or can hardly be obtained in large numbers such as CD34 + cells (Sato et al., 1993 and chapter IV), basophils (chapter IV), HUVEC (chapter IV) and more recently, breast cancer cells (our unpublished observation,). The overexpression of receptors in some leukaemic cells suggests that aberrant receptor expression plays a role in these disorders (reviewed by Lowenberg and Touw, 1993).

(II). **Functional domains of cytokine receptors**: Based on the alignment of conservative residues of IL-3Rα, GMRα, IL-5Rα and β_c with the GH receptor (Bazan, 1990, Goodall *et al.,* 1992), functional domains and residues were

predicted. The predicted domains or residues were then mutated and the activity of these mutants were examined by ligand binding analysis. Several mutants (reviewed in chapter I) have been found to diminish the ligand binding or receptor activation. For example, it was suggested that some mutants in the B'-C' or F'-G' loops of domain 4 of β_c could abolish the binding of IL-3, GM-CSF or IL-5 in different patterns, raising the importance of these loops in receptor binding and activation (Woodcock *et al.,* 1994; 1996).

(III). **Receptor activation and signal transduction**: During receptor activation, it was suggested that the initial event following ligand binding is the receptor α and β chain dimerization. Once the receptor is activated it leads to the very rapid phosphorylation of the β_c and then to the activation of various signalling molecules that are part of the Ras (Satoh *et al.,* 1992; Duronio *et al.,* 1992) and the JAK-STAT (reviewed by Darnell *et al.,* 1994) pathways. The regulation and precise function of these kinase pathways is being currently under investigation.

However, although these receptors have been studied extensively, some fundamental questions are still unknown, such as the overall pattern of receptor expression and distribution, the ligand binding sites on the α or β chain, and the precise mechanism of receptor chain association and activation. A greater understanding of these receptors will help to address these important issues correctly and may also has implication for developing receptor antagonists.

In this thesis, I examined the receptors for IL-3, GM-CSF and IL-5 by means of MoAbs. Beside the development of four groups of MoAbs to the α and β chains of these receptors, I also isolated two neutralising MoAbs to IL-3Rα and the β_c respectively. These neutralising MoAbs could completely inhibit the binding and function of the appropriate cytokines accordingly. These MoAbs also open the potential of directly antagonising the cytokine functions in many clinical situations. More importantly, the epitope-mapping of the neutralising MoAbs

provides strong evidence of the location of functional domains of the α and β chains.

Chapter III commences this project by describing the production of more than 20 MoAbs specific to each of the α-chains and β-chain of GM-CSF, IL-3 and IL-5 receptors. These MoAbs comprise a unique group of reagents in the field of cytokine receptor both for their quantity and quality. They complement ligand binding experiments in detecting the expression of these receptors by introducing quick, stable and specific assays. The anti-β_c MoAbs are even more superior to the ligand binding assays in measuring β_c as β_c cannot bind the ligand on its own. As I have developed several MoAbs to each of the receptor subunits and these MoAbs may recognise different epitopes in the same protein molecule, these MoAbs may complement each other in identifying the appropriate receptor subunits. In addition, most of these MoAbs can also recognise the appropriate receptor subunits in denatured forms, which expands the application of these MoAbs.

The results in chapter IV examine the distribution, expression and regulation of receptor chains for IL-3, GM-CSF and IL-5 because the presence of these receptor subunits on the cell surface is the prerequisite for the response to the appropriate cytokine. The expression of these receptor chains in haemopoietic cells seems to follow a specific pattern: progenitor cells such as CD34+ cells express more α-chains but very limited β_c; while mature cells such as neutrophils, eosinophils, monocytes and basophils express relatively more β_c. It seems that the differentiation of the haemopoietic cells favours the up-regulation of receptors, especially for the β_c. This finding correlates well the the sequential expression of cytokine receptors in which stem cells are regulated by a net work of cytokines during the process of haemopoietic differentiation. Among the haemopoietic cells, the expression of cytokine receptors in neutrophils, monocytes and eosinophils was analysed in detail. The findings were in conformity with the previous studies with ligand binding assays, namely that eosinophils have all the receptor

chains, monocytes were deprived of IL-5Rα, but fresh neutrophils only express GMRα and β_c. In addition, for the first time I show here the pattern of cytokine receptor expression in human basophils. Basophils express a very high level of IL-3Rα and to a lesser degree the β_c. Such a characteristic has made it possible to purify basophils with the anti-IL-3Rα MoAbs. I also show here that some non-haemopoietic cells such as human endothelial cells also have cytokine receptors such as IL-3 receptor. However, the expression of these receptor on the cell surface is a dynamic process rather than a static one. For example, when the neutrophils were stimulated with GM-CSF, they began to express IL-3Rα (Smith *et al.*, 1995). Such an expression of IL-3Rα also led to the formation of high affinity receptors and the potential for receptor activation. The expression of IL-3 receptor on HUVEC can also be up and down-regulated by certain cytokines. Previous studies (Korpelainen *et al.*, 1993; 1995) have confirmed that the activation of endothelial cells by IL-3 lead to several biological activities in the process of inflammation such as the expression of MHC class II and production of chemokine IL-8.

The unique MoAbs described here also provide useful tools to analyse the α and β chain association and β chain phosphorylation by immunoprecipitation with anti-α-chain MoAb followed by western blotting analysis with anti-β_c MoAb, or anti-phosphotyrosine MoAb. We have established that the α- and β-chain heterodimerization of the IL-3 receptor is IL-3 induced (Stomski *et al.*, 1996) while that of the GM-CSF receptor seems to be at least partly pre-formed (Stomski, personal communication). The pattern of IL-5 receptor dimerization is under current investigation. These data indicate that the mechanisms of GM-CSF, IL-3 and IL-5 receptor activation are different despite all them having a predicted similar structure and a shared receptor component.

Chapter V and VI described two neutralising MoAbs to IL-3Rα and β_c respectively. The anti-IL-3Rα MoAb, 7G3, can inhibit the binding and function of IL-3 in various situations. The anti-β_c MoAb, QP1, on the other hand, can block

binding and functions of all three ligands, GM-CSF, IL-3 and IL-5, as they all use β_c in their receptor complex. Both neutralising MoAbs could induce complete blocking activity. However, the MoAb QP1 is 500~1000 less potent in antagonising IL-3 compared with MoAb 7G3. This neutralising dose difference is not surprising. Since IL-3 binds to IL-3Rα with low affinity so it is much easier for neutralising MoAb to compete with IL-3 to the α chain. However IL-3, GM-CSF and IL-5 bind to their α and β complex with much higher affinity, so more anti-β_c MoAb is needed to fulfil the same antagonism as MoAb to the α chain.

Both of the two neutralising MoAbs would be useful in *in vitro* functional analysis of GM-CSF, IL-3, IL-5 and their receptor. They also have potential significance in antagonising the ligand activities in many clinical settings. Many studies have indicated that the overstimulation by a variety of cytokines may lead to many disease conditions as described in chapter I. So the availability of the two neutralising MoAbs make it possible to control the excessive stimulation by GM-CSF, IL-3 or IL-5 by blocking their receptor. In terms of a neutralising dose, the anti-β_c MoAb may be less potent than the anti-α chain MoAbs. However, the anti-β_c MoAb may be superior in producing a satisfactory antagonising effect in some disease conditions, where GM-CSF, IL-3 and IL-5 may function simultaneously *in vivo*. Neutralising antibodies have been applied successfully as a therapeutic reagent in some disease, which opens a new field. For example an anti-IL-6 MoAb (Wendling *et al.*,, 1993) and anti-TNF-α MoAb (Elliott *et al.*, 1993) have been shown to be beneficial in treating rheumatoid arthritis (RA). A double-blind, placebo-controlled clinical trial had shown that *in vivo* blockage of TNF-α by the infusion of a chimaeric neutralising anti-TNF-α antibody, CA2, has proven to be highly effective in the treatment of RA (Elliott *et al.*,, 1994).

However, antibodies often need to be modified before conducting any clinical application in order to reduce its immunogenicity, which may be achieved by two approaches. The first is to produce Fab or Fv fragment of the neutralising MoAb.

The second is to make the MoAb more "humanised" with a molecular approach, such as generating human-murine chimaeric antibodies, or increasing the affinity of the antibody by affinity maturation and though phage display.

The work presented in Chapter V and VI is also in an attempt to identify the functional domains in cytokine receptors. Since both neutralising MoAbs compete directly with the ligand to the same or very close binding site, the epitope of these neutralising MoAbs may provide strong indication of the ligand binding site of these receptors. I mapped the epitopes of the two neutralising MoAbs. One of the striking findings is that location of epitopes of anti-IL-3Rα MoAb 7G3 is on the *N*-terminal domains of IL-3Rα, suggesting that the *N*-terminal domain may be involved in ligand binding. Although the GM-CSF, IL-3 and IL-5 receptor α-chains share certain structures among the cytokine receptor superfamily, the α-chain present a rather unique feature which has an additional *N*-terminal domain beside one copy of CRM. The *N*-terminal domains also demonstrate significant sequence similarity (16-24% identity; 39-47% similarity) among GM-CSF, IL-3 and IL-5 receptor α-chains (Goodall *et al.*, 1993). Importantly, each *N*-terminal domain possesses an odd number of cysteine residues, raising the possibility of forming disulphide bonds with either α-chain itself or with β_c. However, as the growth hormone receptor does not contain an additional *N*-terminal domain, modelling of the α-chain on the growth hormone receptor structure gives no indication of the structure or role of the *N*-terminal domain. The mapping data of MoAb 7G3 strongly suggested that the *N*-terminal domain of IL-3R α-chain participates in ligand binding. A further study with mutant forms of IL-3Rα also demonstrated that a truncated IL-3Rα with the *N*-terminal domain deleted, still form high and low affinity binding and the affinity for IL-3, but the high affinity is 14-fold decreased. These data suggest that the *N*-terminal domain of IL-3Rα is involved in ligand binding although is not essential. However, there is also the possibility that IL-3Rα *N*-terminal domain forms disulphides bonds with β_c as part of its high affinity binding site. As for the IL-5Rα, Cornelis *et al.* (1995).had reported that the *N*-terminal domain of IL-

5Rα was also necessary for the IL-5 binding. Since the *N*-terminal domains of GM-CSFR IL-3R and IL-5R α-chains shared a predicted similar structure (Goodall *et al.*, 1993), it is proposed that *N*-terminal domain of GM-CSFRα is also a functional domain.

Another interesting finding in this thesis is that the epitope of MoAb QP1 was on domain 4, more precisely in the B'-C' and F'-G' loops of β_c. The β_c is a key component of receptor activation, the identification of the ligand binding sites on the β_c has been a major interest among several research groups. Although the β_c contains two copies of CRM in its extracellular portion, it was thought that the ligand binding determinants may lie on the membrane proximal CRM, save for the known structure of GH receptor. From the crystal structure of the GH-GHR complex, three regions represented by the E'-F', B'-C' and F'-G' loops have been identified to be the contact sites between the ligand and the receptor (de Vos *et al.,* 1992). Mutagenesis studies in this region on the β_c suggested the ligand binding site on the β_c may have a different pattern compared with that of GHR. The mutagenesis of the E'-F'of β_c failed to show any effect on high affinity GM-CSF or IL-3 binding (Woodcock, personal communication). In contrast, the B'-C' and F'-G' loops of β_c appear to be involved in the ligand binding. Mutagenesis in these two regions have revealed that some residues such as His^{367} in the B'-C' loop and Tyr^{421} in the F'-G' loop are essential for the binding of one or all three ligands (Woodcock *et al.*, 1994, 1996; Lock *et al.*, 1994). The mapping data of MoAb QP1 gives strong evidence that B'-C' and F'-G'loops of β_c may lie close to each other in the three dimensional structure consistent with also forming the ligand binding site.

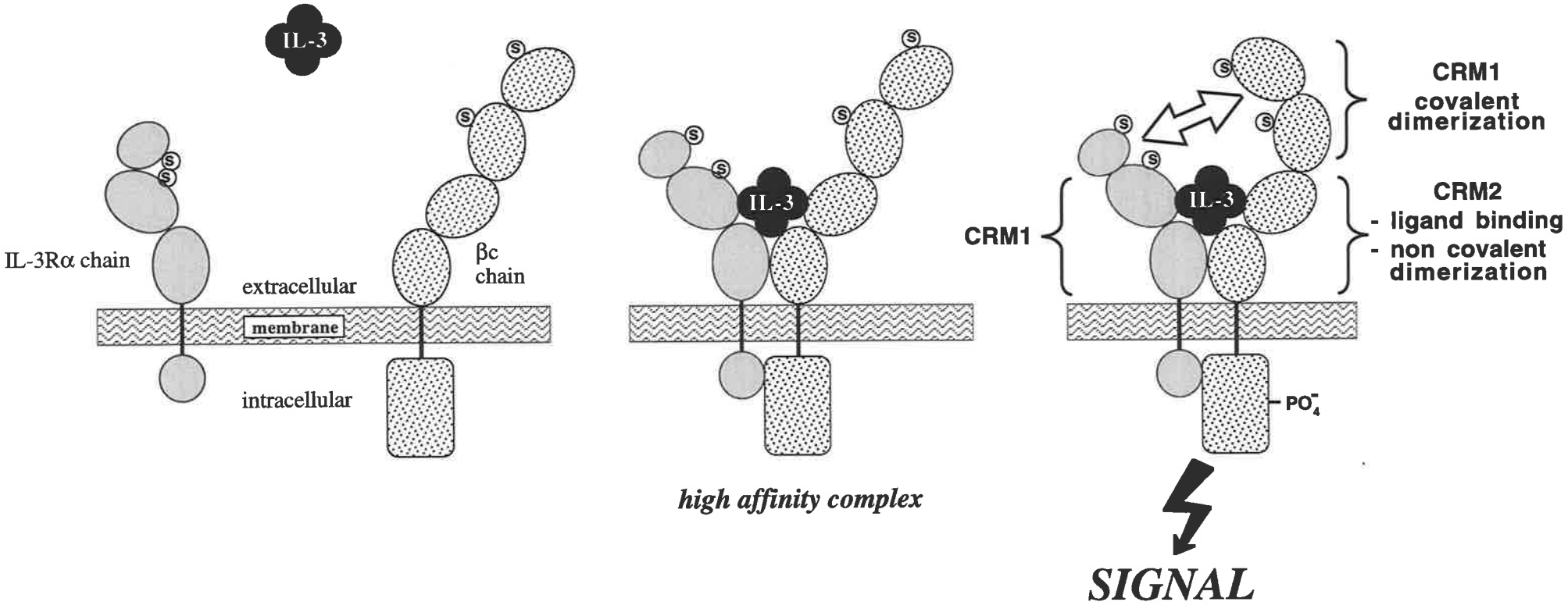
In addition, the neutralising MoAbs 7G3 and QP1 may help to determine the structure of the ligand binding sites by providing an approach with which to co-crystallise appropriate receptor subunits. Such attempts may favour the development of reagents that may cross-link the ligand-binding sites and may mimic the ligand to produce agonising or antagonising activities. An example of

this notion came from the EPO study (Liunak *et al.*, 1996) which showed that a 20-residue cyclic peptide, which was unrelated to the native EPO, targeted to the B'-C' and E'-F' loops of the EPO receptor and produced functional mimicry.

On the basis of the data presented above and the other data from our studies, I propose a model describing the activation of the IL-3R complex (figure7-1). In this model the *N*-terminal domain lies close to the domain 1 of IL-3Rα, save for the fact that both the *N*-terminal domain and domain 1 of IL-3Rα have an odd number of cysteine residues and may form a disulphide-linked interaction with each other. The *N*-terminal domain and domains 1, 2 of the CRM form the ligand binding cleft. The IL-3 binding to its binding site on the α-chain then induces interactions with the β_c and thus forms a ligand and receptor complex. In that complex, the disulphide-link between the *N*-terminal domain and domain 1 of IL-3R may break apart due to the structural modification resulting from IL-3 binding, which favours the new disulphide bond formation between the α- and the β_c. Such a hypothesis may explain why the disulphide link between the IL-3Rα and β chains is essential for receptor activation but not high affinity binding (Stomski *et al.*, 1996). However, although the overall tertiary structure is highly conserved among members of the cytokine receptor superfamily, and deapite the fact that IL-3Rα, GM-CSFRα and IL-5Rα chains are close by related to each other, further studies are needed to investigate weather such a model may be extended to GM-CSFRα or IL-5Rα.

In summary, in this thesis I have 1) developed specific tools to monitor receptor expression in a ligand-independent manner; 2) demonstrated that receptor expression is not static and can be modulated by cytokines; 3) identified strong evidence in defining the *N*-terminal domain of IL-3R α-chain and B'-C' and F'-G' loops of domain 4 of β_c as functional domains involved in ligand binding and function; and 4) provided novel potential therapeutics.

Figure 7-1. Model of human IL-3 receptor activation. In the absence of IL-3, IL-3Rα and β_c are not associated on the cell surface. The two unpaired cystines in the *N*-terminal domain and domain 1 of CRM may form a disulphide-bound. The presence of IL-3 triggers IL-3 binding to IL-3Rα initially and then β_c. IL-3 binding to the IL-3Rα may release the preformed disulphide-bound in the IL-3Rα. IL-3 binding to β_c occurs through the cytokine receptor module CRM2 and triggers dimerization. Two complementary mechanisms of receptor heterodimerization are proposed: a non covalent one probably involving the A-B loop in membrane proximal domains of the CRM2 of each chain analogous to the growth hormone receptor, and a covalent association probably involving unpaired Cys in the *N*-terminal region and domain 1 of IL-3Rα interacting with hitherto unpaired Cys in the CRM1 of β_c. IL-3 is associated with the receptor dimer but is not covalently attached to it. Disulphide-linked receptor heterodimerization leads to phosphorylation of the dimerized β_c, but not of non-covalently associated monomeric β_c or IL-3Rα, an event that leads to cellular activation.


IL-3
IL-3Rα chain
βc chain
extracellular
membrane
intracellular
IL-3
high affinity complex
CRM1
covalent dimerization
CRM2
- ligand binding
- non covalent dimerization
CRM1
IL-3
PO4-
SIGNAL

FUTURE WORK

The work presented in chapter IV provides insight into the expression of cytokine receptors in earlier haemopoiesis. Obviously, further investigation is needed to explore the overall pattern of cytokine receptor expression of the haemopoietic stem cells (HSC), and more importantly the mechanisms of the response of HSCs to a variety of cytokines *in vivo*.

The two neutralising MoAbs to IL-3Rα and the β_c may have clinical potential to control overstimulation by these cytokines in certain disease conditions. So further work to study the significance of these MoAbs using animal model or on clinical trial may be appropriate.

Finally, the data from chapter V and VI give strong evidence that the *N*-terminal domain of IL-3Rα and B'-C' and F'-G' loops of β_c are functional receptor domains. Further exploration to MoAb QP1 is necessary by means of affinity maturation or through phage display in order to increase its affinity to B'-C' and F'-G' loops. Such an attempt may also favour the creation of more powerful receptor antagonist with therapeutic importance.

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