INTERLEUKIN-3 RECEPTOR EXPRESSION AND FUNCTION IN NON-HEMOPOIETIC CELLS

Eija Korpelainen

Thesis submitted for the degree of Doctor of Philosophy in The University of Adelaide

(Faculty of Medicine)

Divison of Human Immunology, Institute of Medical and Veterinary Science
Adelaide, South Australia

June, 1995

Awarded 1996

TABLE OF CONTENTS

ABSTRACT
DECLARATION
ACKNOWLEDGMENTS
PUBLICATIONS ARISING
ABBREVIATIONS

CHAPTER 1: INTRODUCTION

1.1	Preface	2
1.2	Interleukin-3	2
1.3	The IL-3 receptor	5
1.4	Endothelial cells	10

CHAPTER 2: MATERIALS AND METHODS

2.1	Reagents	16
2.2	Cell isolation and culture	17
2.3	RNA extraction	20
2.4	RNase protection assay	20
2.5	mRNA half-life determinations	21
2.6	Flow cytometry	22
2.7	Binding experiments	22
2.8	Neutrophil transmigration assay	24
2.9	Quantitation of cytokines	25
2.10	Glucose uptake assay	25

CHAPTER 3: ENDOTHELIAL CELLS EXPRESS FUNCTIONAL IL-3 RECEPTORS

Introduction		29
Results		31
3.1	HUVEC express mRNA for the IL-3R α and β chains	31
3.2	Surface expression of the IL-3R subunits on HUVEC	31
3.3	HUVEC bind IL-3 with high affinity	34
3.4	IL-3 induces c-fos expression in HUVEC	34
Discussion		38
Summary		41

CHAPTER 4: ENDOTHELIAL IL-3 RECEPTOR EXPRESSION IS REGULATED BY CYTOKINES

Introduction 43

Results 45

4.1 Expression of the IL-3R mRNAs in HUVEC is enhanced by cytokines 45

4.2 Increased IL-3R α and β chain expression is not due to mRNA stabilisation 48

4.3 TNF-α, IFN-γ and IL-4 regulate the surface expression of IL-3R subunits on HUVEC 48

4.4 Binding of IL-3 to HUVEC is induced by TNF-α and IFN-γ 50

Discussion 54

Summary 57

CHAPTER 5: REGULATION OF ENDOTHELIAL CELL FUNCTION BY IL-3

Introduction 59

Results 61

5.1 IL-3 enhances TNF-α induced IL-8 secretion, E-selectin expression and neutrophil transmigration 61

5.2 IL-3 potentiates the IFN-γ induced MHC class II expression in HUVEC 62

5.3 IL-3 synergises with IFN-γ in selectively inducing the production of IL-6 and G-CSF but not GM-CSF 64

5.4 IL-3 does not alter the stability of IL-6 mRNA 66

Discussion 70

Summary 74

CHAPTER 6: IL-3 RECEPTOR EXPRESSION IN OTHER MESENCHYMAL CELLS

Introduction 76

Results 78

6.1 IL-3R mRNA expression in mesenchymal cells 78

6.2 Surface expression of the IL-3R subunits 78

6.3 Functionality of the IL-3R 80

Discussion 85

Summary 88

CHAPTER 7: DISCUSSION 90

BIBLIOGRAPHY 100

ABSTRACT

Interleukin-3 receptor expression and function in non-hemopoietic cells

Interleukin-3 (IL-3) is a T cell-derived hemopoietic growth factor which stimulates the production and functional activity of blood cells. IL-3 exerts its functions through binding to specific high-affinity receptors (IL-3R) consisting of a ligand-specific α chain and a common β chain, which is shared between the related receptors for IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and is required for signalling. The work described in this thesis aimed to examine whether the actions of IL-3 are restricted to blood cells, or whether non-hemopoietic cells also express receptors for IL-3 and can functionally respond to this cytokine.

Interestingly, IL-3Rs were found to be expressed in several vascular and connective tissue-type cells including human umbilical vein endothelial cells (HUVEC), dermal fibroblasts (HFF), smooth muscle cells (SMC) and bone marrow stromal cells (BMSC). These cells exhibited marked differences in the basal expression of the IL-3R subunits: while HUVEC expressed both IL-3R chains, SMC expressed only the β chain, and no expression was found on HFF and BMSC. However, an induction of the IL-3R α chain was observed in all the cell types examined in response to IFN-γ and TNF-α. These inflammatory cytokines also enhanced expression of the β chain in HUVEC and BMSC, but down-regulated it in SMC, and had no effect in HFF which remained β chain negative. IL-3R mRNA expression correlated with protein expression on the cell surface, as judged by flow cytometry with anti-IL-3R α chain and anti-β chain mAbs, and by binding of ^{125}I-labelled IL-3. The receptor expression was IL-3 specific, as α chains for the related GM-CSF and IL-5 receptors were absent in parallel experiments.

The HUVEC IL-3R is functional, as shown by the induction of the immediate-early gene c-fos in response to IL-3 treatment. In contrast, IL-3 failed to stimulate c-fos expression in HFF, SMC or BMSC, which may be due to insufficient β chain expression in these cells. The biological role of the endothelial IL-3R was further

characterised using different functional assays. IL-3 was found to enhance production of the chemokine IL-8 in TNF-α -activated HUVEC and, in keeping with this, increase transmigration of neutrophils across endothelial monolayers. IL-3 also enhanced the IFN-γ -induced MHC class II expression and production of the hemopoietic cytokines G-CSF and IL-6.

The findings described in this thesis identify a novel site of action for IL-3, and suggest that IL-3 can influence immune and inflammatory responses and hemopoiesis by acting not only on hemopoietic cells but also on vascular endothelium.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution 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 copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed: ______________________ Date: 30.6.1995

ACKNOWLEDGMENTS

The work described in this thesis was performed in the Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide. I am grateful to the IMVS for enabling me to pursue my studies, and to the University of Adelaide for my research scholarship.

I am indebted to my supervisors Associate Professor Angel Lopez and Professor Mathew Vadas for their guidance, support and encouragement throughout my candidature. I am also grateful for the significant help and advice given by Dr Jennifer Gamble.

I would like to thank everybody in the department for providing a friendly working environment, and especially the large number of people from various laboratories who have contributed to this work, including:

Leanne Noack for her tireless efforts in providing endothelial cells; Mara Dottore for binding assays; Sun Qiyu for the receptor antibodies; Dr Joanna Woodcock for advice with molecular biology techniques; Dr William Smith for IL-8 measurements and neutrophil transmigration assays; Dr Pritinder Kaur and Jill Furze for provision of fibroblasts and smooth muscle cells respectively; Dr Paul Simmons, Sharon Paton and Stan Gronthos (Department of Hematology) for provision of bone marrow stromal cells; Drs Gregory Goodall and Richard D'Andrea, and Paul Moretti for the IL-3R cDNAs and helpful discussions; Dr Judith Layton (Ludwig Institute, Melbourne) for the G-CSF measurements, and Dr Heddy Zola (Adelaide Children's Hospital) for the MHC class II antibody. I would also like to thank the IMVS library staff, Mark Fitzgerald and the staff of the IMVS photography department, and the delivery ward at the Queen Victoria Hospital for providing umbilical cords.

I warmly thank Cathy Lagnado for her friendship and technical advice during these years, as well as for assistance with English expression in this thesis.

Finally, I wish to express my gratitude to Philippe Rua for his endless support and understanding during the period of my enrolment for this degree.

PUBLICATIONS ARISING

Publications arising from the work included in this thesis, and from related collaborative work, are:

1. Korpelainen EI, Gamble JR, Smith WB, Goodall GJ, Qiyu S, Woodcock JM, Dottore M, Vadas MA, Lopez AF: The receptor for interleukin-3 is selectively induced in human endothelial cells by tumor necrosis factor α and potentiates interleukin 8 secretion and neutrophil transmigration. Proc. Natl. Acad. Sci. USA. 90: 11137-11141, 1993

2. Korpelainen EI, Gamble JR, Smith WB, Dottore M, Vadas MA, Lopez AF: Interferon-γ up-regulates interleukin-3 (IL-3) receptor expression in human endothelial cells and synergizes with IL-3 in stimulating major histocompatibility complex class II expression and cytokine production. Blood, in press.

3. Smith WB, Guida L, Qiyu S, Korpelainen E, Gillis D, van den Heuvel C, Hawrylowicz CM, Vadas MA, Lopez AF: Neutrophils activated by GM-CSF express receptors for interleukin-3 which mediate major histocompatibility complex class II expression. Blood, in press.

4. Khew-Goodall Y, Butcher CM, Litwin MS, Newlands S, Korpelainen EI, Noack LM, Berndt MC, Lopez AF, Gamble JR, Vadas MA: Chronic expression of P-selectin on endothelial cells stimulated by the T cell cytokine interleukin-3. Blood, submitted.

5. Sun Q, Woodcock JM, Rapoport A, Stomski FC, Korpelainen EI, Bagley CJ, Goodall GJ, Smith WB, Gamble JR, Vadas MA, Lopez AF: Monoclonal antibody 7G3 recognises the N-terminal domain of the human interleukin-3 receptor α chain and functions as a specific IL-3 receptor antagonist. Blood, submitted.

ABBREVIATIONS

actD	actinomycin D
APC	antigen presenting cell
AU	absorbance unit
BMSC	bone marrow stromal cell
BSA	bovine serum albumin
CNTF	ciliary neurotrophic factor
DMSO	dimethyl sulfoxide
DOG	deoxyglucose
DTH	delayed type hypersensitivity
DRB	5,6-dichloro-1-ribofuranosylbenzimidazole
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks balanced salt solution
HFF	human foreskin fibroblast
HUVEC	human umbilical vein endothelial cell
IFN-γ	interferon-γ
IL-*n*	interleukin-*n*
K_d	dissociation constant
LIF	leukemia inhibitory factor
LPR	late phase reaction
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
OSM	oncostatin M
PBS	phosphate-buffered saline
R	receptor
RNase	ribonuclease
RT-PCR	reverse transcription - polymerase chain reaction
SA	specific activity
SMC	smooth muscle cell
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α

CHAPTER 1

INTRODUCTION

1.1 Preface

Interleukin-3 (IL-3) is a hemopoietic growth factor which stimulates the production and functional activity of various blood cell types. The aim of the work described in this thesis was to examine whether the actions of IL-3 are restricted to blood cells, or whether non-hemopoietic cells also express receptors for and respond to this cytokine. Identifying the target cells offers insight into the physiological function of IL-3 and is also of clinical importance, as IL-3 is used in bone marrow reconstitution following cancer therapy.

Interestingly, IL-3 receptors (IL-3R) were found to be expressed on various non-hemopoietic cells of mesenchymal origin including endothelial cells. This finding raises the potential for IL-3 to have a much wider role than previously expected, prompting investigations into the function of the IL-3R in these cells. A brief introduction to IL-3 and its receptor, as well as to endothelium and its functions, is given here.

1.2 Interleukin-3

Interleukin-3 was initially identified as one of the active components in T-cell conditioned medium which is able to stimulate colony formation of hemopoietic progenitor cells in semisolid cultures (reviewed by Metcalf, 1988). Biochemical purification of the IL-3 molecule (Ihle *et al.*, 1982) was followed by expression cloning of the mouse IL-3 cDNA (Fung *et al.*, 1984, Yokota *et al.*, 1984) and the human homologue (Yang *et al.*, 1986). Human IL-3 is a 133 amino acid glycoprotein with a mass ranging from 15 to 30 kDa, depending on the degree of glycosylation. Bacterially-derived recombinant human IL-3 is biologically active, indicating that glycosylation is not necessary for function. The gene for IL-3 is located on human chromosome 5 and mouse chromosome 11, and is in close proximity to the genes for GM-CSF and IL-5 (reviewed by Arai *et al.*, 1991). Although these three hemopoietic growth factors have little sequence homology, they share a similar tertiary structure and functional properties.

IL-3 is predominantly produced by mitogen- or antigen-stimulated T lymphocytes (Niemyer *et al.*, 1989). Both T_h1 and T_h2 type $CD4^+$ cells, as well as $CD8^+$ cells have been shown to make IL-3 (Mossman 1987, Kelso *et al.*, 1988). In addition to T lymphocytes, mast cells also can release IL-3 upon IgE-stimulation (Plaut *et al.*, 1989, Wodnar-Filipowicz, 1989). The cellular source and tight regulation of IL-3 production suggest a role for this cytokine in immunological and allergic inflammations.

In vitro activities of IL-3

IL-3 stimulates the proliferation and differentiation of hemopoietic progenitor cells, giving rise to granulocytic, monocytic, erythroid, mast cell, megakaryocytic and mixed colonies *in vitro* (Sieff *et al.*, 1987, Lopez *et al.*, 1988, Metcalf, 1988). The ability to stimulate such a wide spectrum of lineages is unique to IL-3, and is reflected in its alternative name "multipotential colony-stimulating factor" (multi-CSF). IL-3 acts not only on lineage-committed progenitor cells but also on the more primitive pluripotent stem cells, functioning as a primer for other hemopoietic growth factors.

In addition to hemopoietic precursors, IL-3 also activates certain mature blood cells including monocytes, eosinophils and basophils, suggesting a role in the inflammatory process. The monocyte functions induced by IL-3 include cytotoxicity, proliferation, adherence to endothelium and expression of MHC class II molecules (Cannistra *et al.*, 1988, Elliott *et al.*, 1989, Elliott *et al.*, 1990, Sadeghi *et al.*, 1992). IL-3 has also been shown to activate phagocytosis, degranulation and proteoglycan production by eosinophils (Lopez *et al.*, 1988, Fujisawa *et al.*, 1990, Rothenberg *et al.*, 1988A) and histamine release from basophils (Haak-Frendscho *et al.*, 1988). In addition to acting on myeloid cells, IL-3 can stimulate the proliferation of and antibody secretion by activated mature B lymphocytes (Tadmori *et al.*, 1989, Xia *et al.*, 1992).

Fundamental to the effects mentioned above is the ability of IL-3 to enhance cell survival. This has been observed with both hemopoietic progenitor cells and mature

monocytes and eosinophils (Metcalf, 1988, Elliott *et al.*, 1989, Rothenberg *et al.*, 1988B) and is likely to occur via suppression of apoptosis (Williams *et al.*, 1990).

<u>The function of IL-3 *in vivo*</u>

The *in vivo* effects of IL-3 largely reflect those documented *in vitro*. An increase in hemopoietic progenitors and mature blood cells of different lineages has been observed in various hemopoietic tissues following administration of IL-3 in mice (Metcalf *et al.*, 1986). On the other hand, mice reconstituted with IL-3-overproducing bone marrow cells develop a non-neoplastic myeloproliferative syndrome (Chang *et al.*, 1989). The ability of IL-3 to stimulate hemopoiesis has been studied in several animal models with encouraging results, culminating in the clinical use of IL-3 in treatment of bone marrow failure following cancer therapy. Administration of IL-3 to humans induces a multilineage response, with an increase in neutrophil, eosinophil, monocyte, reticulocyte and platelet numbers in the peripheral blood (Ganser *et al.*, 1990). IL-3 is relatively well tolerated, the immediate side effects being mainly fever and headaches. However, most patients treated with IL-3 develop bone marrow fibrosis (Orazi *et al.*, 1992). The origin of this fibrosis, characterised by endothelial cell proliferation, increased reticulin and collagen content and an increase in fibroblast numbers, is not understood at present. The possibility that IL-3 may act directly on endothelial cells and fibroblasts has been examined in this thesis.

Despite the observed hemopoietic activities of IL-3 *in vitro* and *in vivo*, the role of endogenous IL-3 in hemopoiesis remains controversial for several reasons. Firstly, athymic mice and humans appear to have normal hemopoiesis (Ohsawa *et al.*, 1977), and secondly, IL-3 is not detectable in the bone marrow or in the circulation (Garland *et al.*, 1983, Crapper *et al.*, 1984, Cheers *et al.*, 1988, Cluitmans *et al.*, 1995). One possible way in which to address the question of the physiological role of IL-3 would be to create IL-3 -deficient mice through targeted disruption of the IL-3 gene in embryonic stem cells. Although such IL-3 "knock-out" mice have not yet been reported, transgenic mice

with down-regulated IL-3 production have been obtained using an antisense strategy. Interestingly, the mice expressing antisense IL-3 mRNA have normal hemopoiesis but develop a B-cell lymphoproliferative syndrome or neurologic dysfunction (Cockayne *et al.*, 1994).

1.3 The IL-3 receptor

IL-3 exerts its biologic activities through binding to specific high-affinity receptors on the cell surface. The human IL-3R consists of at least two subunits, a 70 kDa α chain which is specific for IL-3 and binds IL-3 with low affinity ($K_d \approx 10^{-7}$ M) (Kitamura *et al.*, 1991A), and a 120 kDa β chain (Hayashida *et al.*, 1990), which does not detectably bind IL-3 by itself but confers high affinity binding ($K_d \approx 10^{-10}$ M) when co-expressed with the α chain. The β chain can also couple with the α chains of the related receptors for GM-CSF and IL-5 (Gearing *et al.*, 1989, Tavernier *et al.*, 1991) and is critical for signalling (Kitamura *et al.*, 1992). The fact that the IL-3, GM-CSF and IL-5 receptors use the same β chain as an affinity-converter and signal-transducer explains why these cytokines exhibit binding cross-competition and elicit an almost identical set of functions on target cells such as eosinophils (reviewed by Lopez *et al.*, 1992). The human IL-3, IL-5 and GM-CSF receptor system is illustrated in figure 1.1. The mouse IL-3R differs from its human counterpart in having two alternative β chains, one of which is unique to the IL-3R and itself also binds IL-3 with low-affinity (Hara *et al.*, 1992, Itoh *et al.*, 1990).

The IL-3R α and β chains are membrane glycoproteins with a single hydrophobic transmembrane domain. Although soluble GM-CSFR and IL-5R α chains, generated by alternative splicing, have been found (Raines *et al.*, 1991, Tavernier *et al.*, 1991), no such soluble forms of either IL-3R subunit have been detected. Structurally the IL-3, IL-5 and GM-CSF receptors belong to the type I cytokine receptor superfamily, which also includes the receptors for IL-2, IL-4, IL-6, IL-7, IL-9, G-CSF, LIF, CNTF, erythropoietin, oncostatin M, prolactin and growth hormone (Bazan, 1990, Taga *et al.*,

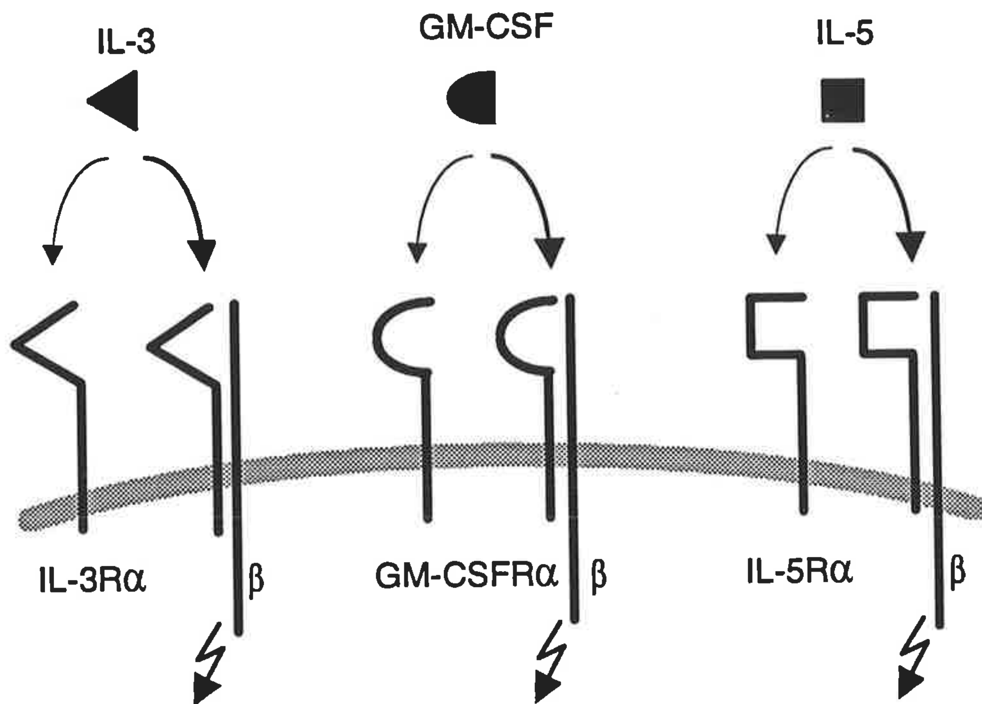


Fig. 1.1 Schematic presentation of the human IL-3, IL-5 and GM-CSF receptor system. High-affinity binding is represented by broad arrows and low-affinity binding by thin arrows, and the flashes indicate signalling.

1992). The extracellular regions of these receptor chains are characterised by the presence of fibronectin type III modules (Patthy, 1990), containing four well conserved cysteine residues and a unique Trp-Ser-X-Trp-Ser motif located close to the transmembrane region. The cytoplasmic domains are less conserved and do not contain any motifs characteristic of signalling molecules.

Expression of the IL-3 receptor

Expression of the IL-3R is a prerequisite for a biological response to IL-3. As expected from the functional data, specific binding of IL-3 has been detected to bone marrow cells, monocytes, macrophages, eosinophils and basophils but not to neutrophils (Park *et al.*, 1989, Lopez *et al.*, 1989, Lopez *et al.*, 1990). The results obtained with radiolabelled ligand binding have since been confirmed at the molecular level using the cloned IL-3R cDNAs and specific antibodies. Both the IL-3R α and β chains are expressed on early $CD34^+$ hemopoietic progenitor cells and in various lineages with myeloid markers such as $CD13^+$, $CD14^+$, $CD15^+$ or $CD33^+$, as well as on cells with the B cell marker $CD19^+$ (Sato *et al.*, 1993). Although IL-3 receptors are not detectable on human T lymphocytes, both the IL-3R α and β chains have been shown to be present on a subset of murine $CD4^+$ T cell clones (Mueller *et al.*, 1994). Unlike the GM-CSFR, which is also found on some non-hemopoietic cells (Bussolino *et al.*, 1989, Baldwin *et al.*, 1989, Baldwin *et al.*, 1993), IL-3R expression appears to be restricted to cells of hemopoietic origin.

It has become increasingly recognised that expression of the cytokine receptors, including that of IL-3, is not static but can be dynamically regulated by various humoral factors. Cytokine responses may therefore be controlled not only at the level of cytokine production, but also at the level of target cell responsiveness. Hemopoietic cells of different lineages and maturation states exhibit differences in the regulation of IL-3R expression. For example, the IL-3R α chain has been shown to be up-regulated by G-CSF in CD34+ cells (Sato *et al.*, 1993) but not in neutrophils, which respond to GM-CSF instead (Smith *et al.*, in press). Similarly, β chain expression is enhanced by

TNF-α in CD34+ hemopoietic progenitor cells (Sato *et al.*, 1993) and TF-1 cells (Watanabe *et al.*, 1992) but not in mature monocytes, where the up-regulating signal is IFN-γ (Hallek *et al.*, 1992). Interestingly, down-regulation of IL-3R expression in response to TGF-β has been observed in murine progenitor cells (Jacobsen *et al.*, 1993).

The molecular mechanisms which regulate the cell-type specificity and level of IL-3R expression are only beginning to be elucidated. While the promoters of the human IL-3R subunits are not yet cloned, the proximal promoter of the mouse IL-3R α chain has been shown to contain potential binding sites for several transcription factors, such as GATA, Ets, c-myb, Sp1, AP-2 and ICSBP (IFN-γ consensus sequence binding protein), and direct transcription in a cell type-specific manner (Miyajima *et al.*, 1995). The mouse β chain promoters also contain a GATA sequence, as well as recognition sites for PU.1 and AP-3, an octamer sequence and two IFN-γ responsive elements (Gorman *et al.*, 1992).

<u>IL-3 receptor signal transduction</u>

The signalling pathways which mediate the functional response following binding of IL-3 to its receptor have been the subject of intense investigation during the last three years. Although the IL-3R does not contain any detectable catalytic domains, rapid phosphorylation of several cellular proteins, including the β chain itself, has been observed following IL-3 binding (Duronio *et al.*, 1992). Analysis of the kinases and substrates involved has identified two major pathways stimulated by IL-3, namely the Ras pathway and, more recently, the JAK-STAT pathway.

Ras is a membrane-associated GTP-binding protein which cycles between the GTP-bound active and GDP-bound inactive states. The switching is controlled by nucleotide exchange factors such as Sos, or by activators of Ras' intrinsic GTPase activity such as GAP. Interestingly, IL-3 has been shown to induce tyrosine phosphorylation of Shc (Matsuguchi *et al.*, 1994), a small adaptor protein which can associate with Sos and bind to receptor phosphotyrosines through its SH2 domains, thus bringing Sos in close

proximity to Ras. IL-3 also induces tyrosine phosphorylation of Vav (Mui *et al.*, 1992), a hemopoietic-cell-specific molecule with nucleotide exchange activity. Both Shc and Vav may account for the observed activation of the Ras signalling cascade by IL-3, which involves activation of Raf and MAP kinases, leading ultimately to induction of the immediate-early genes c-fos and c-jun (reviewed by Miyajima *et al.*, 1993).

The JAK-STAT pathway was first discovered in studies of interferon signalling, but has since been shown to be utilised by many other cytokines (reviewed by Darnell *et al.*, 1994). The JAK-family of tyrosine kinases, often physically associated with the receptors, become phosphorylated and activated upon cytokine stimulation. Activation of JAKs is followed by tyrosine phosphorylation of latent cytoplasmic molecules called STATs (*S*ignal *T*ransducers and *A*ctivators of *T*ranscription). Phosphorylated STAT proteins can form complexes and translocate to the nucleus, where they act as transcription factors. Several lines of evidence suggest involvement of the JAK-STAT pathway in IL-3 -signalling. IL-3 stimulation results in rapid phosphorylation and activation of JAK2 kinase (Silvennoinen *et al.*, 1993), which has been shown to physically associate with the β chain (Quelle *et al.*, 1994). IL-3 also activates p72 tyrosine kinase which associates with STATs (Matsuda *et al.*, 1994). Finally, IL-3 has been shown to induce the phosphorylation and DNA-binding activity of two STAT-family members termed STAT5A and STAT5B (also known as STF-IL-3a p80), as well as activate transcription from promoters containing binding sites for these factors (Mui *et al.*, 1995, Azam *et al.*, 1995).

In addition to the signalling pathways mentioned above, IL-3 activates protein kinase C and the hemopoietic-cell-specific kinases Lyn, Fyn, Fps, Pim-1 and Tec (Miyajima *et al.*, 1993, Mano *et al.*, 1995). Although much research has focussed on the kinases involved in IL-3 signalling, the action of phosphatases is also important. Interestingly, the hemopoietic cell phosphatase SHPTP1 has been shown to associate with the β chain and down-regulate IL-3 -induced tyrosine phosphorylation and mitogenesis (Yi *et al.*,

1993). Recent results suggest that IL-3 also activates the related but more ubiquitously expressed tyrosine phosphatase SHPTP2 (Welham *et al.*, 1994).

The transcription factors involved in the stimulation of specific target genes by IL-3 remain largely unknown. So far only the promoters of the immediate-early genes c-fos and egr-1 have been studied in this regard. The serum response element (SRE) in the c-fos promoter has been shown to be responsible for activation by IL-3 (Hatekeyama *et al.*, 1992), suggesting the involvement of SRF and associated factors. In the case of the egr-1 promoter, both the SRE and CRE (cAMP response element) are required for IL-3 - induced transcription, and binding of CREB to the CRE site has been documented (Sakamoto *et al.*, 1994).

1.4 Endothelial cells

Endothelial cells line the vessels of the vascular system, forming an interface between circulating blood components and the extravascular space. Once considered as an inert barrier, vascular endothelium is now known to dynamically respond to a wide variety of stimuli and play an active role in immune and inflammatory responses and hemopoiesis. For example, endothelial cells have the potential to present antigens, recruit leukocytes from the blood to the site of inflammation, regulate coagulation, control vascular permeability, growth, and tone and produce hemopoietic growth factors. The diverse functions of endothelium are based on its ability to adjust phenotypically to environmental requirements, signalled by humoral mediators, cell-to-cell adhesion pathways, or physical stimuli such as shear stress.

Cytokines have an important role in the interplay of endothelium with other cell types. Endothelial cells not only secrete cytokines, but also respond to a wide variety of factors. These include the monokines TNF-α and IL-1, the lymphokines IFN-γ and IL-4, and several angiogenic stimuli such as FGF (fibroblast growth factor) and the tumor cell derived VEGF (vascular endothelial growth factor) (reviewed by Litwin *et al.*, 1995). It has also been suggested that endothelial cells express receptors for and

respond to certain hemopoietic growth factors such as G-CSF, GM-CSF and erythropoietin (Bussolino *et al.*, 1989, Anagnostou *et al.*, 1990). This may reflect the possible common origin of endothelial cells and blood cells. Both cell types are derived from the embryonic mesoderm and develop in close spatial and temporal relationship, forming the blood islands of the yolk sac which are the first recognisable hemopoietic system (Doetschman *et al.*, 1985).

The work described in this thesis investigated the possibility that endothelial cells express receptors for the hemopoietic growth factor IL-3. As this was found to be the case, investigations were extended to the function of the IL-3R in these cells. The effect of IL-3 was studied on various endothelial responses, including those involved in leukocyte recruitment, antigen presentation and hemopoiesis. A brief introduction to the role of endothelial cells in these processes is given here.

Before discussing the biological activitities of endothelium further, it should be noted that although all endothelial cells resemble each other in their general organisation, their morphology and functions vary considerably in different parts of the body and in different types of vessels (Page *et al.*, 1992). Most *in vitro* studies on human endothelial cells have been performed using cells derived from the umbilical vein (HUVEC). While offering a useful model system, HUVEC may not reflect the properties of endothelial cells in all vessels.

Endothelial control of leukocyte infiltration

An effective inflammatory response requires infiltration of the appropriate cell types from the blood to the site of inflammation in a temporally correct manner. This process is largely controlled by the endothelium, which can selectively recruit leukocytes from the circulation by expressing different adhesion molecules and by secreting chemoattractants.

Leukocyte-endothelial recognition consists of at least three sequential steps (reviewed by Springer, 1994). The initial interaction of leukocytes with endothelium, often referred to as "rolling", is mediated by vascular selectins and their cognate carbohydrate ligands. This is followed by activation of the leukocyte integrins by chemoattractants, small soluble molecules that can also direct the subsequent leukocyte migration. Activated integrins bind their immunoglobulin superfamily ligands on endothelium, resulting in firm adhesion, which can be followed by transmigration. The combinatorial use of different molecules at each step generates specificity for distinct leukocyte subpopulations. The main mediators of leukocyte-endothelial recognition are briefly introduced here.

Selectins

The selectin family of adhesion molecules which mediate the primary adhesion of leukocytes to endothelium includes E- and P-selectin, expressed on endothelial cells, and L-selectin, expressed on leukocytes (reviewed by Bevilacqua *et al.*, 1993). Endothelial expression of E-selectin is induced by inflammatory stimuli such as TNF-α, IL-1 and LPS, and P-selectin by histamine and thrombin. E- and P-selectin recognise sialylated carbohydrate determinants related to sialyl Lewis^x in their counterreceptors, which are expressed on certain leukocytes including neutrophils, monocytes and lymphocyte subsets. The leukocyte L-selectin is constitutively expressed and contributes to adhesion by recognising the CD34 molecule on endothelial cells (Baumhauer *et al.*, 1993).

Chemoattractants

Leukocytes have the ability to sense chemical gradients and migrate in the direction of increasing concentration of chemoattractants, a process termed chemotaxis. The classical chemoattractants such as formylated bacterial peptides and platelet-activating factor act broadly, whereas the members of the newly discovered chemokine family are specific for leukocyte subsets (reviewed by Miller *et al.*, 1992). Based on sequence similarities, this family can be divided into the neutrophil-specific C-X-C chemokines

such as IL-8, and C-C chemokines such as RANTES and MIP-1α which act on monocytes, eosinophils and/or lymphocytes. Endothelial production of IL-8 is induced by TNF-α, IL-1 and LPS (Sica *et al.*, 1990), whereas the expression of RANTES is stimulated by TNF-α and IFN-γ (Devergne *et al.*, 1994). In addition to endothelial cells, chemokines are also produced by other cells involved in the inflammatory response such as T lymphocytes and monocytes.

Integrins and their vascular ligands

The leukocyte integrins are heterodimers of non-covalently linked α and β subunits. They include the β_2 integrins LFA-1 (lymphocyte function-associated antigen-1) and Mac-1 (macrophage antigen-1) which are widely expressed in different leukocyte populations, and the β_1 integrin VLA-4 which is expressed on monocytes, eosinophils, basophils, lymphocytes and NK cells but not on neutrophils (reviewed by Larson and Springer, 1990). The adhesive function of integrins can be activated by chemoattractants and is likely to involve conformational changes. For example, treatment of neutrophils with IL-8 rapidly activates Mac-1 (Detmers *et al.*, 1990). The endothelial ligands for integrins are members of the immunoglobulin superfamily. They include the intercellular adhesion molecule (ICAM)-1, which can interact with LFA-1 and Mac-1, ICAM-2 which interacts with LFA-1 only, and vascular cell adhesion molecule (VCAM)-1 which is the ligand for VLA-4 (reviewed by Bevilacqua, 1993). Both ICAM-1 and -2 are constitutively expressed on endothelium, and the expression of ICAM-1 can be further enhanced by TNF-α, IL-1 and LPS. In addition to these stimuli, the expression of VCAM-1 is also strongly induced by IL-4.

Antigen presentation by endothelial cells

Endothelial cells have the potential to function as antigen presenting cells in immune reactions. Like most cell types, they express the MHC class I antigens required for activation of $CD8^+$ lymphocytes in CTL responses, and this expression can be further enhanced by TNF-α and IFN-γ (Doukas *et al.*, 1990). Interestingly, endothelial cells can

also be recruited to express MHC class II molecules, which are normally expressed on the cells of the immune system, by stimulation with IFN-γ (Pober *et al.*, 1983A). Functional activation of $CD4^+$ T lymphocytes by endothelial cells has been observed *in vitro,* and endothelium may play an important role in initiating or propagating T cell activation in immunologically mediated inflammation and allograft rejection *in vivo* (reviewed by Pober *et al.*, 1991).

Endothelial cells in hemopoiesis

Endothelial cells have been implicated in the control of hemopoiesis for several reasons. They secrete, either constitutively or in response to inflammatory stimulation, hemopoietic growth factors such as G-CSF, GM-CSF, M-CSF, IL-6 and SCF (Seelentag *et al.*, 1987, Jirik *et al.*, 1989, Buzby *et al.*, 1994). Endothelial cells also form an important component of the bone marrow stromal cell population (Tavassoli, 1992, Hasthorpe *et al.*, 1992), which plays a critical role in hemopoiesis by providing hemopoietic growth factors and adhesive interactions. Techniques to isolate human bone marrow microvascular endothelial cells have been recently developed and, interestingly, these cells have been shown to support adhesion of $CD34^+$ hemopoietic progenitors *in vitro* (Rafii *et al.*, 1994). Finally, the anatomic location of the bone marrow microvascular endothelium suggests that it may control the release of blood cells from the marrow into the circulation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

Tissue culture reagents

HDMEM, RPMI 1640, FCS: Gibco Laboratories, Glen Waverly, Australia

M199 medium with Earle's Salts, non-essential amino acids, HEPES, trypsin/EDTA: Cytosystems, Sydney, Australia

α-MEM: ICN Flow laboratories, Irvine, Scotland

Endothelial cell growth supplement: Collaborative research, Bedford, MA

Heparin, soybean trypsin inhibitor: Sigma, St. Louis, MO

Collagenase, elastase: Worthington Biochemicals, Templestowe, Australia

Dispase, fibronectin: Boehringer Mannheim, Germany

Ficoll (Lymphoprep), Metrizamide: Nycomed, Oslo, Norway

Penicillin: Commonwealth Serum Laboratories, Melbourne, Australia

Gentamicin: Delta West, Bentley, Australia

Streptomycin: Servipharm, Basel, Switzerland

Cytokines

IFN-γ ($2x10^7$U/mg), TNF-α ($6.27x10^7$U/mg): gifts from Genentech, San Fransisco, CA

IL-3: a gift from Genetics Institute, Cambridge, MA

IL-1, IL-4: gifts from Immunex, Seattle, WA

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

LPS was undetectable in all the cytokine preparations used, as judged by the *Limulus* amoebocyte assay (detection limit 100 pg/ml).

Antibodies

7.G3 (anti-IL-3R α chain mAb), 4.F3 (anti-β chain mAb), 8.G6 (anti-GM-CSFR α chain mAb): made in the laboratory by Sun Qiyu as previously described (Woodcock *et al.,* 1994, Qiyu *et al.,* submitted)

49-1B11 (anti-E-selectin mAb): made in the laboratory by Joe Wrin as previously described (Cockerill *et al.*, 1994)

FMC52 (anti-MHC class II mAb): a gift from Heddy Zola, Adelaide Children's Hospital

FITC-conjugated rabbit anti-mouse IgG: Silenius, Hawthorn, Australia

anti-α -SMC actin: Sigma, St. Louis, MO

Radiochemicals

^{3}H-uridine (26 Ci/mmol): Amersham, Little Chalfont, England

^{3}H-deoxyglucose (30.6 Ci/mmol): DuPont, NEN products, Boston, MA

Na^{125}I (2500 Ci/mmol): DuPont Australia, Melbourne, Australia

[α-^{32}P]UTP (400 Ci/mmol): Bresatec, Adelaide, Australia

Other chemicals

RNA polymerases SP6, T3 and T7, ribonucleotides, RNasin: Promega, Madison, WI

RNase A: Pharmacia, Uppsala, Sweden

Guanidine thiocyanate: Fluka, Buchs, Switzerland

E.coli-derived LPS: Difco, Detroit, MI

2.2 Cell isolation and culture

HUVEC

HUVEC were isolated by collagenase treatment of umbilical veins (Wall *et al.*, 1978) within 24 h of delivery. The cannulated vein was flushed with RPMI medium containing antibiotics, digested with collagenase for 13 min at 37°C, then washed and the cells collected by centrifugation. HUVEC were cultured in gelatin-coated plastic flasks in M199 medium containing Earle's Salts, 20% FCS, 20 μg/ml endothelial cell growth supplement, 2 mM glutamine, heparin, non-essential amino acids, sodium pyruvate, sodium bicarbonate, penicillin and gentamicin. Cells were used between passages 2 and 6.

Human foreskin fibroblasts (HFF)

Pieces of foreskin tissue were treated with 4 mg/ml dispase overnight at 4°C to separate the epidermis and dermis. The dermis was then finely cut, treated with collagenase for 90 min at room temperature, and the cells collected by centrifugation and plated in HDMEM culture medium containing 20 mM HEPES, 10% FCS, 2 mM glutamine, sodium bicarbonate, penicillin and gentamicin. HFF were passaged up to 30 times.

Bone marrow stromal cells

Human bone marrow samples were obtained by aspiration from the posterior iliac crest or sternum of normal volunteers. The low-density bone marrow mononuclear cells were isolated by density centrifugation over Ficoll, and cultured in α-MEM medium containing 20% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, penicillin and streptomycin. After 48 h, the nonadherent cells were removed by changing the medium. The adherent stromal cells were refed with fresh medium and passaged several times prior to their use in experiments.

Smooth muscle cells

Smooth muscle cells (SMC) were extracted from human saphenous veins by a procedure modified from Chamley-Campbell *et al.,* (1981). Briefly, the endothelial layer was removed by scraping with a scalpel blade, and the tunica intima peeled off with forceps to avoid contaminating adventitial fibroblasts. The vessel was then cut into small pieces and digested with collagenase and soybean trypsin inhibitor for 1 h. The segments were then incubated in 1 mg/ml elastase for 1 h and again with collagenase for 1-3 h prior to centrifugation. The resultant cells were seeded into T25 flasks in M199 medium containing Earle's salts, 10% FCS, 4 mM glutamine, non-essential amino acids, sodium pyruvate, sodium bicarbonate, 10 mM HEPES, penicillin and gentamicin. The medium was changed every 2-3 days and the cells used after 4-6 weeks of growth. The identity of the cells was confirmed by staining with anti-α-SMC actin.

TF-1 cells

TF-1 cells (Kitamura *et al.*, 1989) were maintained in RPMI 1640 medium containing 10% FCS, 2 ng/ml rhGM-CSF, 2 mM glutamine, sodium bicarbonate, penicillin and gentamicin.

Neutrophils

Neutrophils were isolated from the freshly collected blood of healthy volunteers. Citrated blood was dextran sedimented, the buffy coat separated by Ficoll gradient centrifugation, and the remaining red cells removed by hypotonic lysis. The purity of neutrophils was >93%, as judged by morphological examination of Wright's stained cytocentrifuge preparations.

Monocytes

Monocytes were isolated from the blood of healthy donors, obtained from the Red Cross transfusion service (Adelaide, South Australia), by density centrifugation and counter-current elutriation (Elliott *et al.*, 1990). Briefly, after centrifugation through a cushion of Ficoll, mononuclear cells were collected from the interface, washed in RPMI 1640 and resuspended in HBSS containing 0.1% FCS. The cells were separated in a Beckman JE-6B elutriator using the Sanderson chamber, with a rotor speed of 2050 rpm and a flow rate of 11.8 ml/min. The cells remaining in the chamber after 30 min were collected, washed in RPMI 1640, and used immediately. Elutriation resulted in a monocyte purity of >91%.

Eosinophils

Eosinophils were obtained 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). Eosinophils were obtained from the 24% cushion and were >92% pure.

2.3 RNA extraction

Total cellular RNA was isolated using the guanidinium thiocyanate method (Chomczynski *et al.,* 1987). Briefly, cells were lysed in solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl and 0.1 M β-mercaptoethanol) and extracted with acidic phenol. The RNA was isopropanol-precipitated, resuspended in solution D, reprecipitated with isopropanol, washed with 70% ethanol and dissolved in water. All aqueous solutions used in RNA extraction (and in the RNase protection assay) were treated with diethylpyrocarbonate (DEPC). The RNA was quantitated spectrophotometrically, assuming that 1 AU_{260nm} = 40 μg/ml.

2.4 RNase protection assay

RNase protection assays were used to study the expression of the cytokine receptor subunit, hemopoietic growth factor and immediate early gene c-fos mRNAs. A simultaneous probing technique was developed, allowing detection of 5 different mRNAs (IL-3Rα, β, GM-CSFRα, IL-5α and GAPDH) in one sample, and was particularly useful for studying primary human cells such as HUVEC and SMC as it significantly reduced the amount of sample RNA required. Briefly, the antisense RNA probes were designed to protect fragments which are considerably different in size in order to avoid overlapping signals. Care was also taken to avoid regions of the receptor transcripts which are known to be susceptible to alternative splicing. Each probe was individually tested for its optimal RNase digest conditions, and a set of probes with similar requirements was chosen for further use.

cDNAs were subcloned into the following transcription vectors for probe synthesis: pGEM1 (IL-5R α, GM-CSFR α, common β chain, G-CSF, IL-6), pGEM2 (IL-3R α, c-fos), pGEM4Z (GM-CSF) or Bluescript KS (GAPDH). The probes protect the following fragments of the mRNA, with the enzymes used to linearise the transcription template shown in parentheses: 1119-1280 (Pst I) for the IL-3R α chain, 937-1191 (Pvu II) for the IL-5R α chain, 606-731 (Rsa I) for the GM-CSFR α chain, 833-1032 (Bal I)

for the common β chain, 1233-1508 (Mlu I) for G-CSF, 910-1128 (Rsa I) for IL-6, 549-756 (Nco I) for GM-CSF, 3612-3830 (StuI) for c-fos and 707-810 (Sty I) for GAPDH.

RNase protection assays were performed as described (Goodall *et al.* 1990) with a few modifications. Briefly, the probes were prepared from linearised template constructs by *in vitro* transcription using [α-^{32}P]UTP at a specific activity of 400 Ci/mmol (excepting the GAPDH internal control, 4 Ci/mmol), and purified by gel electrophoresis. A 10 or 20 μg sample of total RNA was hybridised with the probes (20 000 Cerenkov cpm each) for 16 h at 45°C. The remaining single-stranded RNA (including the unhybridised probe) was then removed by digestion with RNase A (20 μg/ml) for 40 min at 26°C. The reaction was stopped by incubation with proteinase K and SDS for 15 min at 37°C. The probe-mRNA duplexes were phenol-extracted, ethanol-precipitated and analysed on 8 M urea-6% polyacrylamide gels, using ^{32}P-labelled HpaII-digested pUC19 DNA as a size marker. Gels were visualised by phosphorimaging and quantitated using ImageQuant software (Molecular Dynamics), the GAPDH mRNA being used as an internal control.

2.5 mRNA half-life determinations

The effect of various stimuli on the stability of the IL-3R and IL-6 mRNAs (chapters 4 and 5) was examined by measuring the half-lives of these transcripts. HUVEC were stimulated as described in the text, then ongoing transcription was blocked by the addition of 5 μg/ml actinomycin D (actD). Total RNA was extracted at various times thereafter, and the decay of the mRNA of interest followed by RNase protection. The gels were quantitated by phosphorimager analysis, using the GAPDH mRNA as an internal control. The half-lives were also similarly determined using the alternative transcription inhibitor 5,6-dichloro-1-ribofuranosylbenzimidazole (DRB) at 100 μM. These two inhibitors differ in their mode of action: actD binds to DNA and blocks the movement of RNA polymerases, whereas DRB specifically interferes with RNA polymerase II transcription.

To confirm that the concentrations of actD and DRB used were sufficient to totally block transcription in HUVEC, the incorporation of ^{3}H-labelled uridine was measured. In brief, ^{3}H-uridine (4 μCi/ml) was added to HUVEC culture medium 15 min prior to the addition of actD (5 μg/ml) or DRB (100 μM). At various times thereafter, the cell layers were washed four times with cold 5% TCA, twice with ethanol, air-dried and then dissolved in 0.3 M NaOH. Following neutralisation with HCl, the incorporated ^{3}H-uridine was determined by scintillation counting. Uridine incorporation was completely blocked in the presence of actD or DRB (fig. 2.1), indicating that both inhibitors totally inhibited transcription at the concentrations used.

2.6 Flow cytometry

The surface expression of the cytokine receptor subunits, E-selectin and MHC class II was examined by flow cytometry. Cells were cultured on 24-well plates, stimulated as described in the text, and stained with the relevant antibody for 30 min at 37°C. After three washes with RPMI 1640 containing 2.5% FCS, the cells were further incubated with FITC-conjugated rabbit anti-mouse IgG at 4°C, washed three times, detached with trypsin/EDTA (0.05%/0.02%) and fixed in 0.4% formaldehyde. A minimum of 10^{4} cells was analysed using the EPICS-Profile II (Coulter Electronics) flow cytometer. The relative mean fluorescence intensity values (MFI) (obtained directly from the instrument's data set), were used for quantitative analysis after subtraction of the background fluorescence, which was determined using an irrelevant antibody of matching isotype. All samples were analysed in duplicate.

2.7 Binding experiments

Binding experiments were performed as previously described (Lopez et al., 1989) using ^{125}I-labelled IL-3 (^{125}I-IL-3). In brief, *E. coli*-derived rhIL-3 was radioiodonated to high specific activity (0.6×10^{6}-1.3×10^{6} cpm/pmol) using iodine monochloride, and the radiolabelled cytokine separated from iodide ions by chromatography on Sephadex G-25 (Pharmacia) in PBS containing 0.02% Tween 20. Prior to use, the radiolabelled

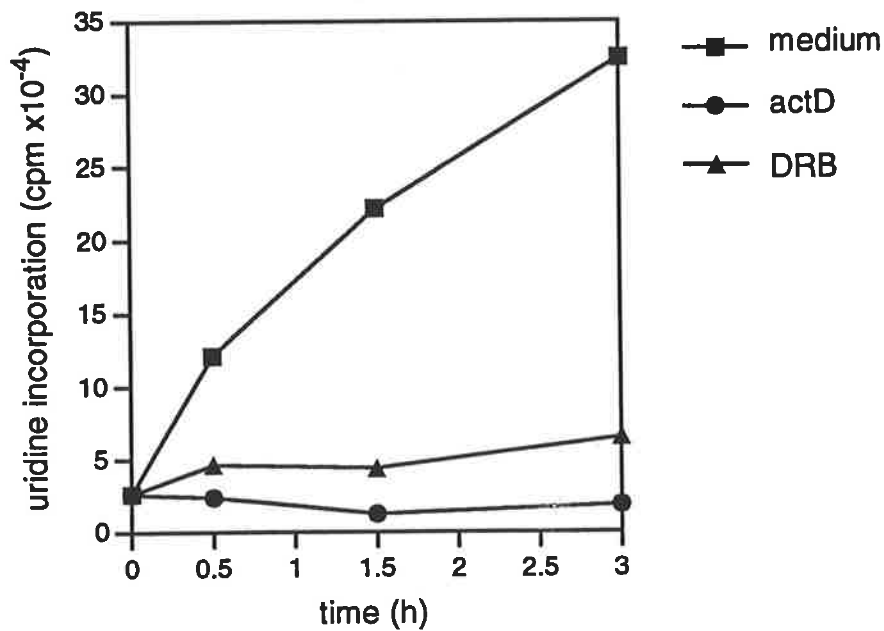


Fig. 2.1 Effect of the transcription inhibitors actinomycinD and DRB on ^{3}H-uridine incorporation in HUVEC. HUVEC were cultured in the presence of ^{3}H-uridine and treated with actD (5 µg/ml) or DRB (100 µM) for the times indicated. The cells were extracted with TCA, and the ^{3}H-uridine incorporation determined by scintillation counting.

protein was purified from non-protein-associated radioactivity by cation-exchange chromatography using carboxymethyl-Sepharose CL-6B.

The Scatchard transformation (Scatchard, 1949) was derived from a saturation binding curve using 2 x 10^6 HUVEC per sample, and six different concentrations of ^{125}I-IL-3 ranging from 80 pM to 1.4 nM. HUVEC were detached with EDTA (40 mM) and chondroitin sulphate (100 µg/ml) prior to incubation with ^{125}I-IL-3 for 3 hours at 22°C in 0.15 ml of binding medium (RPMI 1640, pH 7.4, supplemented with 20 mM HEPES and 0.5% BSA) containing 0.1% sodium azide to prevent receptor internalisation. Cell suspensions were then overlayed onto 0.2 ml of FCS and centrifuged for 30 s at maximum speed in a Beckman Microfuge 12. The visible cell pellet was recovered by cutting the tube, and the radioactivity determined in a Packard Auto Gamma 5650. Specific binding was determined by subtracting the cpm from parallel samples containing a 100-fold excess of unlabelled IL-3. All samples were analysed in duplicate. Binding data were analysed by EBDA and LIGAND software (Elsevier-BIOSOFT, Cambridge, UK). Specific ^{125}I-IL-3 binding to HUVEC during cytokine time-courses (chapter 4) was measured in a similar fashion, except that a fixed concentration of ^{125}I-IL-3 (either 1 nM or 4 nM) was used.

2.8 Neutrophil transmigration assay

Neutrophil transendothelial migration was measured as previously described (Smith *et al.*, 1991) using HUVEC cultured on fibronectin coated transwells (Costar) with 3µm pores. HUVEC were incubated with medium alone or TNF-α (100 U/ml) for 24 h, IL-3 (30 ng/ml) being added for the last 6 h. Two hours prior to the addition of neutrophils the medium was changed. 10^6 neutrophils were then added to the upper compartment by replacing the medium, the HUVEC conditioned medium remaining in the lower compartment. The number of neutrophils which had migrated through the endothelium into the lower compartment was determined after 45 min using a Coulter counter (Herts, UK), transmigration being expressed as a percentage of the total cells added.

2.9 Quantitation of cytokines

IL-6, G-CSF and GM-CSF were measured in the culture supernatants of HUVEC (5×10^5 per well) which had been stimulated as described in chapter 5. Immunoreactive IL-6 was detected using an ELISA (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. G-CSF measurements were kindly performed by Dr J. Layton (Ludwig Institute for Cancer Research, Melbourne) by an ELISA method, as described (Zoellner *et al.*, 1992). GM-CSF was detected using a sandwich ELISA method. In brief, the mouse anti-human GM-CSF mAb 4.D4 was used to coat 96-well plates before samples and standards were added. An avidin-biotin peroxidase system was then used to detect the second stage rabbit anti-human GM-CSF polyclonal Ab. For IL-8 measurements, HUVEC were stimulated as described in the text, the medium changed and supernatants collected after 1 h. Immunoreactive IL-8 was quantified by a sandwich-type ELISA (Van Zee *et al.*, 1991) in which a polyclonal rabbit anti-human IL-8 antibody is used as both capture and detection layer.

2.10 Glucose uptake assay

Glucose uptake by HFF fibroblasts (chapter 6) was measured as the intracellular accumulation of ^{3}H-2-deoxyglucose (^{3}H-2-DOG) (Bird *et al.*, 1990), a glucose analog which is recognised by the glucose transporter and phosphorylated, but not further metabolised. Cells were cultured on 24-well plates and incubated in glucose-free buffer (HBSS containing 20 mM HEPES and 0.1% BSA) in the presence or absence of IL-3 (200 ng/ml) for 30 min. The uptake measurement was initiated by adding 2 μCi/ml ^{3}H-2-DOG and unlabelled deoxyglucose to 0.1 mM. After a 10-min incubation at 37°C the wells were rapidly washed with ice-cold PBS, and the cells lysed in 0.05% SDS. The ^{3}H-2-DOG content of the lysates was determined by scintillation counting.

To confirm that glucose uptake was linear during the 10-min pulse, a time-course was performed. The accumulation of ^{3}H-2-DOG was linear for at least 20 min (figure 2.2)

and could be competed by 10 mM D-glucose (data not shown), indicating that uptake is likely to occur via facilitated diffusion through the glucose transporter.

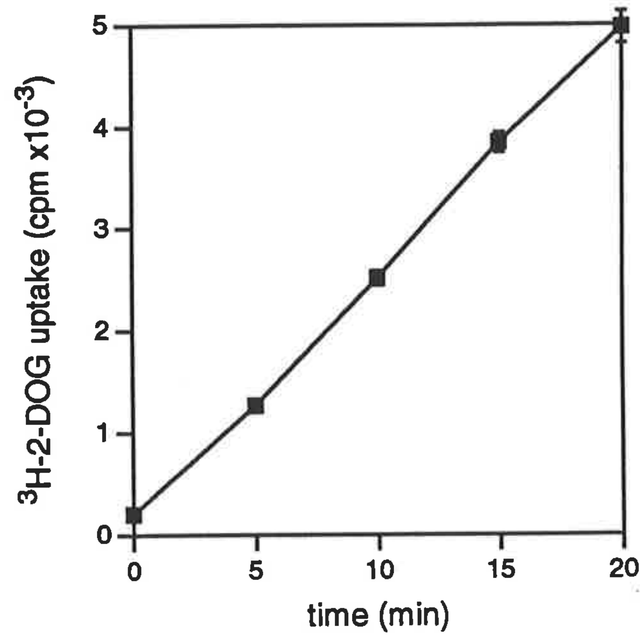


Fig. 2.2 Time-course of ^{3}H-2-DOG uptake in HFF fibroblasts. The cells were incubated in glucose-free buffer for 30 min, then pulsed with ^{3}H-2-DOG for the indicated times. Each point represents the mean±SD determined in duplicate samples.

CHAPTER 3

ENDOTHELIAL CELLS EXPRESS FUNCTIONAL IL-3 RECEPTORS

INTRODUCTION

IL-3 stimulates hemopoietic progenitor cells to proliferate and differentiate into various blood cell types (Lopez *et al.*, 1988, Sieff *et al.*, 1987), a property which has led to its clinical use in bone marrow reconstitution after cancer therapy. In addition, IL-3 has been found to functionally activate some mature blood cells, including monocytes, eosinophils and basophils (Elliott *et al.*, 1990, Lopez *et al.*, 1988, Haak-Frendscho *et al.*, 1988), suggesting that it may play a role in the inflammatory process.

The aim of the work described in this thesis was to examine whether the target cell population is restricted to blood cells, or whether IL-3 can also act on non-hemopoietic cells. Endothelial cells are of particular interest as they play a central role in the inflammatory process, controlling leukocyte infiltration and releasing inflammatory mediators (Vadas *et al.*, 1992). They also secrete hemopoietic growth factors and are a major component of the bone marrow stromal cell population (Seelentag *et al.*, 1987, Jirik *et al.*, 1989, Rafii *et al.*, 1994). Endothelial cells resemble hemopoietic cells in certain aspects, which may reflect the possible common origin of these two cell types (Doetschman *et al.*, 1985). For example, they express CD25 (the α subunit of the IL-2 receptor), the early hemopoietic progenitor marker CD34 and the leukocyte common antigen CD45 (Chen *et al.*, 1991, Fina *et al.*, 1990, Forsyth *et al.*, 1993). Furthermore, they are the only non-hemopoietic cells found to express receptors for erythropoietin and thrombopoietin (c-mpl) (Anagnostou *et al.*, 1990, Methia *et al.*, 1993). Expression of the G-CSF and GM-CSF receptors has also been reported (Bussolino *et al.*, 1989, Colotta *et al.*, 1993) although these findings remain controversial (Yong *et al.*, 1991).

The first step in investigating whether endothelial cells could respond to IL-3 was to determine whether they express receptors for this cytokine. As this was found to be the case, it was important to examine whether these receptors are functional in a non-hemopoietic environment. Induction of the immediate early gene c-fos was used as a

functional read-out, as it has been shown that IL-3 induces c-fos in blood cells and that the ubiquitous Ras pathway is likely to be involved (reviewed by Miyajima *et al.*, 1993).

In addition to the IL-3R α and β chains, HUVEC were also examined for expression of the α chains for the related GM-CSF and IL-5 receptors, which is important as these receptors share the β subunit of the IL-3R and mediate similar functions on target cells. Expression of the GM-CSFR α chain in HUVEC is of particular interest, due to the controversy in the literature as to whether these cells bind and functionally respond to GM-CSF (Bussolino *et al.*, 1989, Yong *et al.*, 1991, Colotta *et al.*, 1993).

RESULTS

3.1 HUVEC express mRNA for the IL-3R α and β chains

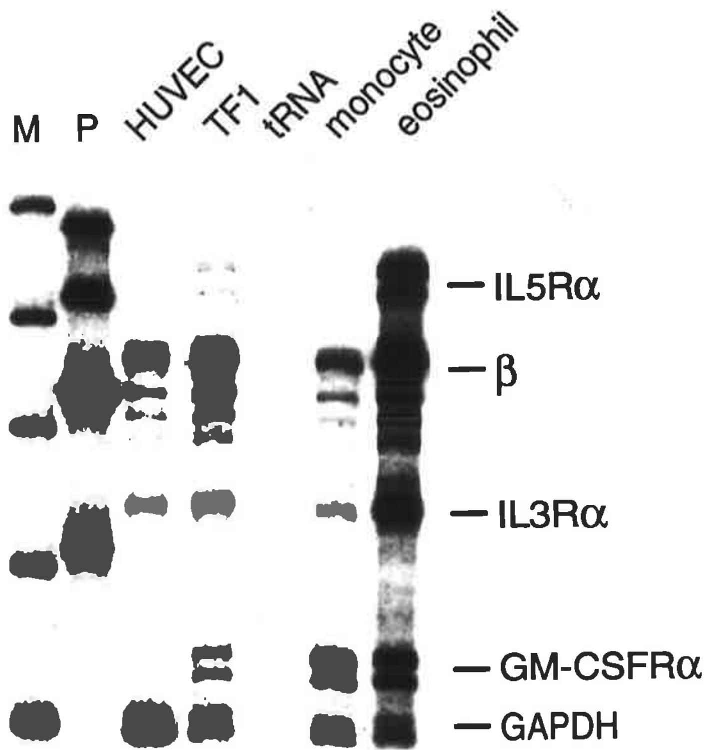
HUVEC were examined for the presence of the IL-3R α and β chain mRNAs as well as those for the related GM-CSFR and IL-5R α chains by using an RNase protection assay. Simultaneous analysis of the different receptor subunit mRNAs was achieved by designing the antisense RNA probes to protect fragments of different sizes. RNA from various hemopoietic cells, including monocytes, eosinophils and TF-1 cells (Kitamura *et al.,* 1989), was used as a positive control for the receptor mRNAs. As shown in figure 3.1, freshly explanted, unstimulated HUVEC expressed substantial amounts of the common β chain mRNA and small amounts of the IL-3R α chain mRNA. In contrast, no expression of the mRNAs for the related GM-CSFR and IL-5R α chains was detected.

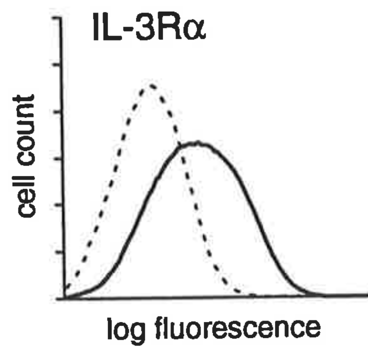
3.2 Surface expression of the IL-3R subunits on HUVEC

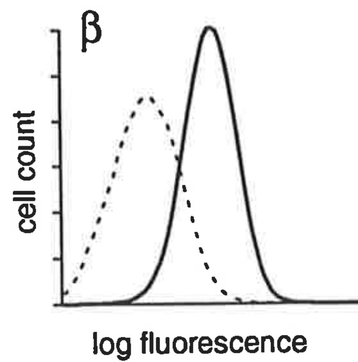
To examine whether expression of the IL-3R mRNA was accompanied by protein expression on the cell surface, HUVEC were stained with the anti-IL-3Rα chain mAb 7.G3 and the anti-β chain mAb 4.F3, and analysed by flow cytometry. These mAbs were developed using mice immunised with COS cells transfected with IL-3R α and β chain expression plasmids, and screened on CHO cell lines which permanently express the appropriate receptor subunit (Woodcock *et al.,* 1994, Qiyu *et al.,* submitted). The anti-GM-CSFR α chain mAb 8.G6 was developed in a similar fashion.

Fig. 3.2 shows representative histograms of HUVEC stained with the mAbs directed against the IL-3Rα, β and GM-CSFRα chains. Staining with 7.G3 resulted in a broad curve, indicating variable levels of IL-3R α chain expression, whereas the curve corresponding to the β chain staining was narrower, indicating a more homogenous expression pattern. Interestingly, the histograms obtained with the anti-GM-CSFR α

Fig. 3.1 HUVEC express mRNA for the IL-3R α and β chains. RNase protection assay of the IL-3R, GM-CSFR and IL-5R subunit mRNAs in HUVEC, monocytes and eosinophils. The samples were also probed for GAPDH mRNA which is used as an internal control. RNA from TF-1 cells was used as a positive control and tRNA as a negative control. Undigested probes (P) and DNA size markers (M; ^{32}P-labelled HpaII-digested pUC19 DNA) are also shown.


M
P
HUVEC
TF1
tRNA
monocyte
eosinophil
IL5Rα
β
IL3Rα
GM-CSFRα
GAPDH





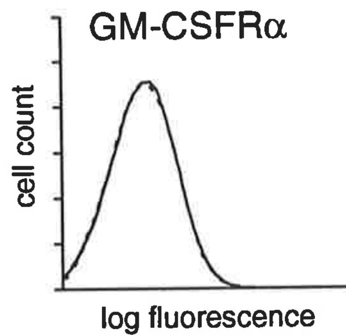


Fig. 3.2 Surface expression of the IL-3R α, β and GM-CSFR α chains on HUVEC.
Flow cytometry analysis of HUVEC stained with the anti-IL-3Rα chain mAb 7.G3, the anti-β chain mAb 4.F3 or the anti-GM-CSFRα chain mAb 8.G6. The isotype control is represented by the dashed histogram.

chain mAb 8.G6 and the negative control Ab overlapped completely, indicating that HUVEC do not express the GM-CSFR α chain protein.

In order to confirm that the small shifts observed in both the IL-3Rα and β chain histograms were significant, several HUVEC lines were tested and the results analysed statistically. The mean fluorescence intensity value (MFI) for cells stained with the negative antibody was 0.432±0.018, whereas that for the IL-3Rα chain was 0.967±0.059, and the β chain 0.975±0.133 (mean±SEM, n=8). Although low, both the IL-3Rα and β chain MFI values were significantly different from that obtained with the negative control antibody ($p<0\cdot0001$ and $p<0.005$ respectively) as judged by student's t-test.

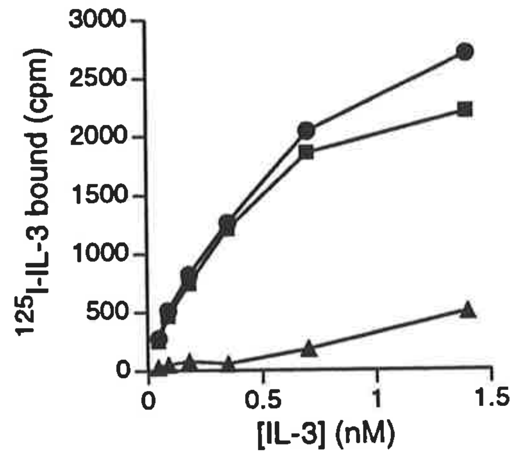
3.3 HUVEC bind IL-3 with high affinity

The ability of HUVEC to specifically bind IL-3 was investigated using ^{125}I-labelled IL-3. Scatchard transformation was derived from a saturation binding curve using six different concentrations of ^{125}I-IL-3 ranging from 40 pM to 1.4 nM, specific binding being determined by subtracting the cpm from parallel samples containing a 100-fold excess of unlabelled IL-3. All samples were analysed in duplicate, using 2×10^6 HUVEC per sample. Endothelial cell lines varied in their ability to bind IL-3, and the number of IL-3Rs per cell, as determined by Scatchard analysis, ranged from undetectable to 2000 (Fig. 3.3). The receptors detected were of the high-affinity class only (K_d=640 pM). In keeping with the lack of GM-CSFR α chain mRNA and protein in HUVEC, no specific binding of ^{125}I-GM-CSF was detected in four separate experiments (data not shown).

3.4 IL-3 induces c-fos expression in HUVEC

To investigate whether the IL-3Rs on HUVEC are capable of transducing a signal, induction of the immediate-early gene c-fos was measured in response to IL-3. HUVEC were incubated in medium containing 0.5% FCS without supplementary growth factors

A



B

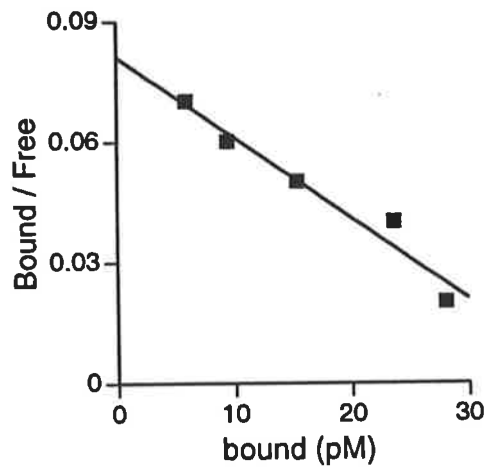
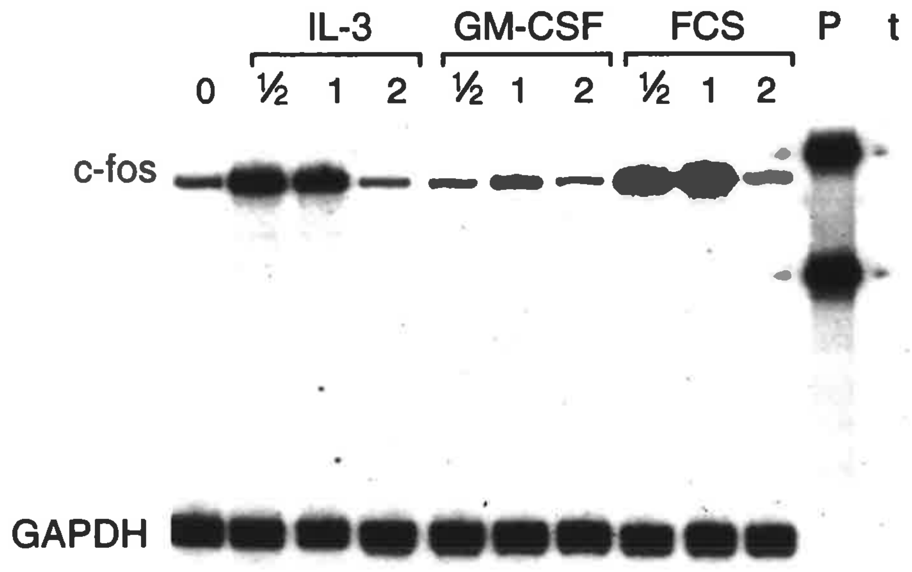


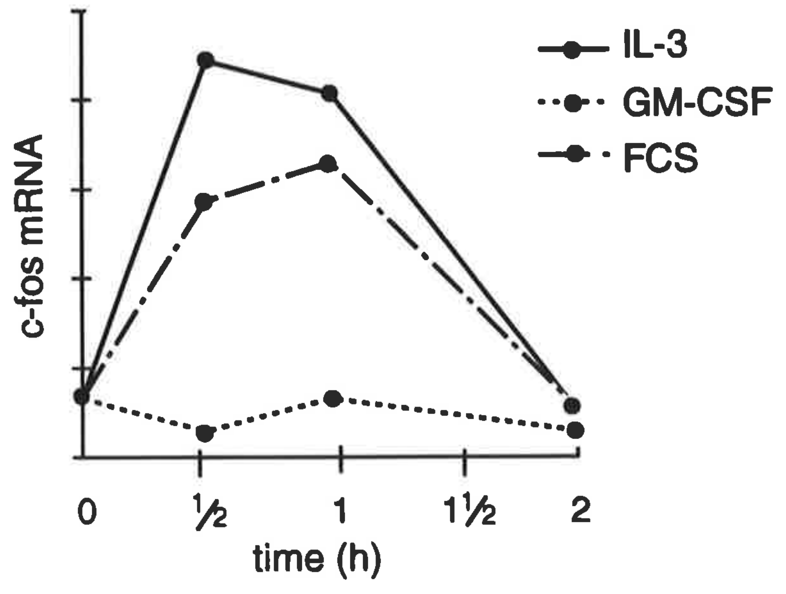
Fig. 3.3 Characterization of ^{125}I-IL-3 binding to HUVEC. a) Saturation binding curve using ^{125}I-IL-3 at 40pM-1.4nM and 2 x 10^6 HUVEC per sample. Specific binding (■) was determined by subtracting non-specific binding (▲) from total cpm (•). The points are averages of duplicate samples. b) Analysis of the data by Scatchard transformation.

for 24 h prior to IL-3 stimulation. IL-3 (30 ng/ml) was then added, total RNA extracted at various times thereafter, and expression of the c-fos mRNA analysed by RNase protection. The induction of c-fos was similarly examined in response to GM-CSF (30 ng/ml) and FCS (20 %), which is a well-characterised inducer of c-fos. As shown in figure 3.4, IL-3 treatment resulted in a rapid and transient induction of c-fos, which reached maximal levels at 30-60 min, and declined back to the basal level by 120 min. This was very similar to the induction observed with FCS. In contrast, GM-CSF did not affect the expression of c-fos (Fig. 3.4).

Fig. 3.4 Induction of the immediate-early gene c-fos in HUVEC in response to IL-3.

RNase protection assay of c-fos mRNA in HUVEC stimulated with IL-3 (30 ng/ml), FCS (20%) or GM-CSF (30 ng/ml) for various times as indicated. The samples were also probed for GAPDH mRNA which is used as an internal control. tRNA was used as a negative control (t) and lane P represents the undigested probes. The gel was quantified by phosphorimaging, and the amount of c-fos mRNA relative to the GAPDH signal is shown in the graph below. Shown is a representative experiment of three.


IL-3
GM-CSF
FCS
P
t
0
½
1
2
½
1
2
½
1
2
c-fos
GAPDH


IL-3
GM-CSF
FCS
c-fos mRNA
0
½
1
1½
2
time (h)

DISCUSSION

This chapter shows that expression of the IL-3R is not restricted to hemopoietic cells as previously thought, but that endothelial cells also express receptors for IL-3. Importantly, the IL-3Rs on HUVEC are able to bind IL-3 with high-affinity and transduce a signal, as shown by the induction of c-fos. The finding of functional IL-3Rs on endothelial cells suggests a novel role for this hemopoietic growth factor in vascular biology.

The presence of the IL-3R was detected using a combination of approaches. RNase protection analysis indicated that there were low levels of both the IL-3R α and β chain mRNAs in HUVEC, which correlated with the modest protein expression detected on the cell surface by flow cytometry. HUVEC were able to specifically bind ^{125}I-IL-3 and, as shown by Scatchard analysis, only high-affinity receptors were present, consistent with the similar levels of IL-3R α and β chain surface expression. HUVEC isolated from different donors showed a great degree of variability in the number of IL-3Rs, ranging from undetectable to 2000 per cell. This line-to-line variation is characteristic of primary HUVEC and may reflect individual differences, as well as the activation state of the endothelium. The presence of IL-3Rs on HUVEC has been recently shown by two other groups. In agreement with our observations, low levels of the IL-3R α and β chain mRNAs have been detected by RT-PCR (Colotta *et al.*, 1993) and by northern blotting following cyclohexamide treatment (Brizzi *et al.*, 1993). Also in keeping with our results, Brizzi *et al.* (1993) have shown that HUVEC bind IL-3 with high affinity.

IL-3 caused a rapid and transient induction of the immediate-early gene c-fos in HUVEC, indicating that hemopoietic cell-specific factors are not required for signalling by the IL-3R. This is consistent with the finding that exogenously expressed human GM-CSFRs are capable of initiating signal transduction in NIH 3T3 fibroblasts (Watanabe *et al.*, 1993, Eder *et al.*, 1993). It remains to be determined whether the IL-3R utilises the same signalling pathway in both HUVEC and hemopoietic cells, or

whether it operates through different mechanisms in different cell types. The biological consequences of c-fos induction result from its ability to complex with members of the c-jun family, forming the heterodimeric transcription factor AP-1. Both Fos and Jun proteins have been shown to be involved in the regulation of cell growth, using anti-sense RNA and microinjection of Fos and Jun antibodies into cells (Nishikura *et al.*, 1987, Riabolow *et al.*, 1988, Kovary *et al.*, 1991). Interestingly, IL-3 has been shown to stimulate the proliferation of endothelial cells *in vitro* (Brizzi *et al.*, 1993), and cause bone marrow fibrosis, characterised by endothelial proliferation, *in vivo* (Orazi *et al.*, 1992).

As shown in this chapter, HUVEC selectively express mRNA for the IL-3R α and β chains but not for the related GM-CSFR and IL-5R α chains. The lack of detectable GM-CSFR α chain mRNA was somewhat surprising, as HUVEC have been reported to bind and functionally respond to GM-CSF (Bussolino *et al.*, 1989; Colotta *et al.*, 1993). However, in our study neither GM-CSFR α chain protein (as judged by mAb staining), nor specific ^{125}I-GM-CSF binding was found. In keeping with the lack of receptor expression, GM-CSF was unable to induce c-fos in HUVEC. This is in contrast to the reports that HUVEC specifically bind GM-CSF with high affinity and respond to it by c-fos induction, proliferation and migration (Bussolino *et al.*, 1989; Colotta *et al.*, 1993). Others have been unable to replicate these results, although high levels of non-specific ^{125}I-GM-CSF binding to HUVEC has been observed (Yong *et al.*, 1991), consistent with our results (data not shown). The reason for this discrepancy is not clear, but it suggests that the ability of HUVEC to bind and respond to GM-CSF is critically dependent on the culture conditions. It should be noted that despite observing GM-CSF binding and functional activation of HUVEC, Colotta *et al.* (1993) also failed to detect any mRNA for the GM-CSFR α chain, prompting them to speculate that HUVEC may express a different, novel kind of GM-CSF receptor. However, in light of our results and those of Yong *et al.*, (1991) one should not assume that GM-CSF influences endothelial cell function *in vivo*. The leukocyte margination observed following GM-CSF administration

(Devreux *et al.*, 1989) is thus likely to be due to a direct effect on neutrophils and monocytes, rather than on the endothelium.

Vascular endothelium is a multifunctional organ, participating in the control of immune and inflammatory phenomena and in the regulation of hemopoiesis. The finding of IL-3Rs on endothelial cells opens a wider role for IL-3 than previously expected. Activation of the endothelium by IL-3 could be envisaged in pathological conditions which involve infiltration of activated T cells, such as chronic inflammation. Indeed, IL-3 mRNA expression has been detected in activated T lymphocytes at the site of inflammation *in vivo* (Kay *et al.*, 1991). The finding that IL-3 is not exclusively a hemopoietic growth factor but also acts on vascular endothelium may aid in understanding the side-effects of IL-3 administration.

SUMMARY

IL-3 is a T cell-derived hemopoietic growth factor which not only stimulates hemopoiesis but also functionally activates mature blood cells, such as monocytes, eosinophils and basophils. This chapter demonstrates that the actions of IL-3 are not restricted to hemopoietic cells, as endothelial cells are also able to bind and functionally respond to this cytokine.

HUVEC were found to selectively express mRNA for the IL-3R α chain as well as for the common β chain, but not for the related GM-CSFR and IL-5R α chains. The mRNA expression correlated with protein expression on the cell surface, as judged by mAb staining and by the ability of HUVEC to specifically bind ^{125}I-IL-3 with high affinity. IL-3 caused a rapid and transient induction of the immediate-early gene c-fos in HUVEC, indicating that the IL-3R is functional in endothelial cells and that hemopoietic cell-specific factors are not required for signal transduction by the IL-3R. In contrast to some previous reports, HUVEC did not bind or functionally respond to GM-CSF.

The finding that endothelial cells express functional IL-3 receptors suggests a wider role for IL-3 than previously thought. Vascular endothelium is a multifunctional interface between the blood and tissues, and participates actively in the control of immune and inflammatory phenomena as well as the regulation of hemopoiesis. The effect of IL-3 on endothelial functions related to these processes will be discussed in the following chapters. The activation of endothelial cells by IL-3 (but not by GM-CSF) is of particular clinical relevance, as both of these cytokines are used in bone marrow reconstitution following cancer therapy.

CHAPTER 4

ENDOTHELIAL IL-3 RECEPTOR EXPRESSION IS REGULATED BY CYTOKINES

INTRODUCTION

IL-3 is a multifunctional cytokine with activities ranging from stimulation of hemopoiesis to activation of mature blood cells. As described in the previous chapter, endothelial cells also express receptors for IL-3 and have the capacity to functionally respond to it. The production of IL-3 by activated T cells and mast cells is strictly controlled, and the mechanisms involved in this regulation have been extensively investigated. However, the *in vivo* effect of IL-3 does not solely depend on the extent of IL-3 production, but also on the responsiveness of the target cells to this factor.

Responsiveness to IL-3 could be regulated at several levels, including receptor expression and signal transduction. Unlike signalling molecules, the receptors are IL-3 -specific, thus offering the possibility to selectively control IL-3 responsiveness. Indeed, recent reports suggest that the expression of cytokine receptors, including that of IL-3, can be regulated and that the regulation has functional importance. For example, IL-1 has been shown to increase β chain expression in TF-1 cells (Watanabe *et al.*, 1992), which may account for the synergistic effect of IL-1 on the proliferation of TF-1 cells in response to IL-3, IL-5 and GM-CSF (Kitamura *et al.*, 1991B). Expression of the β chain is also enhanced by IFN-γ in monocytes (Hallek *et al.*, 1992) and by TNF-α in $CD34^+$ hemopoietic progenitor cells (Sato *et al.*, 1993), and TNF-α has been shown to increase the expression of the IL-3 and GM-CSF receptors on AML cells (Elbaz *et al.*, 1991). On the other hand, IL-3R down-regulation has been observed with TGF-β in murine bone marrow cells, correlating with inhibition of IL-3 -induced proliferation (Jacobsen *et al.*, 1993).

The regulation of IL-3R expression has so far been documented only in hemopoietic cells. The work described in this chapter aimed to examine whether endothelial IL-3R expression is also regulatable by cytokines, and whether the stimuli and responses are similar to those observed for blood cells. Control of IL-3R expression by humoral factors, including those associated with the inflammatory response, could potentially

modulate the responsiveness of endothelium to IL-3 *in vivo*. The factors examined include the pro-inflammatory stimuli TNF-α, IL-1 and LPS, as well as the lymphokines IFN-γ and IL-4 which, like IL-3 itself, are produced upon T cell activation. All of these factors are known to activate endothelial cells functionally, although the set of functions they induce are qualitatively different. For example, TNF-α, IL-1 and LPS increase expression of ICAM-1 and E-selectin and production of IL-8, thus producing an endothelial phenotype promoting neutrophilic infiltration (reviewed by Springer, 1994). IL-4 increases endothelial adhesiveness for monocytes, eosinophils and lymphocytes, rather than neutrophils, by inducing the expression of VCAM (Schleimer *et al.*, 1992) and it also stimulates production of monocyte chemoattractant protein (MCP-1) (Rollins *et al.*, 1991). IFN-γ in turn induces an endothelial phenotype characterised by enhanced expression of the MHC molecules (Pober *et al.*, 1983A), thus favouring immunological events.

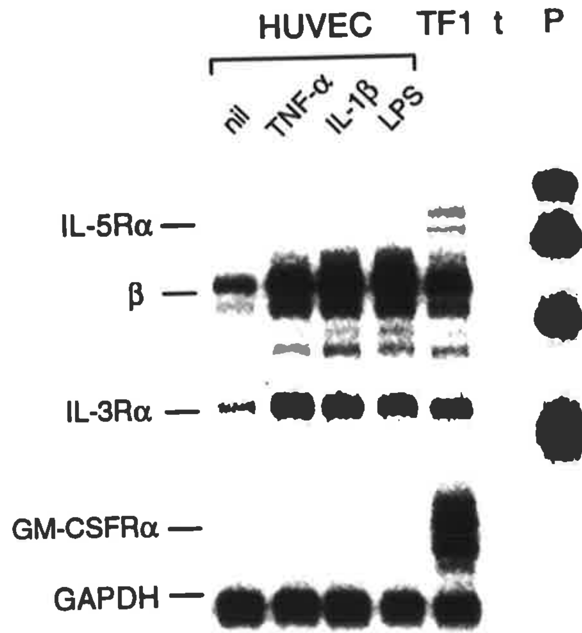
RESULTS

4.1 Expression of the IL-3R α and β chain mRNAs in HUVEC is enhanced by cytokines

A panel of factors, including TNF-α, IL-1β, LPS, IFN-γ and IL-4, were examined for their potential to regulate IL-3R mRNA expression in HUVEC. Total RNA was extracted from HUVEC treated with medium alone, TNF-α (100 U/ml), LPS (100 ng/ml), IFN-γ (100 U/ml) or IL-4 (10 ng/ml) for 24 h, or with IL-1β (100 U/ml) for 12 h, and probed for the IL-3R α and β chain mRNAs using an RNase protection assay. The samples were also simultaneously probed for the mRNAs for the related GM-CSFR and IL-5R α chains, as well as for GAPDH, which was used as an internal control. RNA from TF1 cells (Kitamura *et al.*, 1989) was used as a positive control for the different receptor mRNAs. As demonstrated in figure 4.1, treatment of HUVEC with TNF-α, IL-1β, LPS or IFN-γ strongly enhanced the expression of both the IL-3R α and β chain mRNAs. In contrast to this coordinate up-regulation of both IL-3R subunits, IL-4 selectively increased only the β chain mRNA. None of the factors tested induced detectable expression of the GM-CSFR and IL-5R α chain mRNAs.

The induction of the IL-3R mRNA by TNF-α and IFN-γ was further characterised by time-course and titration experiments. The expression of the IL-3R α and β chains was analysed by RNase protection assay and quantitated by phosphorimaging, using GAPDH as an internal control. Both TNF-α and IFN-γ transiently up-regulated expression of the IL-3R α and β chains in a dose-dependent fashion. With IFN-γ, levels of both the IL-3R transcripts were increased by 2 h, maximal at 12-24 h, then slowly decreased (Fig. 4.2a). Dose-response experiments showed that the maximal effect of IFN-γ was achieved at a concentration of 100-1000 U/ml (Fig. 4.2b). When HUVEC were stimulated with TNF-α, the IL-3R α and β chain mRNA levels increased at 4 h, reached a maximum at 16-36 h, and slowly decreased thereafter (Fig. 4.2c), the optimal dose being 100-1000 U/ml (Fig. 4.2d). In five experiments performed with different HUVEC primary cultures, the IL-3R α chain mRNA expression increased 2.4±0.4

Fig. 4.1 Selective induction of the IL-3R mRNA in HUVEC by a) the pro-inflammatory stimuli TNF-α, IL-1 and LPS and b) the lymphokines IFN-γ and IL-4. RNase protection assays of the IL-3R α and β chain mRNAs in HUVEC stimulated with either medium alone, TNF-α (100 U/ml), LPS (100 ng/ml), IFN-γ (100 U/ml) or IL-4 (10 ng/ml) for 24 h, or with IL-1 (100 U/ml) for 12 h. The samples were also probed for the mRNAs for the GM-CSFR and IL-5R α chains and GAPDH, which was used as an internal control. RNA from TF-1 cells was used as a positive control (TF1) and tRNA as a negative control (t). Lane P represents the undigested probes.


A
HUVEC
TF1
t
P
nil
TNF-α
IL-1β
LPS
IL-5Rα
β
IL-3Rα
GM-CSFRα
GAPDH

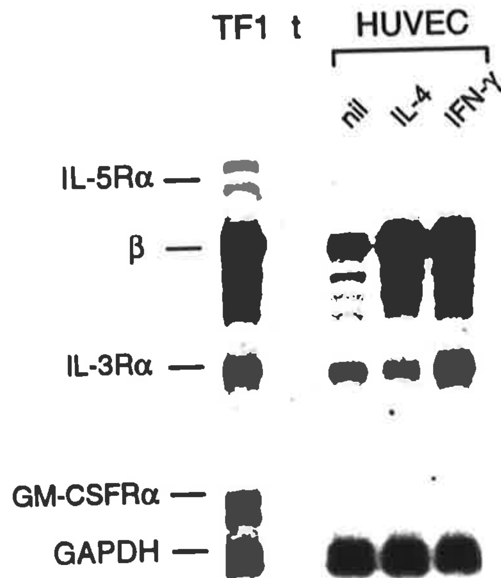
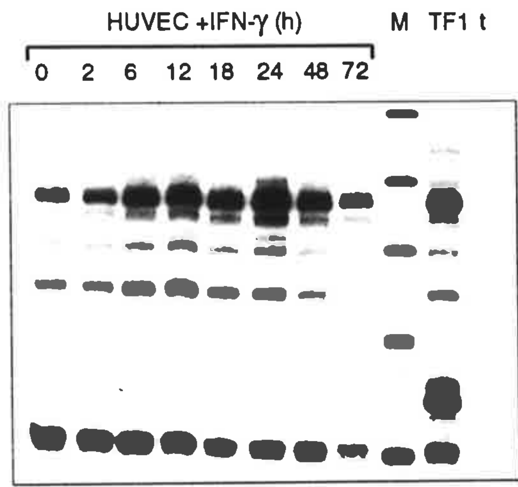
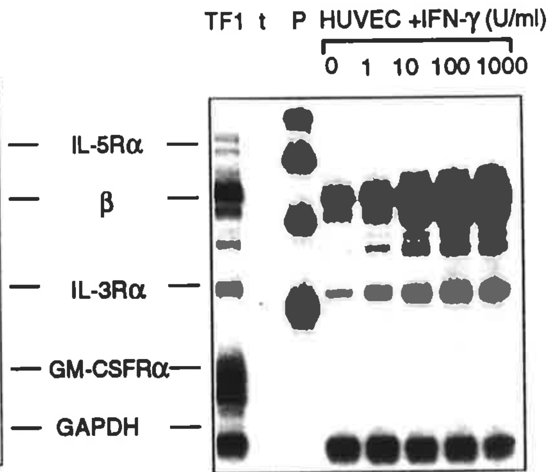

B
TF1
t
HUVEC
nil
IL-4
IFN-γ
IL-5Rα
β
IL-3Rα
GM-CSFRα
GAPDH

Fig. 4.2 IFN-γ and TNF-α enhance expression of the IL-3R α and β chain mRNAs in a time- and dose-dependent manner. RNase protection assay of IL-3R, GM-CSFR and IL-5R mRNAs in HUVEC incubated with IFN-γ (100 U/ml) or TNF-α (100 U/ml) for various times as indicated (a,c), or with varying concentrations of IFN-γ or TNF-α for 24 h (b,d). The samples were also probed for GAPDH mRNA, which is used as an internal control. RNA from TF-1 cells was used as a positive control (TF1) and tRNA as a negative control (t). Also shown is RNA from elutriated mononuclear cells (E). Lanes P and M contain undigested probes and DNA size markers (^{32}P-labeled HpaII-digested pUC19 DNA) respectively.

a

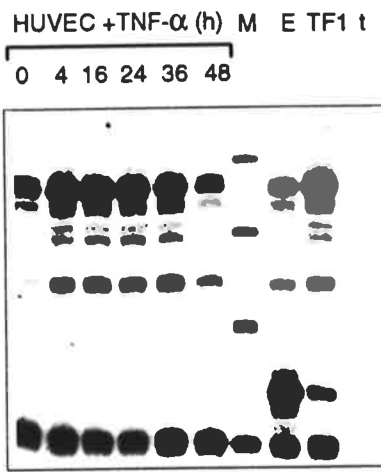
b

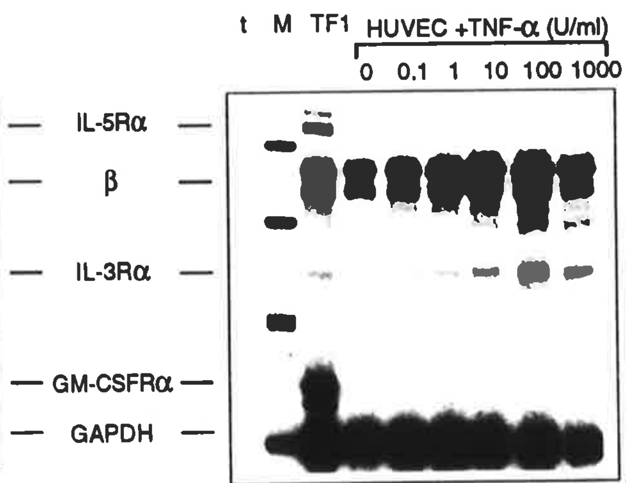




c

d





(mean±SD) -fold in response to IFN-γ and 4.5±0.5 (mean±SD) -fold in response to TNF-α. In parallel experiments the β chain mRNA expression was increased 2.2±0.4 (mean±SD) -fold by IFN-γ and 2.3±0.2 (mean±SD) -fold by TNF-α.

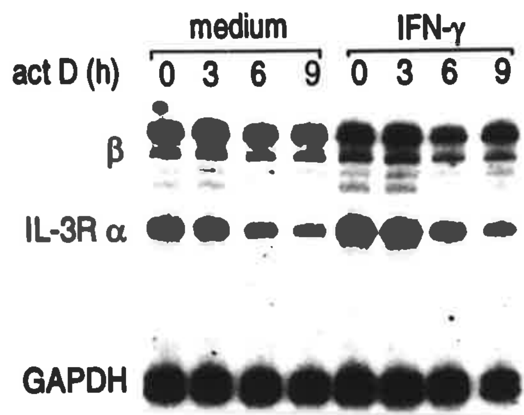
4.2 Increased IL-3R α and β chain mRNA expression is not due to mRNA stabilisation

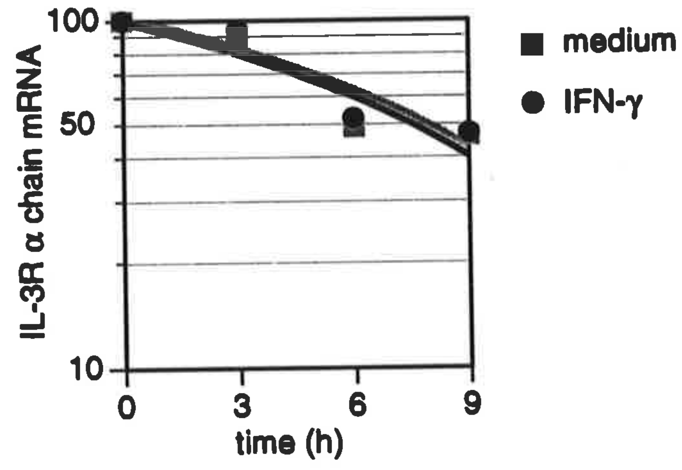
The observed induction of the IL-3R α and β chain mRNA steady-state levels in HUVEC in response to IFN-γ and TNF-α could be mediated by increased transcription and/or mRNA stabilisation. In order to examine the latter possibility, the half-lives of the IL-3R α and β chain mRNAs in HUVEC were determined using the transcription inhibitor actinomycin D (actD). HUVEC were incubated with medium alone or with IFN-γ (100 U/ml) for 4 h, then actD (5 μg/ml) was added and total RNA extracted at various times. IL-3R α and β chain mRNA decay was analysed using an RNase protection assay and quantitated by phosphorimaging, using GAPDH mRNA as an internal control (Fig. 4.3). In 4 separate experiments, all performed with different HUVEC primary cultures, the IL-3R α chain mRNA had a half-life of ≈6 h, and was not altered by IFN-γ treatment. The β chain mRNA was more stable and did not significantly decay during the 9-h actD time-course, in either unstimulated or IFN-γ-stimulated HUVEC. Similarly, TNF-α treatment did not alter the stability of the IL-3R α and β chain mRNAs (data not shown). Identical results were obtained using the alternative transcription inhibitor DRB (100 μM).

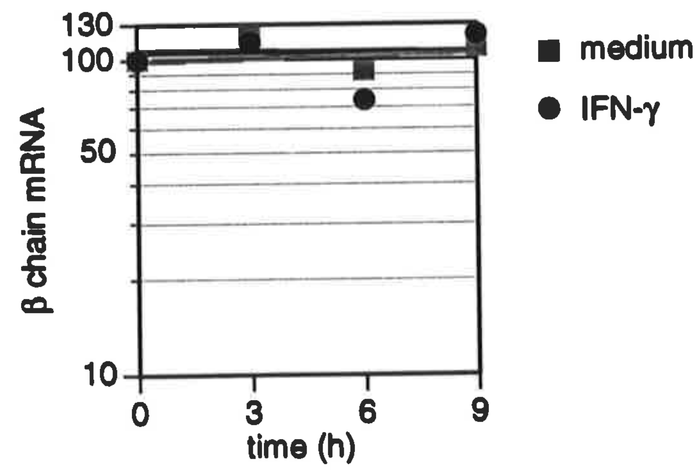
4.3 TNF-α, IFN-γ and IL-4 regulate the surface expression of IL-3R subunits on HUVEC

To examine whether the enhanced IL-3R mRNA levels were accompanied by increased protein levels, the surface expression of the IL-3R subunits on HUVEC was monitored using the specific monoclonal antibodies 7.G3 and 4.F3, directed against the IL-3R α and β chains respectively. HUVEC were stimulated with TNF-α (100 U/ml), IFN-γ (100 U/ml) or IL-4 (10 ng/ml) for 24 h, and the surface expression of the IL-3R α and β chains analysed by flow cytometry. Representative histograms are shown in figure 4.4.

Fig. 4.3 RNase protection analysis of the IL-3R α and β chain mRNA half-lives in HUVEC. HUVEC were pretreated with either medium alone or with IFN-γ (100 U/ml) for 4 h, after which ongoing transcription was inhibited with actinomycin D (5 μg/ml) for various times, as indicated. Phosphorimager quantitation is shown below. Levels of the IL-3R α and β chain mRNAs are expressed as a percentage of the initial IL-3R α/GAPDH or the β/GAPDH ratio.


medium
IFN-γ
act D (h)
0 3 6 9 0 3 6 9
β
IL-3R α
GAPDH


IL-3R α chain mRNA
100
50
10
0 3 6 9
time (h)
medium
IFN-γ

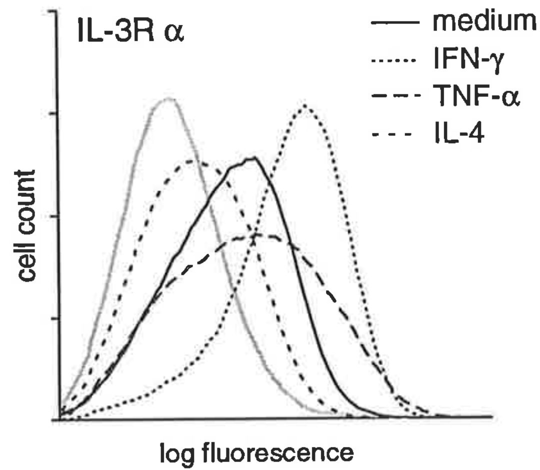

β chain mRNA
130
100
50
10
0 3 6 9
time (h)
medium
IFN-γ

In keeping with the mRNA data, IFN-γ and TNF-α enhanced the surface expression of both IL-3R subunits, whereas IL-4 selectively up-regulated expression of the β chain only. Interestingly, down-regulation of the IL-3R α chain was observed following the treatment with IL-4. None of the factors tested induced detectable expression of the GM-CSFR α chain, as judged by staining with the specific mAb 8.G6 (data not shown).

The regulation of IL-3R α and β chain expression by cytokines was studied in several HUVEC lines, and the results statistically analysed. As shown in figure 4.5, both TNF-α and IFN-γ increased expression of the 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 on IL-3R expression, up-regulating the α chain by ≈14 fold and the β chain by ≈8 fold. This synergism was also observed at the mRNA level (data not shown). Although IL-4 strongly up-regulated expression of the β chain, it consistently down-regulated the IL-3R α chain. The inhibition of IL-3R α chain expression increased with time, being 64±17 % (mean±SD,n=3) at 24 h and 78±19 % (mean±SD,n=3) at 48 h following treatment with IL-4.

4.4 Binding of IL-3 to HUVEC is induced by TNF-α and IFN-γ

The effect of TNF-α and IFN-γ on the ability of HUVEC to specifically bind IL-3 was investigated using ^{125}I-IL-3. HUVEC were incubated with IFN-γ (100 U/ml) or TNF-α (100 U/ml) for various times, and binding assays performed using 1-4 nM ^{125}I-IL-3 and 1.7 x 10^6 HUVEC per sample. In keeping with the increased surface expression of IL-3R subunits, both TNF-α and IFN-γ increased the specific binding of IL-3 to HUVEC (Fig. 4.6). The induction by TNF-α was transient, with maximal binding occurring at 24 h. In contrast, the induction by IFN-γ was more prolonged, reaching a maximum at 20 h and remaining elevated thereafter. Scatchard analysis indicated that neither TNF-α or IFN-γ significantly altered the affinity of IL-3 binding to HUVEC, but rather increased the number of binding sites (data not shown).



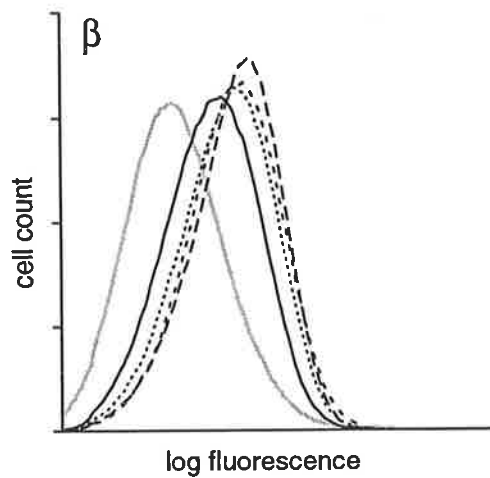


Fig. 4.4 Analysis of regulation of the IL-3R α and β chains on HUVEC. HUVEC were stimulated with either medium alone, TNF-α (100 U/ml), IFN-γ (100 U/ml) or IL-4 (10 ng/ml) for 24 h. The cells were stained with the anti-IL-3Rα chain mAb 7.G3 or with the anti-β chain mAb 4.F3, and indirect immunofluorescence analysed by flow cytometry. The isotype control is represented by the grey histogram.

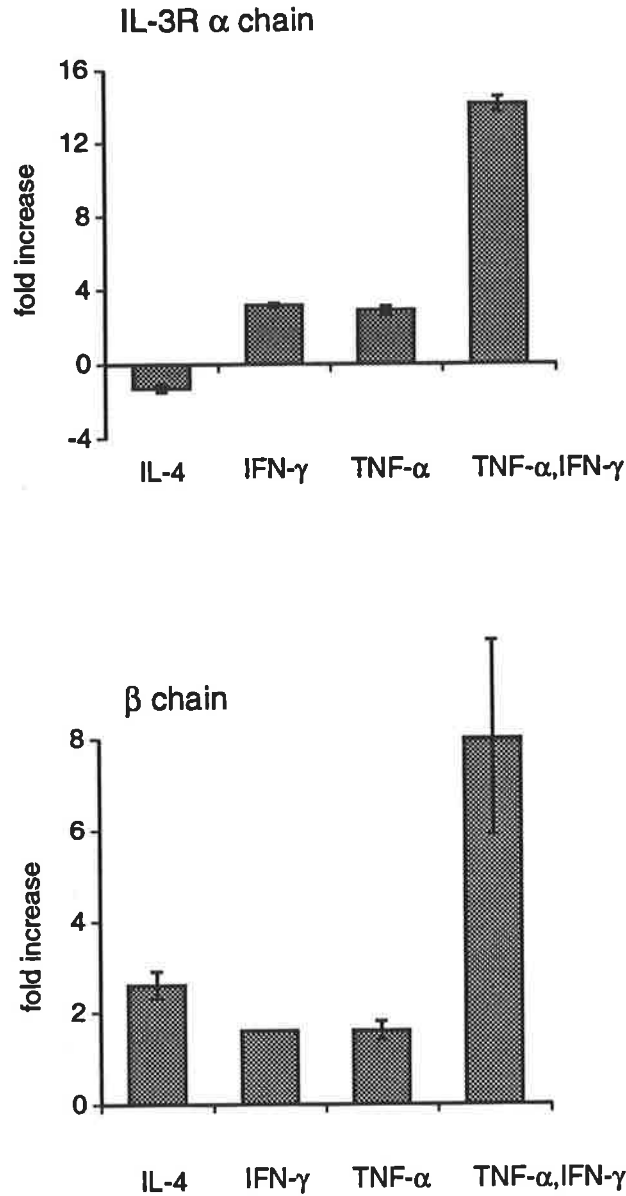
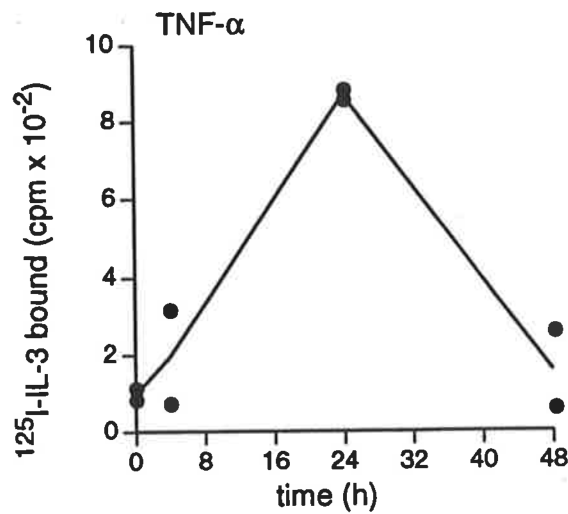


Fig. 4.5 Quantitation of the cytokine-induced IL-3R expression in HUVEC. Cells were stimulated with medium alone, TNF-α (100 U/ml), IFN-γ (100 U/ml) or both factors together for 24 h, or with IL-4 (10 ng/ml) for 24 h. HUVEC were then stained with the anti-IL-3Rα chain mAb 7.G3 or with the anti-β chain mAb 4.F3, and analysed by flow cytometry. The increase in the IL-3R α and β chain surface expression over the basal level was calculated using the mean fluorescense intensity (MFI) values obtained by flow cytometry. Shown is the mean±SEM of four experiments, each performed with different primary HUVEC cultures.



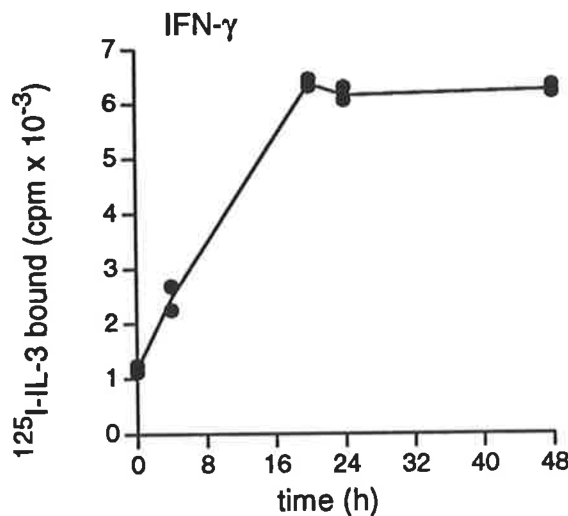


Fig. 4.6 Time-course of specific ^{125}I-IL-3 binding to HUVEC stimulated with TNF-α or IFN-γ. Shown are two separate experiments in which different primary HUVEC lines were stimulated with either a) TNF-α (100 U/ml) or b) IFN-γ (100 U/ml). ^{125}I-IL-3 was used at either a) 4 nM or b) 1 nM with specific activities 0.6 x 10^6 cpm/pmol and 0.9 x 10^6 cpm/pmol respectively. Each experiment is a representative of three.

DISCUSSION

The data in this chapter shows that expression of the IL-3R in endothelial cells is not static, but can be regulated by humoral factors such as the pro-inflammatory stimuli TNF-α, IL-1 and LPS and the lymphokines IFN-γ and IL-4. Control of expression of the IL-3R may serve to modulate the responsiveness of endothelium to IL-3 during inflammatory and immunological responses.

Regulation of expression of the IL-3R in HUVEC was detected using a combination of approaches. Increased levels of IL-3R α and β chain mRNAs were observed after stimulation of HUVEC with TNF-α, IL-1, LPS or IFN-γ. This coordinate up-regulation of both α and β chains is important from the functional point of view, since both subunits are required for IL-3 signalling (Kitamura *et al.*, 1992). The induction of the IL-3R was selective in that the mRNAs for the related GM-CSFR and IL-5R α chains were not detected. In keeping with the mRNA data, TNF-α and IFN-γ selectively enhanced the surface expression of both the IL-3R α and β chains (as judged by mAb staining), and increased ^{125}I-IL-3 binding to HUVEC. The induction of ^{125}I-IL-3 binding by IFN-γ differed from that mediated by TNF-α in being more prolonged, suggesting a role in chronic inflammation. Interestingly, expression of both the IL-3R α and β chains could be further increased by combining IFN-γ and TNF-α, the synergistic effect suggesting that these agents operate through different mechanisms.

In contrast to IFN-γ and TNF-α, which induced the expression of both IL-3R chains, IL-4 selectively up-regulated only the β chain and simultaneously down-regulated the IL-3R α chain. Because endothelial cells do not normally express excess IL-3R α chains (as judged by the lack of low-affinity IL-3 binding sites in scatchard analysis, chapter 3), this down-regulation by IL-4 may seriously impair their responsiveness to IL-3. It is of interest that IL-4 also inhibits E-selectin expression (Thornhill *et al.*, 1990) and IL-8 production (Smith *et al.*, submitted) by endothelial cells, thus dampening the acute inflammatory response involving neutrophil infiltration. IL-4 shares these anti-

inflammatory actions with TGF-β (Gamble *et al.*, 1993, Smith *et al.*, submitted), and the possibility that TGF-β may also down-regulate IL-3R expression in endothelial cells is currently being investigated. Interestingly, TGF-β is to date the only cytokine that has been shown to inhibit IL-3R expression (and IL-3 responsiveness) in hemopoietic cells (Jacobsen *et al.*, 1993). The fact that IL-4 selectively up-regulated the β chain while down-regulating the IL-3R α chain is somewhat surprising, especially as it did not induce any detectable expression of the GM-CSFR and IL-5R α chains. It is possible that the β chain may serve as an affinity-converter and signal-transducer for another, as yet unidentified α chain, or that it has some function of its own. With regard to the latter possibility, it is of interest that the extracellular domain of the β chain bears homology to the type III fibronectin modules (Patthy, 1990), raising the possibility of a role in adhesion.

IFN-γ has previously been shown to selectively up-regulate the β chain but not the GM-CSFR α chain in human monocytes (Hallek *et al.*, 1992), which is in keeping with our results with HUVEC and suggests that the expression of these genes is under similar regulatory control in endothelial and hemopoietic cells. In contrast, the findings that IFN-γ and TNF-α up-regulate the IL-3R α chain in HUVEC but do not affect IL-3R α chain expression in $CD34^+$ hemopoietic progenitor cells (Sato *et al.*, 1993) indicates a different pattern of regulation of the IL-3R α chain in hemopoietic and endothelial cells.

The mechanisms regulating cytokine receptor expression are still poorly understood. Involvement of both transcriptional and post-transcriptional elements have been documented in induction of the β chain by IFN-γ in monocytes, with mRNA stabilisation having a predominant effect (Hallek *et al.*, 1992). Regulation of mRNA stability is less likely to play a role in endothelial cells, because the β chain mRNA is considerably more stable in HUVEC ($t_{1/2}$>>9 h) than in monocytes ($t_{1/2}$=2 h). The IL-3R α chain mRNA is less stable than that of the β chain, but its half-life (≈6 h) was not prolonged in response to either IFN-γ or TNF-α. Taken together, this suggests that induction of the IL-3R α and β chain mRNAs in HUVEC is likely to occur via

transcriptional activation. In order to confirm this hypothesis, the transcription rates should be measured by nuclear run-on assays, but unfortunately these experiments were impractical to perform with primary HUVEC due to the large number of cells required. Cloning of the human IL-3R α and β chain promoters would open up new possibilities to study the transcriptional control of these genes. Interestingly, the promoters for the mouse IL-3R α and β chains have been shown to contain recognition sites for IFN-γ -inducible transcription factors (Miyajima *et al.*, 1995, Gorman *et al.*, 1992).

From the functional point of view, induction of the IL-3R by TNF-α and IFN-γ is especially important in those endothelial cells which express no or very low levels of receptors. Assuming that a certain threshold level of IL-3R expression is necessary to generate a biological response, this receptor up-regulation would allow larger numbers of endothelial cells to respond to IL-3. On the other hand, the inhibition of IL-3R α chain expression by IL-4 may limit the responsiveness of endothelium to IL-3 *in vivo*. The pattern of IL-3R regulation in different, cytokine-induced activation states of endothelium can provide clues to the functional role of IL-3 in vascular biology. For example, IL-3R expression is enhanced by TNF-α and inhibited by IL-4, as are those endothelial functions which favour neutrophil infiltration, suggesting that IL-3 may also be involved in this process. This hypothesis, as well as the possible role of IL-3 in regulating endothelial functions related to immunity and hemopoiesis, will be discussed in the next chapter.

SUMMARY

Regulation of the *in vivo* effect of IL-3 is likely to occur not only at the level of IL-3 production but also at the level of target cell responsiveness to this factor. In hemopoietic cells, control of IL-3 responsiveness is achieved at least in part by regulating expression of the IL-3R. This chapter shows that IL-3R expression is also regulatable in endothelial cells: up-regulation was observed in response to TNF-α and IFN-γ, and down-regulation in response to IL-4.

TNF-α and IFN-γ increased expression of both the IL-3R α and β chains, having a synergistic effect when combined. In contrast to this coordinate up-regulation of both IL-3R subunits, IL-4 selectively enhanced β chain expression and down-regulated the IL-3R α chain. None of the factors tested induced detectable expression of the GM-CSFR and IL-5R α chains. This regulation of IL-3R expression was observed at both the mRNA and protein level, and correlated with the ability of HUVEC to bind IL-3. The mechanism mediating induction of the IL-3R by TNF-α and IFN-γ remains to be investigated, but does not seem to involve mRNA stabilisation, as indicated by the lack of effect of these cytokines on the half-lives of the IL-3R α and β chain mRNAs.

TNF-α and IFN-γ may recruit more endothelial cells to respond to IL-3 by increasing the receptor number over the threshold level required for generating a biological response. On the other hand, IL-4 stimulation could have the opposite effect by inhibiting expression of the IL-3R α chain. Considered together, the control of IL-3R expression by cytokines may serve to modulate the responsiveness of endothelium to IL-3 during inflammatory and immunological responses *in vivo*.

CHAPTER 5

REGULATION OF ENDOTHELIAL CELL FUNCTION BY IL-3

INTRODUCTION

The vascular endothelium is a dynamic interface between the blood and tissues which is involved in the control of immune and inflammatory phenomena and in the regulation of hemopoiesis. Active participation in these diverse processes requires endothelial cells to undergo phenotypic changes, such as alterations in the expression of adhesion and antigen presenting molecules and the production of chemokines and hemopoietic growth factors. These changes are orchestrated to a large degree by cytokines. The finding of functional IL-3 receptors on endothelial cells suggests that IL-3 may also play a role in this regulation. The effect of IL-3 on various endothelial functions related to inflammation, immunity and hemopoiesis has been investigated in this chapter.

The central role of the endothelium in inflammation is based on its ability to control leukocyte trafficking from the blood to the inflammatory site in the tissue. Differential expression of adhesion molecules and chemokines enables the endothelium to selectively promote the adhesion and transmigration of different leukocyte populations (reviewed by Springer, 1994). For example, neutrophil infiltration is favoured by the expression of E-selectin, an adhesion molecule that mediates the initial "rolling" interaction of neutrophils with the endothelium, and by the secretion of IL-8, which enhances adhesion by activating neutrophil integrins, and directs the subsequent transendothelial migration. In keeping with their role in the acute inflammatory response, the expression of both E-selectin and IL-8 is rapidly induced by the pro-inflammatory cytokines TNF-α, IL-1 and bacterial endotoxin (LPS) (Bevilacqua *et al.*, 1987, Sica *et al.*, 1990).

Endothelial cells are an important source of hemopoietic cytokines such as G-CSF, GM-CSF and IL-6, which regulate the inflammatory response in several ways. The granulocyte colony-stimulating factors G-CSF and GM-CSF not only activate effector functions of granulocytes and monocytes, but also stimulate their maturation (reviewed by Demetri *et al.*, 1991 and Gasson, 1991), thereby replenishing the pool of circulating

leukocytes. IL-6 regulates immune responses and the acute phase reaction and also stimulates hemopoietic progenitor growth in synergy with IL-3 (reviewed by Kishimoto *et al.*, 1992). The production of all these factors by the endothelium is induced by the pro-inflammatory stimuli TNF-α, IL-1 and LPS (Seelentag *et al.*, 1987, Jirik *et al.*, 1989).

Endothelial cells express the MHC molecules necessary for antigen presentation in immune reactions. Unlike "professional" APCs, endothelial cells do not express MHC class II molecules constitutively, but the expression is inducible by IFN-γ (Pober *et al.*, 1983A). Functional activation of $CD4^+$ T lymphocytes by endothelial cells has been observed *in vitro* (Geppert *et al.*, 1985, Adams *et al.*, 1992), and the endothelium may play an important role in initiating or propagating T cell activation in immunologically-mediated inflammation and allograft rejection *in vivo*. Endothelial cells also express the MHC class I antigens required for activation of $CD8^+$ lymphocytes in CTL responses, and this expression is further enhanced by TNF-α and IFN-γ (Doukas *et al.*, 1990).

RESULTS

5.1 IL-3 enhances TNF-α -induced IL-8 secretion, E-selectin expression and neutrophil transmigration

The effect of IL-3 on endothelial IL-8 production was studied using both unstimulated cells, and cells which had been pretreated with TNF-α or IFN-γ. As shown in the previous chapter, these cytokines up-regulate expression of the IL-3R in HUVEC, thus possibly increasing their responsiveness to IL-3. HUVEC were stimulated with medium alone, TNF-α (100 U/ml) or IFN-γ (100 U/ml) for 18 h, and IL-3 (30 ng/ml) was then added for 6 h. IL-8 was measured in the culture supernatants using an ELISA method. As shown in table 5.1, IL-3 did not induce IL-8 production by unstimulated HUVEC, but significantly enhanced the production of IL-8 by HUVEC which had been treated with TNF-α, compared to the IL-8 levels elicited by TNF-α alone. The increase in IL-8 expression was also seen at the mRNA level (W. Smith, personal communication). In contrast, when IL-3 was combined with IFN-γ, which does not induce IL-8 production, no enhancing effect was observed (data not shown).

Surface expression of E-selectin was examined in HUVEC stimulated with medium alone, IL-3 (30 ng/ml), TNF-α (100 U/ml), or both factors together for 4 h. As judged by flow cytometry using the anti-E-selectin mAb 49-1B11, IL-3 enhanced E-selectin expression in HUVEC treated with TNF-α by 47%±19% (mean±SEM, n=4) compared to TNF-α stimulation alone.

Since E-selectin induces the initial leukocyte-endothelial interaction known as "rolling" (Springer, 1994) and IL-8 is a powerful chemoattractant for neutrophils (Smith *et al.*, 1991), we examined whether IL-3 could regulate the migration of neutrophils across an endothelial monolayer. HUVEC were cultured in transwells and pretreated with medium alone or TNF-α (100 U/ml) for 18 h, before IL-3 (30 ng/ml) was added for 6 h. Neutrophils were then added to the upper compartment by replacing the medium (thus creating an IL-8 gradient across the HUVEC monolayer), and the number of

transmigrated neutrophils in the lower compartment counted after 45 min. As shown in table 5.1, IL-3 significantly enhanced neutrophil transmigration in TNF-α-stimulated, but not in resting HUVEC.

Table 5.1 IL-3 enhances IL-8 production and neutrophil transmigration in TNF-α - treated HUVEC.

HUVEC Treatment	IL-8 (ng/ml)	Neutrophil Transmigration (%)
Medium	0.04±0.01	7.9±1.2
IL-3 (30ng/ml)	0.06±0.02 (ns)	8.2±1.6 (ns)
TNF-α (100U/ml)	2.62±0.60 (p=0.006)	20.6±1.0 (p<0.001)
IL-3 (30ng/ml), TNF-α(100U/ml)	5.02±0.98 (p<0.01)	27.1±1.8 (p<0.005)

The values represent the mean ±SEM of four different experiments. The p values indicate the significance of the difference between the TNF-α vs medium groups, and between the IL-3+TNF-α vs TNF-α groups, by the student's t-test. The values obtained with IL-3 alone were not significantly different from medium control (ns).

5.2 IL-3 Potentiates the IFN-γ -induced MHC Class II Expression in HUVEC.

In order to investigate the role of the endothelial IL-3R in immune reactions, the effect of IL-3 on expression of the antigen presenting MHC molecules was studied. HUVEC were stimulated with IL-3 (30 ng/ml), IFN-γ (100 U/ml) or both factors together for various times. The expression of MHC class II was detected using mAb FMC52 and quantitated by flow cytometry. As shown in figure 5.1, IL-3 alone did not induce MHC class II on HUVEC, but strongly enhanced the expression induced by IFN-γ. The enhancing effect of IL-3 was first noted after 48 h of stimulation and lasted for at least 72 h. At 48 h the MHC class II expression was increased 3.1±0.7 (mean±SD, n=4) -fold by IL-3, compared to the levels induced by IFN-γ alone. In contrast, IL-3 did not affect MHC class I expression, either alone or in combination with IFN-γ (data not shown).

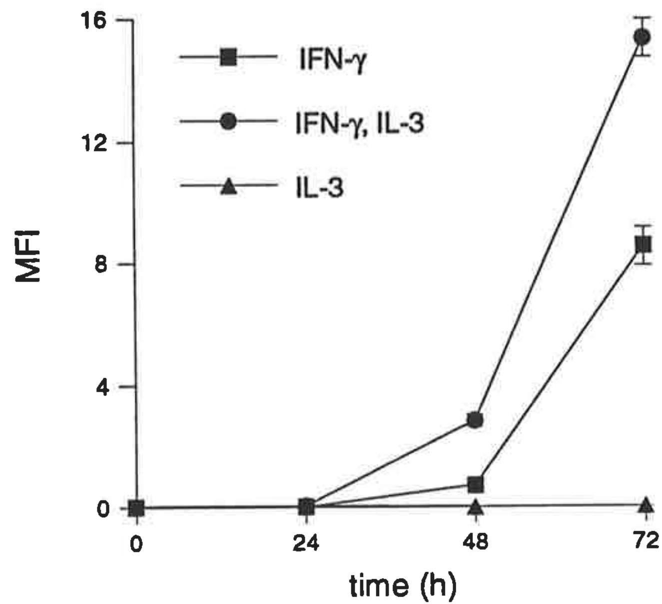


Fig. 5.1 IL-3 synergises with IFN-γ in up-regulating MHC class II antigen expression on HUVEC. Time-course of indirect immunofluorescence, quantified by flow cytometry, of HUVEC stimulated with IFN-γ (100 U/ml), IL-3 (30 ng/ml) or both factors together. The y-axis shows the relative mean fluorescence intensity (MFI) after subtraction of the background fluorescence. Data are from one experiment representative of three, and are expressed as the mean±SD determined in duplicate samples.

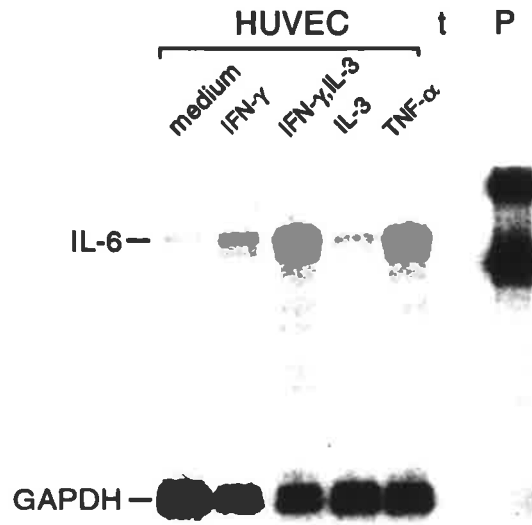
5.3 IL-3 synergises with IFN-γ in selectively inducing the production of IL-6 and G-CSF but not GM-CSF

To further investigate the functional role of the IL-3R in endothelial cells, the production of the hemopoietic growth factors G-CSF, GM-CSF and IL-6 was measured in response to IL-3. HUVEC which had been pretreated with medium alone or with IFN-γ (100 U/ml) for 24 h were stimulated with IL-3 (30 ng/ml) for 24 h. RNA was then extracted and the expression of the G-CSF, GM-CSF and IL-6 mRNAs analysed using an RNase protection assay. As shown in figure 5.2, IL-3 (30 ng/ml) did not increase the IL-6 mRNA in untreated HUVEC, whilst IFN-γ alone had only a modest effect. However, IL-3 strongly enhanced the IL-6 mRNA in HUVEC pretreated with IFN-γ, to similar levels as those observed with TNF-α (100 U/ml), which was used as a positive control. A similar pattern of induction was observed for G-CSF: IL-3 alone did not induce the G-CSF mRNA but demonstrated a synergistic effect when combined with IFN-γ. In contrast, the GM-CSF mRNA was not induced by IL-3, either alone or in combination with IFN-γ. On the other hand, TNF-α strongly induced both the G-CSF and GM-CSF mRNAs (Fig. 5.2). The possibility that IL-3 may also enhance the TNF-α -induced expression of IL-6, G-CSF and GM-CSF was examined, but interestingly no further induction of any of the cytokine mRNAs was observed (data not shown).

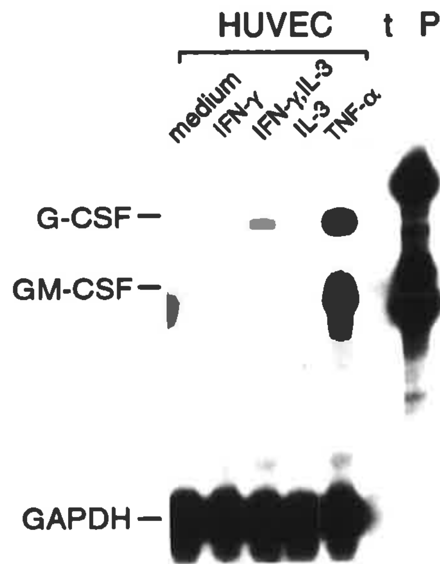
To examine whether increased cytokine mRNA levels resulted in increased protein production, HUVEC supernatants were analysed for the presence of IL-6, G-CSF and GM-CSF using ELISA methods. In keeping with the mRNA results, IL-3 did not induce IL-6 secretion in unstimulated HUVEC but caused a 3.2±1.0 (mean±SD, n=3) -fold increase in IL-6 secretion in HUVEC pretreated with IFN-γ (Fig 5.3). To prove that the induction by IL-3 was specific, a blocking mAb to the IL-3R α chain, 7.G3, was used. This mAb has been shown to completely block binding of IL-3 to its receptor and the IL-3-induced proliferation of TF-1 cells (Qiyu *et al.,* submitted). HUVEC were pre-incubated with mAb 7.G3 or a control mAb 6.H6 (a non-blocking anti-IL-3R α chain mAb) at 100 μg/ml for 2 h prior to the addition of IL-3. As shown in figure 5.4, mAb

Fig. 5.2 Induction of IL-6 and G-CSF but not GM-CSF mRNAs by IL-3 in HUVEC. RNase protection assay probed for IL-6 mRNA (a) or G-CSF and GM-CSF mRNAs (b) using RNA from HUVEC incubated with medium alone, IFN-γ (100 U/ml) for 48 h, IL-3 (30 ng/ml) for 24 h, IFN-γ for 48 h with IL-3 added for the last 24 h, or with TNF-α (100 U/ml) for 24 h. The samples were also probed for GAPDH mRNA, which is used as an internal control. tRNA was used as a negative control (t) and lane P represents undigested probes.

A



B



7.G3 abolished the enhancing effect of IL-3 on IL-6 production, whereas 6.H6 had no effect.

The production of G-CSF was induced in a similar manner to that of IL-6: while IL-3 had no effect on G-CSF secretion in unstimulated HUVEC, it caused a 3.0±1.2 (mean±SD, n=3) -fold increase in G-CSF production in HUVEC which had been treated with IFN-γ, compared to the levels elicited by IFN-γ alone (Fig 5.3). In contrast to IL-6 and G-CSF, GM-CSF production was not induced by IL-3 or IFN-γ, either alone or in combination. Consistent with the GM-CSF mRNA data, immunoreactive GM-CSF could only be detected in the supernatants from TNF-α-stimulated cells (Fig 5.3).

5.4 IL-3 does not alter the stability of IL-6 mRNA

Both transcriptional and post-transcriptional mechanisms have been implicated in the regulation of IL-6 and G-CSF expression (Seelentag *et al.*, 1987, Elias *et al.*, 1990). In order to examine whether the enhancing effect of IL-3 on IL-6 production by IFN-γ -stimulated HUVEC is mediated at least in part by mRNA stabilisation, the half-life of the IL-6 mRNA was measured before and after IL-3 stimulation. The effect of IL-3 on the stability of the G-CSF mRNA could not be reliably examined however, because the level in HUVEC prior to IL-3 stimulation is very low or undetectable.

HUVEC (pre-treated with IFN-γ at 100 U/ml for 24 h) were stimulated with IL-3 (30 ng/ml) or medium alone for 6 h. Transcription was then inhibited by the addition of actinomycin D (5 μg/ml), and RNA extracted at various times thereafter. IL-6 mRNA decay was analysed using RNase protection assay and quantitated by phosphorimaging, using the GAPDH mRNA as an internal control (Fig. 5.5). In 3 separate experiments performed with different HUVEC primary cultures, the half-life of the IL-6 mRNA was 100 min, and was not altered by IL-3 treatment. Similar results were obtained using DRB (100 μM) (data not shown), which inhibits transcription in a different manner.

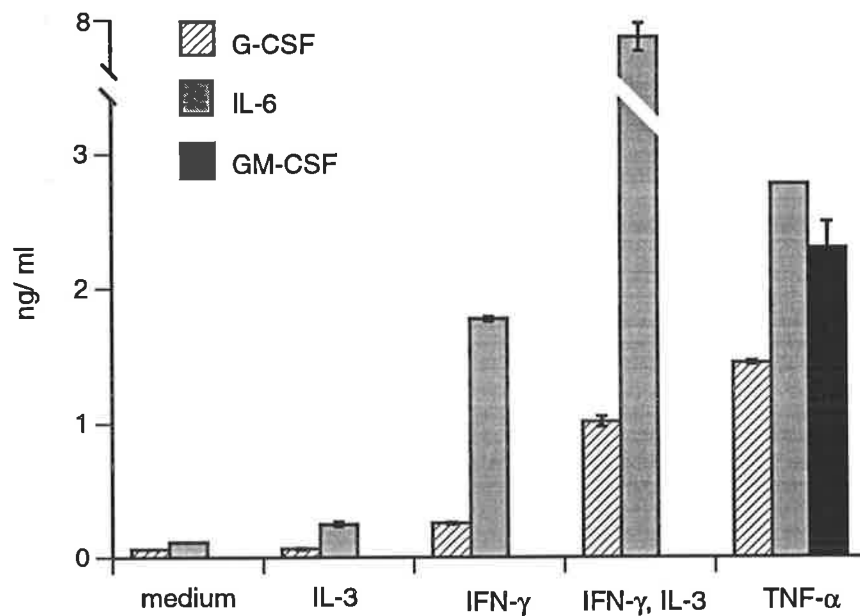


Fig. 5.3 IL-3 enhances IL-6 and G-CSF but not GM-CSF production in IFN-γ treated HUVEC. Immunoreactive IL-6, G-CSF and GM-CSF were measured by ELISA methods in tissue culture supernatants of HUVEC treated with medium alone, IL-3 (30 ng/ml) for 24 h, IFN-γ (100 U/ml) for 48 h, or IFN-γ for 48 h with IL-3 added for the last 24 h. As a positive control HUVEC were stimulated with TNF-α (100 U/ml) for 24 h. Data are from one experiment representative of three, and are expressed as the mean±SEM determined in duplicate wells.

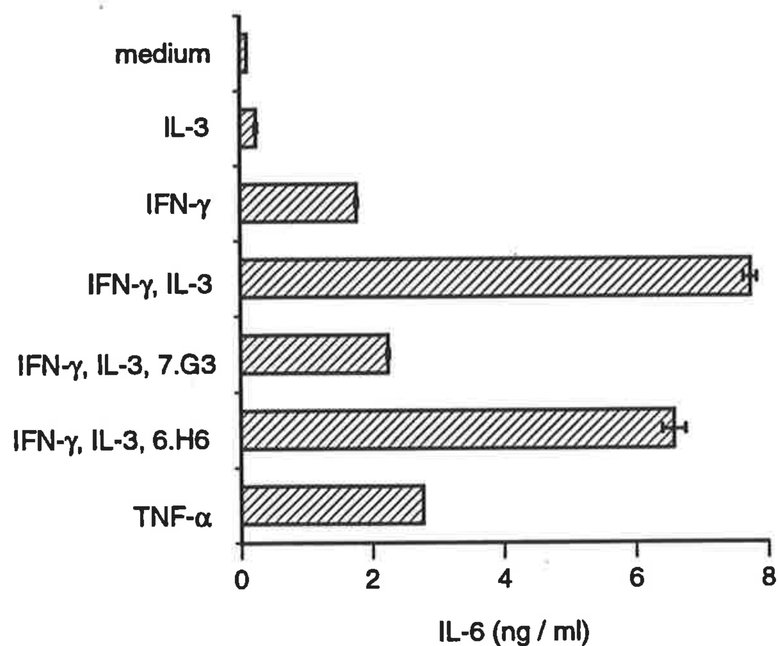
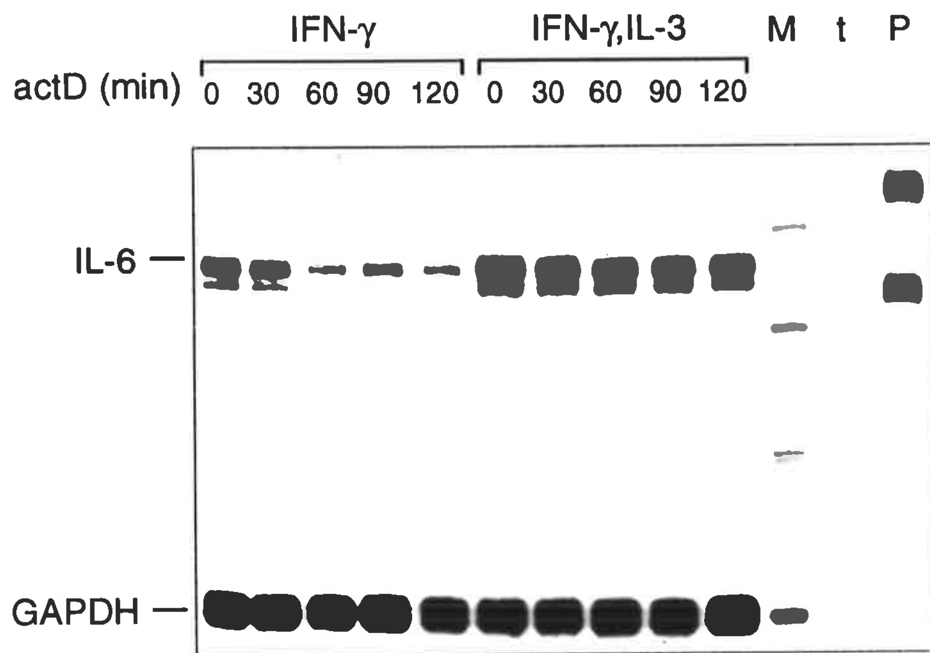
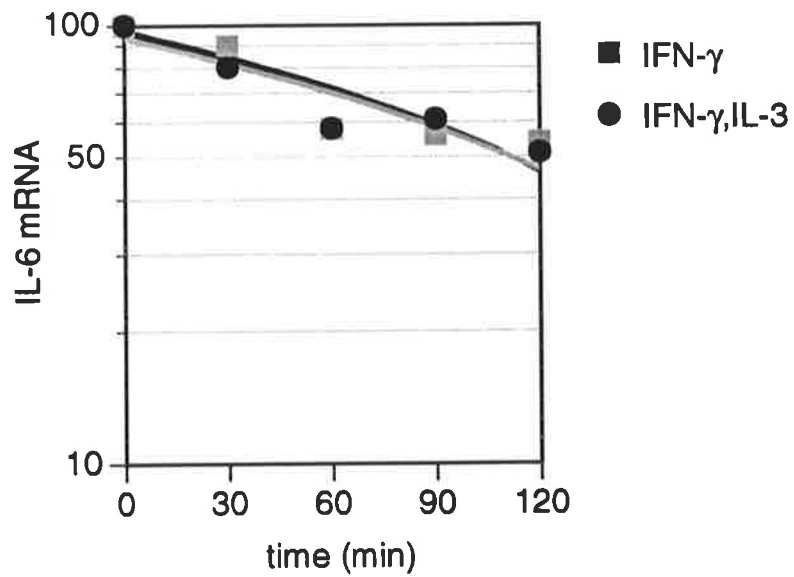


Fig. 5.4 The enhancing effect of IL-3 on the IL-6 production by HUVEC is specifically inhibited by the blocking anti-IL-3R α chain mAb 7.G3. IL-6 was measured by an ELISA method in tissue culture supernatants of HUVEC, stimulated as described in the legend to fig. 5.3, except that anti-IL-3R α chain mAbs 7.G3 (blocking) or 6.H6 (non-blocking) were added at 100 μg/ml 2 h prior to stimulation with IL-3.

Fig. 5.5 RNase protection analysis of IL-6 mRNA half-life in HUVEC. After pre-treatment with IFN-γ (100 U/ml) for 24 h, HUVEC were stimulated with IL-3 (30 ng/ml) or medium alone for 6 h. Ongoing transcription was then inhibited with actinomycin D for various times as indicated, and expression of the IL-6 mRNA analysed by RNase protection. The samples were also probed for GAPDH mRNA, which is used as an internal control. tRNA (t) was used as a negative control, and lanes P and M contain undigested probes and DNA size markers (^{32}P-labeled HpaII-digested pUC19 DNA) respectively. Phosphorimager quantitation of the gel is shown below, with IL-6 mRNA levels expressed as a percentage of the initial IL-6 vs. GAPDH ratio.


IFN-γ
IFN-γ,IL-3
M
t
P
actD (min)
0
30
60
90
120
0
30
60
90
120
IL-6
GAPDH


100
50
10
IL-6 mRNA
0
30
60
90
120
time (min)
IFN-γ
IFN-γ,IL-3

DISCUSSION

This chapter demonstrates that the hemopoietic growth factor IL-3 has a wide spectrum of effects on endothelial cells, ranging from stimulation of IL-8 production and neutrophil transmigration to enhancement of MHC class II expression and the production of G-CSF and IL-6. The demonstration that IL-3 can regulate endothelial functions related to inflammation, immunity and hemopoiesis reveals a novel role for IL-3 in vascular biology.

Stimulation of TNF-α-activated HUVEC with IL-3 led to increased E-selectin expression, suggesting that IL-3 can regulate the adhesion of leukocytes (including neutrophils, eosinophils, monocytes and certain T lymphocytes) to endothelium *in vivo* (reviewed by Bevilacqua, 1993). The enhanced secretion of the chemokine IL-8 could further facilitate recruitment of neutrophils and activated eosinophils to the site of inflammation (Smith *et al.*, 1991, Warringa *et al.*, 1992). This is consistent with the increased neutrophil transmigration observed in these experiments. In addition to its chemoattractant properties, IL-8 also stimulates phagocytosis and various other neutrophil functions (reviewed by Miller *et al.*, 1992). Although IL-3 is not likely to be present at the site of an acute inflammatory reaction, it may contribute to the initial neutrophilic infiltration in DTH reactions, which are orchestrated by activated T cells. On the other hand, as mast cells can produce IL-3 (Plaut *et al.*, 1989, Wodnar-Filipowicz *et al.*, 1989) and are also a rich source of TNF-α (Gordon *et al.*, 1990), the IgE-dependent release of these two factors could regulate leukocyte infiltration in allergen-induced late-phase reactions and asthma.

IL-3 enhanced the IFN-γ -induced MHC class II expression on HUVEC, suggesting that it may regulate the ability of endothelial cells to function as APCs in the cognitive phase of inflammation *in vivo*. IFN-γ is the prototypic inducer of class II antigens on endothelial cells, however this response is notoriously slow, taking several days to develop (Pober *et al.*, 1983B). The enhancing effect of IL-3 could thus serve to

accelerate this process. The expression of MHC antigens on vascular endothelium has important implications because endothelial cells, by virtue of their anatomic location, have access to circulating lymphocytes and intravascular antigens. The concomitant production of IL-3 and IFN-γ upon T cell activation may lead to enhanced MHC class II expression and thus antigen presentation by the endothelium, thereby amplifying the T helper response.

The experiments described in this chapter identified the combination of IL-3 and IFN-γ as a novel inducer of endothelial cytokine production. In contrast to TNF-α and other known inducers, IL-3 and IFN-γ selectively induced the production of G-CSF and IL-6 but not GM-CSF or IL-8. Both G-CSF and IL-6 are early-acting hemopoietic growth factors which, together with IL-3, form part of the optimal cytokine combination for hemopoiesis *in vitro* (Haylock *et al.,* 1992). They also play an important role in inflammation; G-CSF stimulates the functional activity of neutrophils, while IL-6 is the main inducer of the acute phase response (reviewed by Demetri *et al.,* 1991, Kishimoto *et al.,* 1992). Both IL-6 and G-CSF can be found in the circulation under inflammatory conditions and, interestingly, increased serum levels of IL-6 and acute phase proteins have also been detected following clinical administration of IL-3 (Lindemann *et al.,* 1991). The IL-3 and IFN-γ -stimulated release of G-CSF and IL-6 into the circulation by the vascular endothelium could serve to further exacerbate the inflammatory response and stimulate hemopoiesis in the bone marrow, thereby replenishing the pool of leukocytes in the blood. In addition to acting directly on hemopoietic progenitor cells, IL-3 could thus also influence hemopoiesis indirectly. It would be of interest to determine whether endothelial cells isolated from bone marrow (Rafii *et al.,* 1994) respond to IL-3 in a similar manner to HUVEC, allowing IL-3 to regulate cytokine production locally in the hemopoietic microenvironment.

The mechanism(s) by which IL-3 enhances the expression of IL-6 and G-CSF requires further investigation. Both increased transcription and mRNA stability have been implicated in the induction of these cytokines by other stimuli (Seelentag *et al.,* 1987,

Elias *et al.*, 1990). As shown in this chapter, IL-3 does not prolong the half-life of the IL-6 mRNA, suggesting that the observed increase in the steady-state level of IL-6 mRNA is due to transcriptional activation. Unfortunately it was not possible to test this hypothesis by nuclear run-on assays due to the large number of primary HUVEC required. As an alternative, the effect of IL-3 on transcription of the IL-6 and G-CSF genes could be studied using reporter constructs driven by the appropriate cytokine promoters. Interestingly, the promoters of G-CSF and IL-6, as well as that of IL-8, have been recently shown to resemble each other in that they contain functionally linked NF-IL6 and NF-κB binding sites (Dunn *et al.*, 1994, Matsusaka *et al.*, 1993, Kunsch *et al.*, 1994).

The role of IL-3 in stimulating the endothelial functions described here is essentially that of an amplification factor: IL-3 enhances the E-selectin expression and IL-8 production induced by TNF-α, and potentiates the MHC class II expression and production of G-CSF and IL-6 induced by IFN-γ. This is in contrast to monocytes, as IL-3 alone induces most of the above-mentioned functions in these cells (Takahashi *et al.*, 1993, Sadeghi *et al.*, 1992, Oster *et al.*, 1989). It is not clear why a co-stimulant is required for endothelial activation: unstimulated HUVEC appear to be able to respond to IL-3, as shown by the induction of c-fos (chapter 3), and more recently by our finding that IL-3 increases the expression of P-selectin in unstimulated HUVEC (Khew-Goodall *et al.*, submitted). Stimulation of certain endothelial functions may require higher threshold levels of IL-3R expression, which could be achieved by treatment with TNF-α or IFN-γ (chapter 4). However, this alone does not explain the need for a co-stimulant, at least in the case of IL-8: although both TNF-α and IFN-γ up-regulate IL-3R expression, only TNF-α, which is also able to induce IL-8, can function as a co-stimulant with IL-3. The synergistic effects observed with IL-3 and TNF-α or IFN-γ are more likely due to some post-receptor mechanism along the signalling pathway. It is of interest in this regard that IL-3 has been shown to activate JAK2 kinase (Silvennoinen *et al.*, 1993), also implicated in IFN-γ signalling, and to induce DNA binding complexes which recognise

IFN-γ activation site (GAS) elements (Lamb *et al.*, 1994, Mui *et al.*, 1995, Azam *et al.*, 1995).

The findings described in this chapter raise the possibility that IL-3 may be involved in regulating endothelial responses in pathological conditions involving activated T cells and mast cells, such as chronic inflammation, DTH reactions and allergy. *In vivo,* expression of the IL-3 mRNA has been detected in allergen-induced late phase reactions, and in activated T lymphocytes from patients with asthma (Kay *et al.*, 1991, Robinson *et al.*, 1992). The stimulation of endothelial responses by IL-3 not only reveals a novel role for this hemopoietic growth factor in vascular biology but also provides a warning with regard to the clinical administration of IL-3, especially in patients with underlying inflammatory conditions.

SUMMARY

Active participation of the vascular endothelium in various physiological processes requires endothelial cells to undergo phenotypic changes, which are to a large degree coordinated by cytokines. This chapter identifies for the first time the hemopoietic growth factor IL-3 as a potent stimulator of several endothelial functions related to inflammation, immunity and hemopoiesis.

IL-3 stimulation of TNF-α-activated endothelial cells enhanced E-selectin expression, IL-8 production and neutrophil transmigration, indicating that IL-3 can regulate leukocyte adhesion, attraction and transmigration into inflammatory foci. IL-3 also synergised with IFN-γ in stimulating the expression of MHC class II antigens on HUVEC, suggesting that the concomitant production of IL-3 and IFN-γ upon T cell activation *in vivo* may lead to enhanced antigen presentation by endothelial cells. Finally, the combination of IL-3 and IFN-γ was found to selectively induce secretion of the early-acting hemopoietic growth factors G-CSF and IL-6 by endothelial cells. Taken together, IL-3 may maintain and exacerbate pathological conditions involving activated T cells and mast cells *in vivo*.

These results open a novel role for IL-3 in vascular biology, and suggest that care should be taken in the clinical administration of IL-3, especially in patients with underlying inflammatory conditions.

CHAPTER 6

IL-3 RECEPTOR EXPRESSION IN OTHER MESENCHYMAL CELLS

INTRODUCTION

IL-3 receptors were previously thought to be expressed only on hemopoietic cells, where they mediate proliferation and differentiation, or functional activation. This thesis shows that endothelial cells also express receptors for IL-3 and are able to functionally respond to this cytokine. The work described in this chapter investigated whether IL-3 receptors are also expressed on other non-hemopoietic cells, such as fibroblasts, smooth muscle cells and bone marrow stromal cells. These cells are of mesenchymal origin like endothelial cells, and are often located in close relationship to vascular endothelium.

Smooth muscle cells (SMC) are a major component of the vascular wall, by far outnumbering endothelial cells and adventitial fibroblasts in most blood vessel types. Studies of the proliferative and contractile properties of SMC have shown that these cells respond to various cytokines and other humoral factors (Warner *et al.*, 1989, Battegay *et al.*, 1990, Vanhoutte, 1994). However, SMC should not be considered solely as target cells, as they can also secrete cytokines including IL-1, IL-6 and G-CSF (Libby *et al.*, 1986, Loppnow *et al.*, 1990, Zoellner *et al.*, 1992), and are likely to play an active role in various vascular diseases.

IL-3R expression in bone marrow stromal cells (BMSC) is of special interest, as these cells have a critical function in hemopoiesis. They secrete growth factors and express adhesion molecules which can regulate the proliferation and differentiation of hemopoietic progenitor cells. BMSC are a heterogeneous population of fibroblasts, endothelial cells, SMC, adipocytes, osteoblasts and macrophages. Interestingly, clinical administration of IL-3 has been shown to cause bone marrow fibrosis, characterised by endothelial cell proliferation and an increase in extracellular matrix proteins and fibroblasts (Orazi *et al.*, 1992). Most *in vitro* studies on BMSC, including this one, have focussed on the bone marrow fibroblasts, as the isolation and culture conditions strongly favour the growth of these cells.

Fibroblasts have previously been examined for IL-3R expression, with negative results (Hara *et al.*, 1992), however these studies were performed using only unstimulated cells. The experiments described here were designed to also encompass TNF-α and IFN-γ - treated cells, as the results obtained with HUVEC (chapter 4) indicate that IL-3R expression can be up-regulated by these cytokines.

RESULTS

6.1 IL-3R mRNA expression in mesenchymal cells

Several mesenchymal cells, including human foreskin fibroblasts (HFF), saphenous vein -derived SMC and BMSC were examined for their potential to express IL-3R mRNA. Total RNA was extracted from cells treated with medium alone, TNF-α (100 U/ml), IFN-γ (100 U/ml) or both factors together for 24 h, and probed for IL-3R α and β chain mRNAs using an RNase protection assay. The samples were simultaneously probed for mRNA for the related GM-CSFR and IL-5R α chains, as well as for GAPDH, which was used as an internal control. RNA from TF1 cells (Kitamura *et al.*, 1989) was used as a positive control for the different receptor mRNAs.

As shown in figure 6.1, unstimulated HFF did not express either the IL-3R α or β chain mRNAs. Interestingly, expression of the IL-3R α chain could be selectively induced by IFN-γ or TNF-α, and was further increased with both factors together. Unstimulated BMSC expressed low levels of both the IL-3R α and β chain transcripts. Expression of the IL-3R α chain was further enhanced by IFN-γ and TNF-α, and the combination of these two factors also increased expression of the β chain mRNA. Unstimulated SMC expressed only the β chain, however expression of the IL-3R α chain could be induced by stimulation with TNF-α and/or IFN-γ. In contrast, expression of the β chain was down-regulated by TNF-α (alone or in combination with IFN-γ). The GM-CSFR and IL-5R α chain mRNAs were not detected in any of the cell types examined.

6.2 Surface expression of the IL-3R subunits

The next series of experiments examined whether the expression and regulation of IL-3R mRNAs observed in HFF, SMC and BMSC correlated with the expression of protein on the cell surface. Cells were treated with medium alone, TNF-α (100 U/ml), IFN-γ (100 U/ml) or both factors together for 24 h, and the surface expression of the IL-3R subunits analysed by flow cytometry using the specific anti-IL-3Rα chain mAb

Fig. 6.1 Expression of IL-3R mRNA in human dermal fibroblasts (HFF), smooth muscle cells (SMC) and bone marrow stromal cells (BMSC). RNase protection assay of IL-3R α and β chain mRNAs in cells treated with either medium alone, TNF-α at 100 U/ml, IFN-γ at 100 U/ml or both factors together for 24 h. The samples were also probed for the GM-CSFR and IL-5R α chain mRNAs and for GAPDH, which was used as an internal control. RNA from TF-1 cells was used as a positive control (TF1) and tRNA as a negative control (t). Lane P represents undigested probes. The band between the IL-3R α and β chain signals appears consistently under the conditions of the protection assay, and is caused by incomplete digestion of vector sequences in the IL-3R α chain probe.

TF1 t HFF BM stroma SMC P

TNF-α
IFN-γ

IL-5Rα—
β—
IL-3Rα—
GM-CSFRα—
GAPDH—

7.G3 and the anti-β chain mAb 4.F3. Representative histograms are shown in figure 6.2. IL-3R α chains were not detected on unstimulated cells, however expression was induced in all cell types by IFN-γ and TNF-α, and to a lesser extent by IFN-γ alone. Expression of the β chain was observed only on unstimulated SMC. It was decreased by IFN-γ and TNF-α, while the same cytokine combination slightly induced the β chain on bone marrow stromal cells. In keeping with the mRNA data, HFF fibroblasts remained β chain negative. No detectable expression of the GM-CSFR α chain was observed in any cell type, as judged by staining with the specific mAb 8.G6 (data not shown).

6.3 Functionality of the IL-3R

In order to investigate whether the IL-3Rs expressed on HFF, SMC and BMSC are functional, initial experiments examined the induction of the immediate-early gene c-fos in response to IL-3. Cells were incubated in low-serum medium (0.5% FCS) for 24 h and simultaneously treated with IFN-γ (100 U/ml) and TNF-α (100 U/ml) in order to maximise the level of IL-3R expression (SMC were treated with IFN-γ only, as TNF-α decreases expression of the β chain in these cells). IL-3 (200 ng/ml) or FCS (20%) were then added and total RNA extracted after 30 min. Expression of the c-fos mRNA was analysed by RNase protection and quantitated by phosphorimaging.

As shown in figure 6.3, IL-3 had no effect on c-fos expression in any of the cell types examined. In contrast, the FCS used as a positive control strongly induced c-fos both in unstimulated and IFN-γ and TNF-α -stimulated cells, indicating that pretreatment with these cytokines does not inhibit c-fos expression. Flow cytometry analysis performed in parallel confirmed that levels of the IL-3R expression were not affected by the serum starvation conditions used (data not shown).

It was next examined whether IL-3 could stimulate functions which are likely to be mediated by signalling pathways other than those involved in c-fos induction. An obvious candidate in this regard is the uptake of glucose: both IL-3 and GM-CSF are known to stimulate glucose uptake in hemopoietic cells (Hamilton *et al.*, 1988) and it

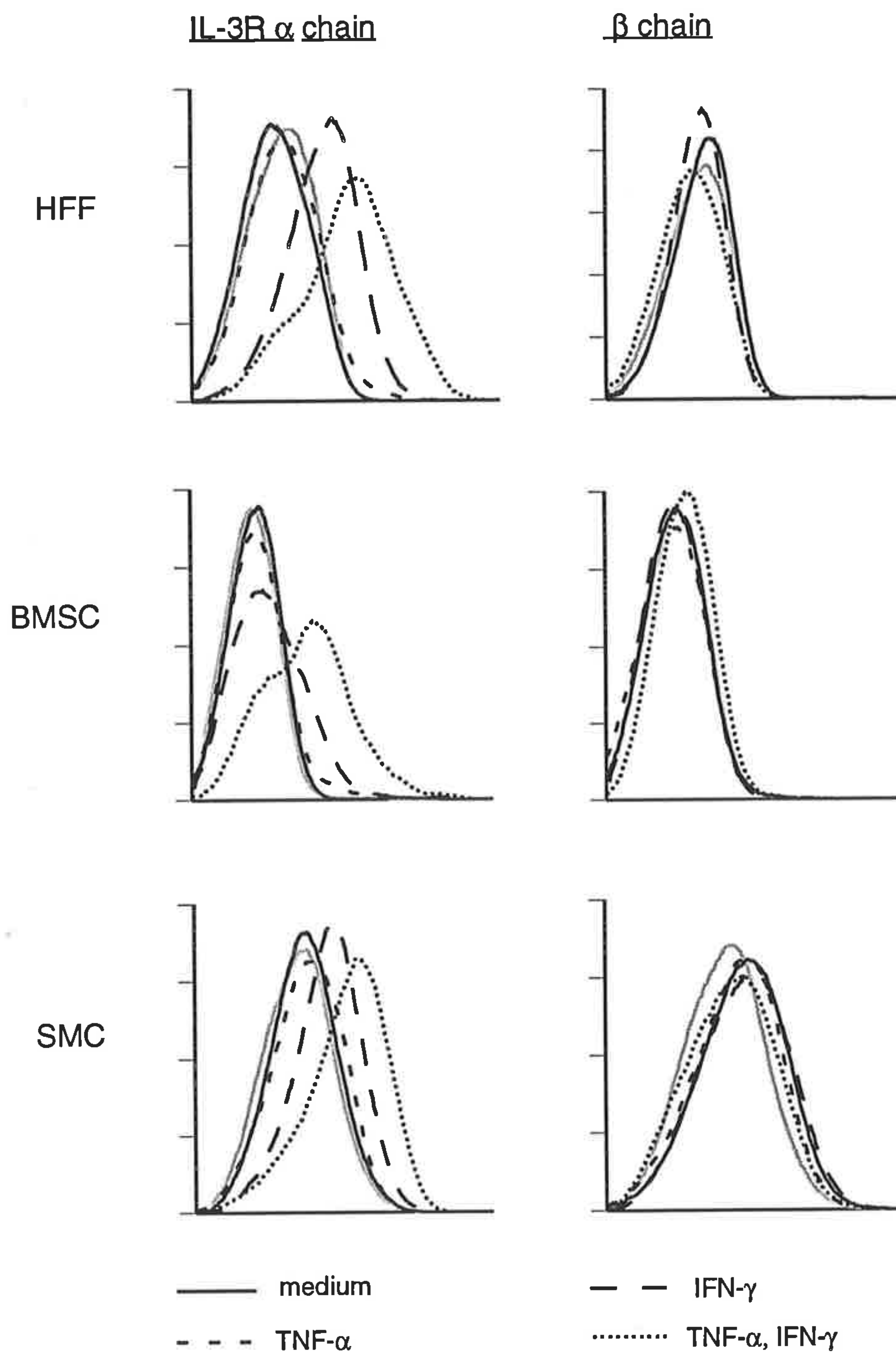
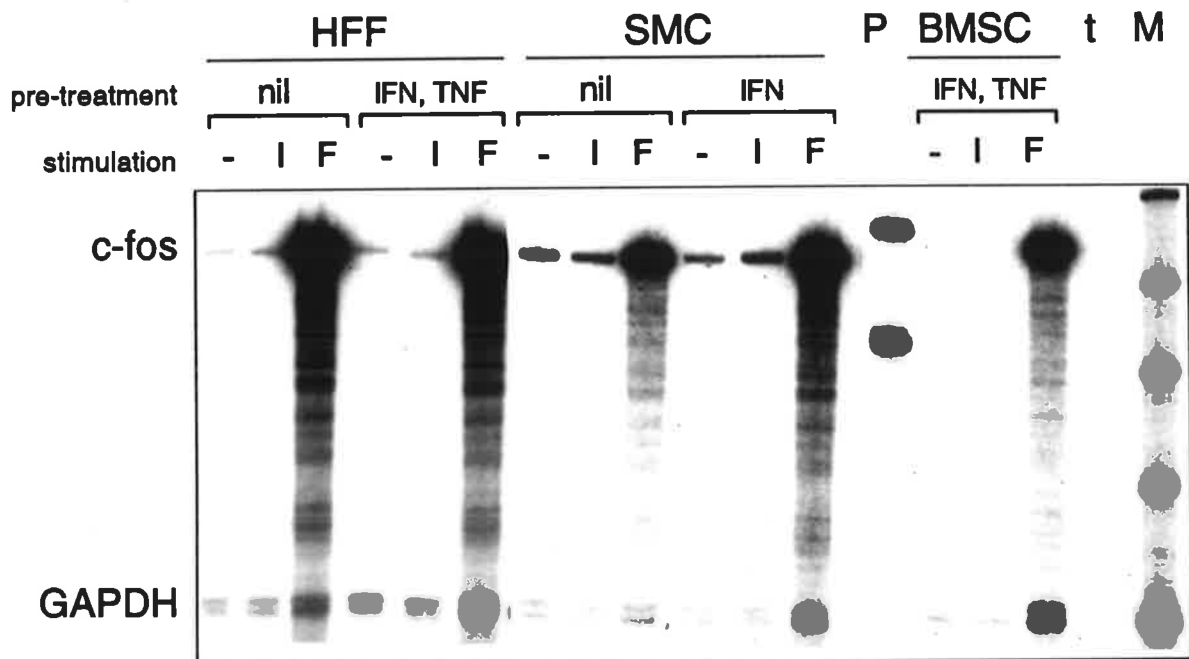


Fig. 6.2 Flow cytometry analysis of the regulation of IL-3R α and β chain expression on HFF, SMC and BMSC. Cells were treated with either medium alone, TNF-α (100 U/ml), IFN-γ (100 U/ml) or both factors combined for 24 h, and stained with the anti-IL-3R α chain mAb 7.G3 or with the anti-β chain mAb 4.F3. Indirect immunofluoresence was analysed by flow cytometry. The isotype control is represented by the grey histogram. Shown is one experiment representative of three.

Fig. 6.3 Inducibility of the immediate-early gene c-fos in response to IL-3 treatment in HFF, SMC and BMSC. The cells, serum-starved for 24 h in the presence or absence of TNF-α (100 U/ml) and IFN-γ (100 U/ml) as indicated, were stimulated with IL-3 at 200 ng/ml (I) or 20% FCS (F) for 30 min. A 10 μg sample of total RNA was analysed for c-fos expression by RNase protection assay. The samples were also probed for GAPDH mRNA, which was used as an internal control. tRNA was used as a negative control (t). Lanes P and M contain undigested probes and DNA size markers (^{32}P-labeled HpaII-digested pUC19 DNA) respectively.


HFF
SMC
P
BMSC
t
M
pre-treatment
nil
IFN, TNF
nil
IFN
IFN, TNF
stimulation
- I F - I F - I F - I F - I F
c-fos
GAPDH

was recently reported that the effect of GM-SCF does not require tyrosine phosphorylation and also occurs in cells which express only low-affinity GM-CSFRs (Spielholz *et al.*, 1995, Ding *et al.*, 1994).

The ability of IL-3 to stimulate glucose uptake was examined in HFF fibroblasts, using either untreated cells or cells treated with IFN-γ (100 U/ml) and TNF-α (100 U/ml) for 24 h. The cells were stimulated with IL-3 (200 ng/ml) for 30 min in glucose-free buffer, pulsed for 10 min with the glucose analog ^{3}H-2-deoxyglucose (^{3}H-2-DOG), then lysed and the uptake of ^{3}H-2-DOG determined by scintillation counting.

As shown in figure 6.4, IL-3 did not affect the uptake of ^{3}H-2-DOG in either untreated HFF, or in HFF pretreated with IFN-γ and TNF-α. However, as the latter had much higher basal level of glucose uptake activity and this could mask any effect of IL-3, ^{3}H-2-DOG uptake was also studied in cells pretreated with IFN-γ alone (the IL-3R α chain expression is induced by IFN-γ, although to a lesser extent than by both factors together). While the basal level of glucose uptake was lower in IFN-γ -treated HFF, no induction was observed in response to IL-3 (data not shown).

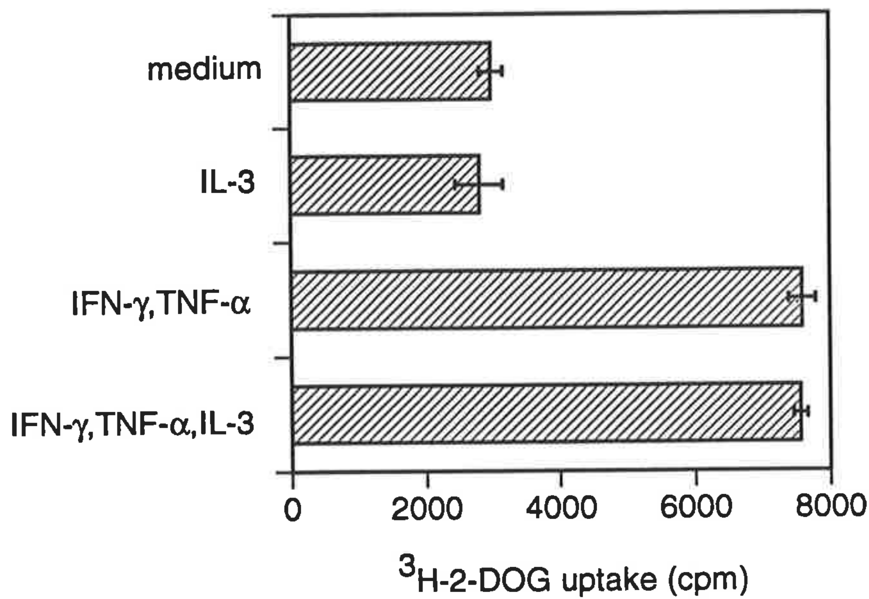


Fig. 6.4 Effect of IL-3 on the uptake of 2-deoxyglucose by HFF. IL-3 (200 ng/ml) was added to untreated cells or cells treated with TNF-α (100 U/ml) and IFN-γ (100 U/ml) for 24 h. Following incubation for 30 min, glucose transport activity was measured by pulsing the cells for 10 min with ^{3}H-2-DOG. Data are from one experiment representative of three, and are expressed as the mean±SD determined in duplicate samples.

DISCUSSION

This chapter shows that expression of the IL-3R outside the hemopoietic system is not limited to endothelial cells, but that fibroblasts, smooth muscle cells and bone marrow stromal cells also have the potential to express receptors for this cytokine when appropriately stimulated. Interestingly, these cells exhibited distinct patterns of expression and regulation of the IL-3R subunits, which differed from that observed in HUVEC.

No expression of either IL-3R subunit could be detected on unstimulated dermal fibroblasts or bone marrow stromal cells (although the latter expressed small amounts of the IL-3R α and β chain mRNAs), while smooth muscle cells expressed only the β chain. Interestingly, expression of the IL-3R α chain could be induced in all cell types by stimulation with IFN-γ and TNF-α. Although both factors induced the IL-3R α chain mRNA, only IFN-γ significantly induced surface expression of the IL-3R α chain protein. However, a synergistic effect similar to that in HUVEC (chapter 4) was observed when the two stimuli were combined. In contrast to the uniform pattern of regulation of the IL-3R α chain, regulation of the β chain exhibited marked differences between the cell types. The combination of IFN-γ and TNF-α slightly increased expression of the β chain in BMSC, had no effect on HFF, and down-regulated the β chain in SMC. This is in contrast to endothelial cells, in which the expression of the IL-3R subunits is regulated in a coordinate manner. Receptor expression appeared to be IL-3 -specific in all cell types examined, as no α chains for the related GM-CSF and IL-5 receptors could be detected. The lack of GM-CSFR α chain expression in BMSC is of particular interest, as GM-CSF has been reported to stimulate proliferation of these cells (Dedhar *et al.*, 1988). However, no effect was found in a more recent study (Gronthos *et al.*, 1995), the controversy perhaps being due to differences in cell purity or the assay conditions used.

The results presented here open several points for discussion. Firstly, they indicate that cells such as fibroblasts, which are seemingly IL-3R -negative, have the potential to become IL-3R -positive when stimulated with inflammatory cytokines. The IL-3R may thus be much more widely expressed than previously anticipated. Secondly, the finding that HFF fibroblasts express only the α chain of the IL-3R is intriguing, since both subunits are required for signalling (Kitamura *et al.,* 1992). Similarly, unstimulated SMC were found to express the β chain but none of the α chains which can associate with it. One could speculate that these individually expressed IL-3R subunits may serve as receptor components for some other, presently unidentified factors. A precedent for this is seen in the case of the low-affinity oncostatin M receptor gp130, which also functions as an affinity converter for the receptors for IL-6, IL-11, LIF and CNTF (Kishimoto *et al.,* 1992, Hilton *et al.,* 1994). Alternatively, an adhesive function could be envisaged, as the extracellular regions of both the IL-3R α and β chains bear homology to the type III fibronectin modules (Patthy, 1990).

Although HFF, SMC and BMSC all expressed the IL-3R, none of these cells could respond to IL-3, as judged by c-fos induction. In keeping with its inability to induce c-fos, IL-3 also failed to stimulate proliferation of HFF (data not shown) and BMSC (P. Simmons, personal communication). This lack of IL-3 responsiveness is not surprising in the case of HFF, as these cells completely lack the β subunit which is required for signalling, and it is possible that the level of β chain expression in BMSC and SMC is below the threshold required for signal transduction. It would be of interest to examine whether exogenous overexpression of the β chain could confer IL-3 responsiveness in these cells. Considering the inducible nature of the IL-3R subunits, it is possible that β chain expression is enhanced under some circumstances *in vivo.* On the other hand, one should not exclude the possibility that IL-3 may activate some other functions not involving c-fos. In this regard, the ability of IL-3 to stimulate glucose transport was examined in HFF fibroblasts. Although no response to IL-3 was observed, these experiments were complicated by the fact that TNF-α and IFN-γ, which are

required for up-regulation of the IL-3R α chain, also strongly affect glucose uptake. It is also not known at present whether the IL-3R α chain is capable of signalling glucose transport in the absence of β chain, as has been demonstrated for its GM-CSFR counterpart (Spielholz *et al.*, 1995, Ding *et al.*, 1994).

The finding of IL-3R expression on smooth muscle cells, fibroblasts and bone marrow stromal cells further widens the population of possible target cells for IL-3. The results presented here also indicate that IFN-γ and TNF-α play an important role in regulating this expression, suggesting a role for the IL-3R in inflammation. It is important to note that the lack of IL-3 responsiveness in the *in vitro* assays used does not exclude the possibility that these cells may respond to IL-3 under the more complex *in vivo* conditions. Furthermore, the subunits of the IL-3R may mediate some other, yet unidentified functions on these cells.

SUMMARY

Expression of the IL-3R is not restricted to blood cells as previously thought, as vascular endothelial cells also express receptors for this hemopoietic growth factor (chapter 3). This chapter shows that endothelial cells are not unique in this regard, as other non-hemopoietic cells of mesenchymal origin such as smooth muscle cells, fibroblasts and bone marrow stromal cells are also capable of expressing the IL-3R.

When unstimulated cells were examined, IL-3R chains could not be detected on HFF fibroblasts or BMSC, while SMC expressed only the β chain. However, expression of the IL-3R α chain could be induced in all cell types by IFN-γ and TNF-α. This was not accompanied by a coordinate up-regulation of the β subunit: while only a slight induction was observed in BMSC, HFF remained β chain -negative, and a down-regulation was observed in SMC. The low β chain levels may explain the lack of IL-3 responsiveness of these cells in functional assays: IL-3 failed to induce expression of the immediate-early gene c-fos and to stimulate glucose transport activity.

The finding of IL-3R expression in smooth muscle cells, fibroblasts and bone marrow stromal cells further widens the population of possible target cells for IL-3. Importantly, the expression is not constitutive but can be strongly induced by the inflammatory stimuli IFN-γ and TNF-α. The biological function of the IL-3R in these cells remains to be investigated. The lack of IL-3 responsiveness in the *in vitro* assays used does not exclude the possibility that these cells may respond to IL-3 under the more complex *in vivo* conditions.

CHAPTER 7

DISCUSSION

DISCUSSION

IL-3 is a hemopoietic growth factor which stimulates bone marrow progenitor cells to proliferate and differentiate *in vitro* into various blood cell types (Sieff *et al.*, 1987, Lopez *et al.*, 1988), a property which has led to its clinical use in bone marrow reconstitution after cancer therapy. Surprisingly however, no IL-3 is found either in the bone marrow or circulation (Garland *et al.*, 1983, Crapper *et al.*, 1984, Cheers *et al.*, 1988, Cluitmans *et al.*, 1995), raising doubts as to the role of IL-3 in normal hemopoiesis. On the other hand, IL-3 has been found to functionally activate mature blood cells including monocytes, basophils and eosinophils (Elliott *et al.*, 1989, Haak-Frendscho *et al.*, 1988, Lopez *et al.*, 1988), suggestive of a role in the inflammatory response. The work described in this thesis sought further clues as to the physiological role of IL-3 by investigating whether this cytokine could also act on non-hemopoietic cells. Interestingly, various vascular and connective tissue-type cells, including endothelial, smooth muscle, fibroblast and bone marrow stromal cells, were found to express receptors for IL-3. Although the basal level of IL-3R expression was low or undetectable in these cells, a strong up-regulation was observed in response to TNF-α and IFN-γ in all the cell types examined. These findings suggested a potentially much wider role for IL-3 than previously anticipated, and prompted investigations into the function of the IL-3R in these cells.

Expression of the IL-3R

The expression of the IL-3R in non-hemopoietic cells differed from that in blood cells in several ways: a) the expression was inducible rather than constitutive, b) the two IL-3R subunits could be independently expressed, and c) no simultaneous expression of the GM-CSFR was found. These differences, and the possible molecular mechanisms underlying them, will be discussed here.

Most of the cell types examined, including fibroblasts, smooth muscle cells and bone marrow stromal cells, were seemingly IL-3R -negative in the resting state. However,

surface expression of the IL-3R could be induced by the inflammatory mediators TNF-α and IFN-γ, which also up-regulated the IL-3R in endothelial cells. Such inducible expression suggests that the role of the IL-3R may be limited to inflammatory conditions in non-hemopoietic cells. In keeping with this, endothelial expression of the IL-3R was down-regulated by IL-4, which also inhibits expression of E-selectin and IL-8 by endothelial cells, and has several anti-inflammatory activities on monocytes (Thornhill *et al.,* 1990, Smith *et al.,* submitted, Standiford *et al.,* 1990, Fenton *et al.,* 1992). This strict control of IL-3R expression in non-hemopoietic cells is consistent with the tight regulation of IL-3 production by T cells and mast cells, thus further ensuring that IL-3 responses should not occur inappropriately. Interestingly, hemopoietic cells express the IL-3R constitutively. It is possible that these differing modes of IL-3R expression serve to keep blood cells, whose primary function is host-defence, on the "alert" at all times, but recruit non-hemopoietic cells to respond to IL-3 only under more severe circumstances. Notably, the cells which produce IL-3 can also potentially regulate IL-3R expression and thus the target cell population: for example, activated T cells can secrete IFN-γ or IL-4 (depending on the T_h subtype) (Mossman *et al.,* 1987), and IgE-activated mast cells are a rich source of TNF-α (Gordon *et al.,* 1990).

An unusual feature of IL-3R expression in non-hemopoietic cells, the significance of which is as yet unclear, is that the IL-3R α and β subunits can be expressed independently of each other. For example, unstimulated SMC express only the β chain, and HFF fibroblasts express only the IL-3R α chain (following treatment with IFN-γ and TNF-α). In addition, in endothelial cells stimulated with IL-4 the subunit balance changes to high β chain and low IL-3R α chain expression. This selective expression of one subunit is in contrast to hemopoietic cells, in which the IL-3R α chain is always accompanied by the β chain, and the β chain is accompanied by at least one of the α chains that can associate with it. In fact, the only documented example of independent expression is that of the IL-3R α chain in ES cells (McClanahan *et al.,* 1993). The role of such single receptor subunits is not clear at present, as IL-3R signalling has been

shown to require both chains (Kitamura *et al.,* 1992). It should be noted, however, that signal transduction is usually assessed by tyrosine phosphorylation and the induction of certain immediate early genes, but these processes may not be required for all IL-3R - mediated functions. For example, the GM-CSFR α chain has recently been claimed to mediate glucose uptake in the absence of tyrosine phosphorylation (Ding *et al.,* 1994). On the other hand, the subunits of the IL-3R may have some other, IL-3-unrelated functions. For example, they could serve as receptor components for other, presently unidentified factors. This would not be surprising, as many receptor molecules are shared between different cytokines and can function both as affinity-converters and ligand binding subunits. This is well illustrated in the case of the gp130 molecule, which is the signal transducer and high-affinity converting subunit of the IL-6, IL-11, CNTF and LIF receptors, and the low-affinity receptor for oncostatin M (Kishimoto *et al.,* 1992, Hilton *et al.,* 1994). Finally, the IL-3R α and β chains could perform some other functions besides being cytokine receptors; for example, the type III fibronectin modules found in their extracellular domains (Patthy, 1990) are suggestive of a potential adhesive role.

Interestingly, no overlapping expression of the GM-CSFR was found in the mesenchymal cells studied. The lack of the GM-CSFR α chain in endothelial cells and bone marrow stromal cells is of particular interest, as the literature concerning the effects of GM-CSF on these cells has been controversial (Bussolino *et al.,* 1989, Dedhar *et al.,* 1988, Yong *et al.,* 1991, Gronthos *et al.,* 1995). It is important to note that the cell types examined, including endothelial cells, fibroblasts, SMC and bone marrow stromal cells, have the capacity to produce GM-CSF (Broudy *et al.,* 1986, Munker *et al.,* 1986, Schrader *et al.,* 1991, Guba *et al.,* 1992), which could thus function as an autocrine factor if the receptor were present. The production of IL-3 is more restricted, requiring activation of T cells or mast cells. Selective expression of the IL-3R (and not the functionally similar GM-CSFR) may thus serve to limit cytokine responses to inflammation of an immunological or allergic origin. Hemopoietic cells which express

both receptors would, in contrast, be able to respond maximally in all situations. In addition to the GM-CSFR α chain, the IL-5R α chain was also undetectable in the cell types examined, suggesting that the actions of this cytokine may be restricted to eosinophils, basophils and some B cells (reviewed by Sanderson *et al.,* 1992).

Although the control of IL-3 production has been extensively studied, relatively little is known about the molecular mechanisms regulating expression of the IL-3 receptor. These mechanisms are likely to be complex to account for the following observations: a) expression of the IL-3R is constitutive in some cell types (eg. hemopoietic progenitor cells, monocytes, eosinophils and endothelial cells) and inducible in others (eg. bone marrow stromal cells and HFF fibroblasts); b) IL-3R expression is regulated by many cytokines including TNF-α, IFN-γ, erythropoietin, G-CSF, GM-CSF, TGF-β, IL-4 (Sato *et al.,* 1993, Liboi *et al.,* 1992, Jacobsen *et al.,* 1993); and c) a given cytokine can have a different effect on expression of the IL-3R on different cells (for example, TNF-α up-regulates the β subunit in HUVEC and $CD34^{+}$ progenitor cells but down-regulates it in SMC). Another level of complexity is added by the fact that the IL-3R subunits can be regulated either coordinately or differentially, depending on the cell type and the stimulus used. Examples of these complex regulation patterns are shown in table 7.1, which summarises the effect of IFN-γ and TNF-α on expression of the human IL-3R α and β chains in various primary cells. Additional insight into the molecular basis of this regulation would be provided by cloning and characterising the promoters of these genes. The promoters for the mouse IL-3R α chain and two β chains (AIC2A and AIC2B) have already been cloned and contain potential binding sites for various transcription factors (Miyajima *et al.,* 1995, Gorman *et al.,* 1992). In keeping with the differential regulation of the IL-3R α and β chains, the 5' flanking sequences of these genes show little similarity, although a GATA motif is present in both subunits. It is tempting to speculate that the GATA proteins would be amongst the factors which are involved in conferring constitutive IL-3R expression, as they are expressed mainly in hemopoietic cells but are also found in endothelial cells (Orkin, 1992). Interestingly, the

promoters of both subunits also contain several IFN-γ responsive elements. A 394 bp fragment of the IL-3R α chain promoter has been shown to direct transcription in a cell type-specific manner, being active in mast cells but not in fibroblasts or T cells (Miyajima *et al.*, 1995). It would be of interest to examine whether transcription could also be activated in fibroblasts following IFN-γ treatment.

In addition to transcriptional control, post-transcriptional mechanisms can also contribute to the regulation of IL-3R expression. For example, the increased expression of the β chain in IFN-γ-treated monocytes has been shown to involve mRNA stabilisation (Hallek *et al.*, 1992). Further control could be exerted at the levels of translation, post-translational modification or membrane transport. There is some precedent to suggest that this may be the case: while both IFN-γ and TNF-α induce the IL-3R α chain mRNA in SMC, only IFN-γ increases the protein expression on the cell surface (chapter 6).

Table 7.1 Regulation of expression of the IL-3R subunits by TNF-α and IFN-γ in different cell types.

	TNF-α		IFN-γ		TNF-α,IFN-γ	
	α	β	α	β	α	β
HUVEC	↑	↑	↑	↑	↑	↑
SMC	↑	↓	↑	-	↑	↓
HFF	↑	-	↑	-	↑	-
BMSC	↑	-	↑	-	↑	↑
CD34+ progenitor[1]	-	↑	-	-	?	?
monocyte[2]	?	-	?	↑	?	?

Up/down-regulation is indicated by arrows and no effect by a dash. Question marks indicate that the effect has not been investigated. [1]Sato *et al.*, 1993, [2]Hallek *et al.*, 1992

IL-3R function in non-hemopoietic cells

The finding of IL-3Rs in non-hemopoietic cells raises the potential for IL-3 to have a much wider role than previously expected. Importantly, hemopoietic cell-specific molecules do not appear to be required for signal transduction by the IL-3R, as IL-3 was able to induce c-fos expression (and various other functions) in HUVEC. However SMC, fibroblasts and bone marrow stromal cells failed to respond to IL-3 in the functional assays used. This may simply be due to the fact that the level of β chain expression is lower, and perhaps thus insufficient, in these cells. Alternatively, it could reflect a lack of an essential component(s) of the signalling pathway. This could be analogous to the "uncoupled" GM-CSFRs in HL60 cells: despite constitutive GM-CSFR expression, these cells are able respond to GM-CSF only after stimulation with DMSO, which increases expression of the src-like kinase hck (Linnekin *et al.,* 1994). In either case, it is possible that cells which do not respond to IL-3 *in vitro* can acquire IL-3 responsiveness *in vivo* in the presence of appropriate stimuli.

The potential of IL-3 to regulate various endothelial responses was investigated, in order to further elucidate the role of this hemopoietic growth factor in vascular biology. An inflammatory function seemed likely, as the IL-3R was strongly up-regulated by TNF-α and IFN-γ. This hypothesis was confirmed by the finding that IL-3 enhanced expression of the adhesion molecule E-selectin and the chemokine IL-8, both of which have been implicated in recruiting neutrophils to inflammatory foci (Springer, 1994). In keeping with this, IL-3 increased the transmigration of neutrophils across endothelial monolayers. IL-3 also enhanced expression of the MHC class II molecules required for antigen presentation, and secretion of the hemopoietic cytokines G-CSF and IL-6. The role of IL-3 in regulating these functions is essentially that of an enhancer: it has no effect on its own, but potentiates the expression induced by TNF-α or IFN-γ. IL-3 did not, however, augment all aspects of the TNF-α or IFN-γ -induced activation of endothelial cells. For example, it did not enhance the TNF-α -induced expression of ICAM-1 or VCAM-1 (J. Gamble, personal communication), or the IFN-γ -induced

expression of MHC class I antigens. This selectivity of IL-3 action is further emphasised in the case of IL-6 and G-CSF expression: although both TNF-α and IFN-γ induce production of these growth factors, only the induction mediated by IFN-γ is further enhanced by IL-3. These stimulatory actions of IL-3 are one example of how the combinatorial use of cytokines can produce different patterns of endothelial cell activation. In addition to its function as a selective enhancer, IL-3 also has independent actions on endothelial cells, as demonstrated by our recent finding that it induces expression of P-selectin (Goodall-Khew *et al.*, submitted).

Endothelial activation by IL-3 could be envisaged in pathological conditions involving activated T cells, such as chronic inflammation and delayed-type hypersensitivity reactions (DTH), in which IL-3 and the required costimuli IFN-γ and TNF-α are likely to be produced concomitantly. A role in allergen-induced late-phase reactions (LPR) is also possible, as mast cells release both IL-3 and TNF-α upon IgE-activation (Plaut *et al.*, 1989, Wodnar-Filipowicz *et al.*, 1989, Gordon *et al.*, 1990). IL-3 could enhance participation of the endothelium in several stages of the inflammatory process; for example, the ability of IL-3 to accelerate the IFN-γ -induced MHC class II expression could recruit more endothelial cells to function as APCs in the cognitive phase of inflammation. IL-3 could also augment the initial neutrophilic infiltration in DTH and LPR by enhancing the TNF-α -induced expression of E-selectin and secretion of IL-8 by endothelial cells. Finally, the enhanced production of IL-6 and G-CSF by endothelial cells in response to IL-3 could further exacerbate the inflammatory response through neutrophil activation and the release of acute phase proteins. Both of these circulating cytokines could also stimulate hemopoiesis in the bone marrow, thereby replenishing the pool of leukocytes in the blood.

It is important to note that although the work presented in this thesis focussed on the transmigration of neutrophils, IL-3 could also facilitate the recruitment of eosinophils: E-selectin mediates the adhesion of eosinophils to endothelium (Weller *et al.*, 1991), and IL-8 has been shown to be chemotactic for eosinophils from patients with atopic

dermatitis and asthma (Bruijnzeel *et al.*, 1993, Warringa *et al.*, 1993). Even eosinophils from normal individuals can respond to IL-8 when primed with IL-3, GM-CSF or IL-5 (Warringa *et al.*, 1992). The fact that IL-3 stimulates both eosinophils (Lopez *et al.*, 1988) and endothelial cells points to an important role for IL-3 in allergic diseases. *In vivo,* elevated levels of IL-3 mRNA have been detected in allergen-induced LPR and in bronchoalveolar T lymphocytes from patients with atopic asthma (Kay *et al.*, 1991, Robinson *et al.*, 1992).

The molecular mechanisms by which IL-3 enhances the expression of G-CSF, IL-6, IL-8, E-selectin and MHC class II in endothelial cells remain to be elucidated. Transcriptional activation seems likely at least in the case of IL-6, as no stabilisation of the IL-6 mRNA was observed. The identity of the transcription factors activated by IL-3 is only beginning to be investigated: CREB (cAMP response element binding protein) has been implicated in the induction of the egr-1 gene, and IL-3 has also been shown to activate factor(s) which recognise the SRE element in the promoters of c-fos and egr-1 (Sakamoto *et al.*, 1994, Hatekeyama *et al.*, 1992). Egr-1 itself is a transcription factor, and c-Fos can complex with c-Jun (also inducible by IL-3) to form the heterodimeric transcription factor AP-1. Interestingly, the promoters for IL-6, IL-8 and E-selectin contain AP-1 recognition sequences (Tanabe *et al.*, 1988, Yasumoto *et al.*, 1992, Collins et *al.*, 1991), suggesting that c-fos induction may be involved in the up-regulation of these genes by IL-3. On the other hand, the G-CSF, IL-6 and IL-8 promoters resemble each other in that they contain functionally linked NF-IL6 and NF-κB binding sites (Dunn *et al.*, 1994, Matsusaka *et al.*, 1993, Kunsch *et al.*, 1994), and an NF-κB site is also found in the E-selectin promoter (Collins et *al.*, 1991). It remains to be investigated whether these sites, which are important for activation by TNF-α, are also involved in activation by IL-3. Of note, IL-3 has recently been shown to activate STAT5 (Mui *et al.*, 1995, Azam *et al.*, 1995), a member of the STAT family of transcription factors which are implicated in IFN-γ signalling. It would be of interest to determine whether STAT5

is expressed in endothelial cells and whether it contributes, either directly or indirectly, to the synergism between IL-3 and IFN-γ.

In summary, the findings described in this thesis expand the role of IL-3, and emphasise its involvement in the inflammatory process. IL-3 not only stimulates the effector functions of leukocytes, but can also regulate their localisation to inflammatory sites by acting on the endothelium. In addition, the ability of IL-3 to enhance MHC class II expression is not limited to monocytes and eosinophils (Sadeghi *et al.*, 1992, Hansel *et al.*, 1992), but also encompasses endothelial cells. Finally, the finding that IL-3 stimulates secretion of the circulating cytokines IL-6 and G-CSF by the vascular endothelium suggests an alternative, indirect mechanism by which IL-3 could influence hemopoiesis in the bone marrow. In addition to endothelial cells, IL-3 may also act on neighbouring cell types such as SMC and fibroblasts, although this possibility remains to be elucidated. This local, inflammation-oriented role for IL-3 is in agreement with the fact that IL-3 is produced at the inflammatory site and is not detectable in the circulation (Garland *et al.*, 1983, Crapper *et al.*, 1984, Cheers *et al.*, 1988). The finding that IL-3 is not exclusively a hemopoietic growth factor but also acts on the vascular endothelium suggests that caution should be taken in the clinical administration of IL-3, especially in patients with underlying inflammatory conditions.

Future work

The work described in this thesis opens a new area for IL-3 research. Further investigation into the distribution and function of the IL-3R in different non-hemopoietic cells would contribute to an understanding of the physiological role of this cytokine, and also provide information of clinical importance. In addition to using isolated cells, it would be of interest to study expression of the IL-3R in tissue sections, as this would better reflect the *in vivo* situation and may also provide clues as to the role of the IL-3R in different pathological conditions. Finally, identifying new target genes for IL-3 and

the transcription factors involved in their regulation would also contribute to elucidating the mechanisms involved in IL-3R signalling.

BIBLIOGRAPHY

Adams PW, Lee HS, Waldman WJ, Sedmak DD, Morgan CJ, Ward JS, Orosz CG: Alloantigenicity of human endothelial cells. J. Immunol. 148: 3753-3760, 1992

Anagnostou A., Lee ES, Kessimian N, Levinson R, Steiner M: Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc. Natl. Acad. Sci. USA. 87: 5978-5982, 1990

Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T: Cytokines: coordinators of immune and inflammatory responses. Annu. Rev. Biochem. 59: 783-836, 1990

Azam M, Erdjument-Bromage H, Kreider BL, Xia M, Quelle F, Basu R, Saris C, Tempst P, Ihle JN, Schindler C: Interleukin-3 signals through multiple isoforms of Stat5. EMBO J. 14: 1402-1411, 1995

Baldwin GC, Gasson JC, Kaufman SE, Quan SG, Williams RE, Avalos BR, Gazdar AF, Golde DW, DiPersio JF: Nonhemopoietic tumor cells express functional GM-CSF receptors. Blood 73: 1033-1037, 1989

Baldwin GC, Benveniste EN, Chung GY, Gasson JC, Golde DW: Identification and characterization of a high-affinity granulocyte-macrophage colony-stimulating factor receptor on primary rat oligodendrocytes. Blood 82: 3279-3282, 1993

Battegay EJ, Raines EW, Seifert EA, Bowen-Pope DF, RossR: TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63: 515-524, 1990

Baumhauer S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA: Binding of L-selectin to the vascular sialomucin CD34. Science 262: 436-438, 1993

Bazan FJ: Haemopoietic receptors and helical cytokines. Immunology Today 11: 350-354, 1990

Bevilacqua MP: Endothelial-leukocyte adhesion molecules. Annu. Rev. Immunol. 11: 767-804, 1993

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA: Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA 84: 9238-9242, 1987

Bird TA, Davies A, Baldwin SA, Saklatvala J: Interleukin 1 stimulates hexose transport in fibroblasts by increasing the expression of glucose transporters. J. Biol. Chem. 265: 13578-13583, 1990

Brizzi MF, Garbarino G, Rossi PR, Pagliardi GL, Arduino C, Avanzi GC, Pegoraro L: Interleukin 3 stimulates proliferation and triggers endothelial-leukocyte adhesion molecule 1 gene activation of human endothelial cells. J. Clin. Invest. 91: 2887-2892, 1993

Broudy VC, Kaushansky K, Segal GM, Harlan JM, Adamson JW: Tumour necrosis factor α stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA 83:7467-7471, 1986

Bruijnzeel PL, Kuijper PH, Rihs S, Betz S, Warringa RA, Koenderman L: Eosinophil migration in atopic dermatitis. I: increased migratory responses to N-formyl-methionyl-leucyl-phenylalanine, neutrophil activating factor, platelet activating factor, and platelet factor 4. J. Invest. Dermatol. 100: 137-142, 1993

Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell C-JS, Aglietta M, Arese P, Mantovani A: Granulocyte and granulocyte-macrophage colony-stimulating factors induce human endothelial cells to migrate and proliferate. Nature 337: 471-473, 1989

Buzby JS, Knoppel EM, Cairo MS: Coordinate regulation of Steel factor, its receptor (Kit) and cytoadhesion molecule (ICAM-1 and ELAM-1) mRNA expression in human vascular endothelial cells of differing origins. Exp. Hematol. 22: 122-129, 1994

Cannistra SA, Vellenga E, Groshek P, Rambaldi A, Griffin JD: Human granulocyte-macrophage colony-stimulating factor and interleukin 3 stimulate monocyte cytotoxicity through a tumor necrosis factor-dependent mechanism. Blood 71: 672-676, 1988

Chamley-Campbell JH, Campbell GR, Ross R: Phenotype-dependent response of cultured aorta smooth muscle cells to serum mitogens. J. Cell. Biol. 89:379-383, 1981

Chang JM, Metcalf D, Lang RA, Gonda TJ, Johnson GR: Nonneoplastic hematopoietic myeloproliferative syndrome induced by dysregulated multi-CSF (IL-3) expression. Blood 73: 1487-1497, 1989

Cheers C, Haigh AM, Kelso A, Metcalf D, Stanley ER, Young AM: Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. Infect. Immun. 56: 247-251, 1988

Chen ZZ, Van Bockstaele DR, Buyssens N, Hendrics D, De Meester I, Vanhoof G, Scharpe SL, Peetermans ME, Berneman ZN: Stromal populations and fibrosis in human long-term bone marrow cultures. Leukemia 5: 772-781, 1991

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidiniun thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-161, 1987

Cluitmans FHM, Esendam BHJ, Landegent JE, Willemze R, Falkenburg JHF: Constitutive in vivo cytokine and hematopoietic growth factor gene expression in the bone marrow and peripheral blood of healthy individuals. Blood 85: 2038-2044, 1995

Cockayne DA, Bodine DM, Cline A, Nienhuis AW, Dunbar CE: Transgenic mice expressing antisense interleukin-3 RNA develop a B-cell lymphoproliferative syndrome or neurologic dysfunction. Blood 84: 2699-2710, 1994

Cockerill GW, Meyer G, Noack L, Vadas MA, Gamble JR: Characterisation of a spontaneously transformed human endothelial cell line. Lab. Invest. 71: 497-509, 1994

Collins T, Williams A, Johnston GI, Kim J, Eddy R, Shows T, Gimbrone MA, Bevilacqua MP: Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule-1. J. Biol. Chem. 266: 2466-2473, 1991

Colotta F, Bussolino F, Polentarutti N, Guglielmetti A, Sironi M, Bocchietto E, De Rossi M, Mantovani A: Differential expression of the common β and specific α chains of the receptors for GM-CSF, IL-3 and IL-5 in endothelial cells. Exp. Cell Res. 206: 311-317, 1993

Crapper RM, Clark-Lewis I, Schrader JW: The in vivo functions and properties of persisting cell-stimulating factor. Immunology 53:33-42, 1984

Darnell JE, Kerr IM, Stark GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415-1421, 1994

Dedhar S, Gaboury L, Galloway P, Eaves C: Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin. Proc. Natl. Acad. Sci. USA. 85:9253-9257, 1988

Demetri GD, Griffin JD: Granulocyte colony-stimulating factor and its receptor. Blood 78: 2791-2808, 1991

Detmers PA, Los K, Olsen-Egbert E, Waltz A, Baggiolini M, Cohn ZA: Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J. Exp. Med. 171: 1155-1162, 1990

Devergne O, Marfaing-Koka A, Schall TT, Leger-Ravet MB, Sadick M, Peuchmaur M, Crevon MC, Kim T, Galanaud M, Emilie D: Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J. Exp. Med. 179: 1689-1694, 1994

Devreux S, Bull HA, Campos-Costa D, Saib R, Linch DC: Granulocyte-macrophage colony-stimulating factor induces changes in cellular adhesion molecule expression and adhesion to endothelium: in vitro and in vivo studies in man. Br. J. Hematol. 71: 323-330, 1989

Ding DXH, Rivas CI, Heaney ML, Raines MA, Vera JC, Golde DW: The α subunit of the human granulocyte-macrophage colony-stimulating factor receptor signals for glucose transport via a phosphorylation-independent pathway. Proc. Natl. Acad. Sci. USA. 91:2537-2541, 1994

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R: The in vitro development of blastocyst-derived emryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morphol. 87: 27-45, 1985

Doukas J, Pober JS: Lymphocyte-mediated activation of cultured endothelial cells. J. Immunol. 145: 1088-1098, 1990

Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF: Requirement for nuclear factor (NF)-κB p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. Blood 83: 2469-2479, 1994

Duronio V, Clark LI, Federppiel B, Wieler JS, Schrader JW: Tyrosine phosphorylation of receptor beta subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. J. Biol. Chem. 267: 21856-21863, 1992

Eder M, Griffin JD, Ernst TJ: The human granulocyte-macrophage colony-stimulating factor receptor is capable of initiating signal transduction in NIH3T3 cells. EMBO J. 12: 1647-1656, 1993

Elbaz O, Budel LM, Hoogerbrugge H, Touw IP, Delwel R, Mahmoud LA, Lowenberg B: Tumor necrosis factor regulates the expression of granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors on human acute myeloid leukemia cells. Blood 77: 989-995, 1991

Elias JA, Lentz V: IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL-6 production and stabilize IL-6 messenger RNA. J. Immunol. 145: 161-166, 1990

Elliott JI, Vadas MA, Cleland LG, Gamble JR, Lopez AF: IL-3 and granulocyte-macrophage colony-stimulating factor stimulate distinct phases of adhesion in human monocytes. J. Immunol. 145: 167-176, 1990

Elliott JI, Vadas MA, Eglinton JM, Park LS, To LB, Cleland LG, Clark SC, Lopez AF: Recombinant human interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) show common biological effects and binding chatacteristics on human monocytes. Blood 74: 2349-2359, 1989

Fenton MJ, Buras JA, Donnelly RP: IL-4 reciprocally regulates IL-1 and IL-1 receptor antagonist expression in human monocytes. J. Immunol. 149: 1283-1288, 1992

Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, Greaves MF: Expression of the CD34 gene in vascular endothelial cells. Blood 75: 2417-2426, 1990

Forsyth KD, Chua K-Y, Talbot V, Thomas WR: Expression of the leukocyte common antigen CD45 by endothelium. J. Immunol. 150: 3471-3477, 1993

Fujisawa T, Abu-Ghazaleh R, Kita H, Sanderson CJ, Gleich GJ: Regulatory effect of cytokines on eosinophil degranulation. J. Immunol. 144: 642-646,1990

Fung MC, Hapel AJ, Ymer S, Cohen DR, Johnson RM, Campbell HD, Young IG: Molecular cloning of cDNA for murine interleukin-3. Nature 307: 233-237, 1984

Gamble JR, Khew-Goodall Y, Vadas MA: Transforming growth factor-β inhibits E-selectin expression on human endothelial cells. J. Immunol. 150: 4494-4503, 1993

Ganser A, Lindemann A, Seipelt G, Ottman OG, Herrman F, Eder M, Frisch J, Schulz G, Mertelsmann R, Hoelzer D: Effects of recombinant human interleukin-3 in patients with normal hematopoiesis and in patients with bone marrow failure. Blood 76: 666-676, 1990

Garland JM, Aldridge A, Wagstaffe J, Dexter TM: Studies on the in vivo production of a lymphokine activity, interleukin 3 (IL-3) elaborated by lymphocytes and a myeloid

leukaemic line in vitro and the fate of IL-3 dependent cell lines. Br. J. Cancer 48:247-259,1983

Gasson JC: Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood 77:1131-1145, 1991

Gearing DP, King JA, Gough NM, Nicola NA: Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J. 8: 3667-3676, 1989

Geppert TD, Lipsky PE: Antigen presentation by interferon-γ treated endothelial cells and fibroblasts: differential ability to function as antigen presenting cells despite comparable Ia expression. J. Immunol. 135:3750-3762, 1985

Goodall GJ, Wiebauer K, Filipowicz W: Analysis of pre-mRNA processing in transfected plant protoplasts. Methods in Enzymol. 181: 148-161, 1990

Gordon JR, Galli SJ: Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. Nature 346: 274-276, 1990

Gorman DM, Itoh N, Jenkins NA, Gilbert DJ, Copeland NG, Miyajima A: Chromosomal localization and organization of the murine genes encoding the β subunits (AIC2A and AIC2B) of the interleukin 3, granulocyte-macrophage colony-stimulating factor and interleukin 5 receptors. J. Biol. Chem. 267: 15842-15848, 1992

Gronthos S, Simmons PJ: The growth factor requirements of STRO-1 -positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 85: 929-940, 1995

Guba SC, Sartor CI, Gottschalk LR, Ye-Hu J, Mulligan T, Emerson SG: Bone marrow stromal fibroblasts secrete interleukin-6 and granulocyte-macrophage colony-stimulating factor in the absence of inflammatory stimulation: demostration by serum-free bioassay, enzyme-linked immunosorbent assay and reverse transcriptase polymerase chain reaction. Blood 80: 1190-1198, 1992

Haak-Frendscho M, Arai N, Arai K-I, Baeza ML, Finn A, Kaplan AP: Human recombinant granulocyte-macrophage colony-stimulating factor and interleukin 3 cause basophil histamine release. J. Clin. Invest. 82: 17-20, 1988

Hallek M, Lepisto EM, Slattery KE, Griffin JD, Ernst TJ: Interferon-γ increases the expression of the gene encoding the β subunit of the granulocyte-macrophage colony-stimulating factor receptor. Blood 80: 1736-1742, 1992

Hamilton JA, Vairo G, Lingelbach SR: Activation and proliferation signals in murine macrophages: stimulation of glucose uptake by hemopoietic growth factors and other agents. J. Cell. Physiol. 134: 405-412, 1988

Hansel TT, de Vries JM, Carballido JM, Braun RK, Carballido-Perrig N, Rihs S, Blaser K, Walker C: Induction and function of eosinophil intercellular adhesion molecule-1 and HLA-DR. J. Immunol. 149: 2130-2136, 1992

Hara T, Miyajima A: Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3). EMBO J. 11: 1875-1884, 1992

Hasthorpe S, Bogdanovski M, Rogerson J, Radley JM: Characterization of endothelial cells in murine long-term marrow culture. Implication for hemopoietic regulation. Exp. Hematol. 20: 476-481, 1992

Hatekeyama M, Kawahara A, Mori H, Shibuya H, Tanigushi T: c-fos gene induction by interleukin 2: Identification of the critical cytoplasmic regions within the interleukin 2 receptor β chain. Proc. Natl. Acad. Sci. USA. 89: 2022-2026, 1992

Hayashida K, Kitamura T, Gorman DM, Arai K-I, Yokota T, Miyajima A: Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor. Proc. Natl. Acad. Sci. USA 87:9655-9659, 1990

Haylock DN, To LB, Dowse TL, Juttner CA, Simmons PJ: Ex vivo expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. Blood 80: 1405-1412, 1992

Hercus TR, Bagley CJ, Cambareri B, Dottore M, Woodcock JM, Vadas MA, Shannon MF, Lopez AF: Specific human granulocyte-macrophage colony-stimulating factor antagonists. Proc. Natl. Acad. Sci. USA 91: 5838-5842, 1994

Hilton DJ, Hilton AA, Raisevic A, Rakar S, Harrison-Smith M, Gough NM, Begley CG, Metcalf D, Nicola NA, Wilson TA: Cloning of a murine IL-11 receptor α-chain; requirement for gp130 for high affinity binding and signal transduction. EMBO J. 13: 4765-4775, 1994

Ihle JN, Keller J, Henderson L, Klein F, Palaszynski E: Procedures for the purification of interleukin 3 to homogeneity. J. Immunol. 129: 2431-2436, 1982

Itoh N, Yonehara S, Schreurs J, Gorman DM, Maruyama K, Ishii A, Yahara I, Arai KI, Miyajima A: Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. Science 24:324-327, 1990

Jacobsen SEW, Ruscetti FW, Roberts AB, Keller JR: TGF-β is a birectional modulator of cytokine receptor expression on murine bone marrow cells. J. Immunol. 151: 4534-4544, 1993

Jirik FR, Podor TJ, Hirano T, Kishimoto T, Loskutoff DJ, Carson DA, Lotz M: Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. J. Immunol. 142: 144-147, 1989

Kay AB, Ying S, Varney V, Gaga M, Durham SR, Moqbel R, Wardlaw AJ, Hamid Q: Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. J. Exp. Med. 173: 775-778, 1991

Kelso A, Gough NM: Coexpression of granulocyte-macrophage colony-stimulating factor, γ interferon, and interleukins 3 and 4 is random in murine alloreactive T-lymphocyte clones. Proc. Natl. Acad. Sci. USA 85: 9189-9193, 1988

Kishimoto T, Akira S, Taga T: Interleukin-6 and its receptor: A paradigm for cytokines. Science 258: 593-597, 1992

Kitamura T, Sato N, Arai K-I, Miyajima A: Expression cloning of the human IL-3 receptor cDNA reveals a shared β subunit for the human IL-3 and GM-CSF receptors. Cell 66: 1165-1174, 1991A

Kitamura T, Miyajima A: Functional reconstitution of the human interleukin 3 receptor. Blood 80: 84-90, 1992

Kitamura T, Takaku F, Miyajima A: IL-1 up-regulates the expression of cytokine receptors on a factor-dependent human hemopoietic cell line TF-1. Int. Immunol. 3: 571-577, 1991B

Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, Piao Y-F, Miyazono K, Urabe A, Takaku F: Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3 or erythropoietin. J. Cell. Physiol: 140, 323-334, 1989

Kovary K, Bravo R: The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. 11: 4466-4472, 1991

Kunsch C, Lang RK, Rosen CA, Shannon MF: Synergistic transcriptional activation of the IL-8 gene by NFκ-B p65 (RelA) and NF-IL-6. J. Immunol. 153: 153-164, 1994

Lamb P, Kessler LV, Suto C, Levy DE, Seidel HM, Stein RB, Rosen J: Rapid activation of proteins that interact with the interferon γ activation site in response to multiple cytokines. Blood 83: 2063-2071, 1994

Larson RS, Springer TA: Structure and function of leukocyte integrins. Immunol. Rev. 114: 181-217, 1990

Libby P, Ordovas JM, Birinyi LK, Auger KR, Dinarello CA: Inducible interleukin-1 gene expression in human vascular smooth muscle cells. J. Clin. Invest. 78: 1432-1438, 1986

Liboi E, Jubinsky P, Andrews NC, Nathan DG, Mathey-Prevot B: Enhanced expression of interleukin-3 and granulocyte-macrophage colony-stimulating factor receptor subunits in murine hematopoietic cells stimulated with hematopoietic growth factors. Blood 80: 1183-1189, 1992

Lindemann A, Ganser A, Hoelzer D, Mertelsmann R, Herrmann F: In vivo administration of recombinant human interleukin-3 elicits an acute phase response involving endogenous synthesis of interleukin-6. Eur. Cytokine Netw. 2: 173-176, 1991

Linnekin D, Howard OMZ, Park L, Farrar W, Ferris D, Longo DL: Hck expression correlates with granulocyte-macrophage colony-stimulating factor -induced proliferation in HL-60 cells. Blood 84: 94-103, 1994

Litwin MS, Gamble JR, Vadas MA: Role of cytokines in endothelial cell functions. In Human cytokines: their role in disease and therapy. Editors Aggarwal BB, Puri RK. Blackwell Science, Cambridge, MA, 1995. pp.101-131.

Lopez AF, Dyson P, To L-B, Elliott MJ, Milton S, Russel J, Juttner C, Yang Y-C, Clark SC, Vadas MA: Recombinant human interleukin-3 stimulation of hematopoiesis in humans: loss of responsiveness with differentiation in the neutrophilic myeloid series. Blood 72: 1797-1804, 1988

Lopez AF, Eglinton JM, Lyons AB, Tapley PM, To LB, Park LS, Clark SC, Vadas MA: Human interleukin-3 inhibits the binding of granulocyte-macrophage colony-stimulating factor and interleukin-5 to basophils and strongly enhances their functional activation. J. Cell. Physiol. 145: 69-77, 1990

Lopez AF, Elliott MJ, Woodcock J, Vadas MA: GM-CSF, IL-3 and IL-5: cross-competition on human haemopoietic cells. Immunology Today 13: 495-500, 1992

Lopez AF, Eglinton JM, Gillis D, Park LS, Vadas MA: Reciprocal inhibition of binding between interleukin 3 and granulocyte-macrophage colony-stimulating factor to human eosinophils. Proc. Natl. Acad. Sci. USA. 86: 7022-7026, 1989

Loppnow H, Libby P: Proliferating or interleukin 1 -activated human vascular smooth muscle cells secrete copious interleukin 6. J. Clin. Invest. 85: 731-738, 1990

Mano H, Yamashita Y, Sato K, Yazaki Y, Hirai H: Tec protein-tyrosine kinase is involved in interleukin-3 signalling pathway. Blood 85: 343-350, 1995

Matsuda T, Hirano T: Association of p72 tyrosine kinase with Stat factors and its activation by interleukin-3, interleukin-6 and granulocyte colony-stimulating factor. Blood 83: 3457-3461, 1994

Matsuguchi T, Salgia R, Hallek M, Eder M, Druker B, Ernst TJ, Griffin JD: Shc phosphorylation in myeloid cells is regulated by granulocyte-macrophage colony-stimulating factor, interleukin-3 and steel factor and is constitutively increased by $p210^{BCR/ABL}$. J. Biol. Chem. 18: 5016-5021, 1994

Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto, Akira S: Transcription factors NF-IL6 and NF-B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc. Natl. Acad. Sci.USA 90: 10193-10197, 1993

McClanahan T, Dalrymple S, Barkett M, Lee F: Hematopoietic growth factor receptor genes as markers of lineage commitment during in vitro development of hematopoietic cells. Blood 81: 2903-2915, 1993

Metcalf D: The molecular control of blood cells. Harvard University Press, Cambridge, Massachusetts, 1988

Metcalf D, Begley CG, Johnson GR, Nicola NA, Lopez AF, Williamson: Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hematopoiesis in normal adult mice. Blood 68: 46-57, 1986

Methia N, Louache F, Vainchenker W, Wendling F: Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. Blood 82: 1395-1401, 1993

Miller MD, Krangel MS: Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. Crit. Rev. Immunol. 12: 17-46, 1992

Miyajima A, Mui AL-F, Ogorochi T, Sakamaki K: Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3 and interleukin-5. Blood 82: 1960-1974, 1993

Miyajima I, Levitt L, Hara T, Bedell MA, Copeland NG, Jenkins NA, Miyajima A: The murine interleukin-3 receptor α subunit gene: chromosomal localization, genomic structure and promoter function. Blood 85: 1246-1253, 1995

Mossman TR, Coffman RL: Two types of mouse helper T-cell clone. Immunology Today 8: 223-227, 1987

Mueller DL, Chen ZM, Schwartz RH, Gorman DM, Kennedy MK: Subset of $CD4^{+}$ T cell clones expressing IL-3 receptor α-chains uses IL-3 as a cofactor in autocrine growth. J. Immunol. 153: 3014-3027, 1994

Munker R, Gasson J, Ogawa M, Koeffler HP: Recombinant human TNF induces production of granulocyte-macrophage colony-stimulating factor. Nature 323: 79-82, 1986

Mui ALF, Cutler R, Alai M, Bustelo X, Barbacid M, Krystal G: Steel factor and interleukin-3 stimulate the tyrosine phosphorylation of p95vav in hematopoietic cell lines. Exp. Hematol. 20: 752, 1992

Mui ALF, Wakao H, O'Farrell AM, Harada N, Miyajima A: Interleukin-3, granulocyte-macrophage colony-stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. EMBO J. 14: 1166-1175, 1995

Niemeyer CM, Sieff CA, Mathey-Prevot B, Wimperis JZ, Bierer B, Clark SC, Nathan DG: Expression of human interleukin-3 (multi-CSF) is restricted to human lymphocytes and T-cell tumor lines. Blood 73: 945-954, 1989

Nishikura K, Murray JM: Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol. Cell. Biol. 7:639-649, 1987

Ohsawa N, Ueyama Y, Morita K, Kondo Y: Heterotransplantation of human functioning tumors to nude mice, in The potentialities and limitations of the nude mouse: proceedings of the second international workshop on nude mice. Editors Nomura T, Ohsawa N, Tamaoki N, Fujisawa K. Gustav Fischer Verlag, Stuttgart, Germany. pp. 395-404, 1977

Orazi A, Cattoretti G, Schiro R, Siena S, Bregni M, Di Nicola M, Gianni AM: Recombinant human interleukin-3 and recombinant granulocyte-macrophage colony-stimulating factor administered in vivo after high-dose cyclophosphamide cancer chemotherapy: Effect on hematopoiesis and microenvironment in human bone marrow. Blood 79: 2610-2619, 1992

Orkin SH: GATA-binding transcription factors in hematopoietic cells. Blood 80: 575-581, 1992

Oster W, Lindemann A, Mertelsmann R, Hermann F: Granulocyte-macrophage colony-stimulating factor and multilineage CSF recruit human monocytes to express granulocyte CSF. Blood 73: 64-67, 1989

Page C, Rose M, Yacoub M, Pigott R: Antigenic heterogeneity of vascular endothelium. Am. J. Path. 141: 673-683, 1992

Park LS, Friend D, Price V, Anderson D, Singer J, Prickett KS, Urdal DL: Heterogeneity in human interleukin-3 receptors. J. Biol. Chem. 264: 5420-5427, 1989

Patthy L: Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. Cell 61: 13-14, 1990

Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP, Paul WE: Mast cell lines produce lymphokines in response to cross-linkage of FceRI or to calcium ionophores. Nature 339: 64-67, 1989

Pober JS, Cotran RS: Immunologic interactions of T lymphocytes with vascular endothelium. Adv. Immunol. 50: 261-302, 1991

Pober JS, Collins T, Gimbrone MA, Cotran RS, Gitlin JD, Fiers W, Clayberger C, Krensky AM, Burakoff SJ, Reis CS: Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. Nature 305: 726-729, 1983A

Pober JS, Gimbrone MA, Cotran RS, Reiss CS, Burakoff SJ, Fiers W, Ault KA: Ia expression by vascular endothelium is inducible by activated T cells and by human γ interferon. J. Exp. Med. 157: 1339-1353, 1983B

Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD, Ihle JN: JAK2 associates with the β chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Moll. Cell. Biol. 14: 4335-4341, 1994

Rafii S, Shapiro F, Rimarachin J, Nachman RL, Ferris B, Weksler B, Moore MAS, Asch AS: Isolation and characterization of human bone marrow microvascular endothelial cells: Hematopoietic progenitor adhesion. Blood 84: 10-19, 1994

Raines MA, Liu L, Quan SG, Joe V, DiPersio JF, Golde DW: Identification and molecular cloning of a soluble human granulocyte-macrophage colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA 88: 8203-8207, 1991

Riabolow KT, Vosatka RJ, Ziff EB, Lamb NJ, Feramisco JR: Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell. Biol. 8: 1670-1676, 1988.

Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay B: Predominant T_{H2}-like bronchoalveolar T-lymphocyte population in atopic asthma. New. Engl. J. Med. 326: 298-304, 1992

Rollins BJ, Pober JS: Interleukin-4 induces the synthesis and secretion of MCP-1/JE by human endothelial cells. Am. J. Pathol. 138: 1315-1319, 1991

Rothenberg ME, Pomerantz JL, Owen WR, Avraham S, Soberman RJ, Austen KF, Stevens RL: Characterization of a human eosinophil proteoglycan and augmentation of its biosynthesis and size by interleukin-3, interleukin-5 and granulocyte-macrophage colony-stimulating factor. J. Biol. Chem. 263: 13901-13908, 1988A

Rothenberg ME, Owen WR, Silberstein DS, Woods J, Soberman RJ, Austen KF, Stevens RL: Human eosinophils have prolonged survival, enhanced functional properties and become hypodense when exposed to human interleukin-3. J. Clin. Invest. 81: 1986-1992, 1988B

Sadeghi R, Feldman M, Hawrylowicz C: Upregulation of HLA class II, but not intercellular adhesion molecule 1 (ICAM-1) by granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 in synergy with dexamethasone. Eur. Cytokine Netw. 3: 373-380, 1992

Sakamoto KM, Fraser JK, Lee HJJ, Lehman E, Gasson JC: Granulocyte-macrophage colony-stimulating factor and interleukin-3 signaling pathways converge on the CREB-binding site in the human egr-1 promoter. Mol. Cell. Biol. 14: 5975-5985, 1994

Sanderson CJ: Pharmacological implications of interleukin-5 in the control of eosinophilia. Adv. Pharmacol. 23: 163-177, 1992

Sato N, Caux C, Kitamura T, Watanabe Y, Arai K-I, Bancherau J, Miyajima A: Expression and factor-dependent modulation of the interleukin-3 receptor subunits on human hemopoietic cells. Blood 82: 752-761, 1993

Scatchard G: The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51: 660-672, 1949

Schleimer RP, Sterbinsky SA, Kaiser J, Bickel CA, Klunk DA, Tomioka K, Newman W, Luscinkas FW, Gimbrone MA, McIntyre BW, Boechner BS: IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J. Immunol. 148: 1086-1092, 1992

Schrader JW, Moyer C, Ziltener HJ, Reinisch CL: Release of the cytokines colony-stimulating factor-1, granulocyte-macrophage colony-stimulating factor and IL-6 by cloned murine vascular smooth muscle cells. J. Immunol. 146: 3799-3808, 1991

Seelentag WK, Mermod J-J, Montesano R, Vassalli P: Additive effects of interleukin 1 and tumour necrosis factor-α on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. EMBO J. 6:2261-2265, 1987

Sica A, Matsushima K, van Damme J, Wang JM, Polentarutti N, Dejana E, Colotta F, Mantovani A: IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. Immunology Today 69: 548-553, 1990

Sieff CA, Niemyer CM, Nathan DG, Ekern SC, Bieber FR, Yang YC, Wong G, Clark SC: Stimulation of human hematopoietic colony formation by recombinant gibbon multi-colony-stimulating factor or interleukin 3. J. Clin. Invest. 80: 818-823, 1987

Silvennoinen O, Witthuhn BA, Quelle FW, Cleveland JL, Yi T, Ihle JN: Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. Proc. Natl. Acad. Sci. USA 90: 8429-8433, 1993

Smith WB, Gamble JR, Clark-Lewis I, Vadas MA: Interleukin-8 induces neutrophil transendothelial migration. Immunology 72: 65-72, 1991

Smith WB, Guida L, Qiyu S, Korpelainen EI, Gillis D, van der Heuvel C, Hawrylowicz CM, Vadas MA, Lopez AF: Neutrophils activated by GM-CSF express receptors for interleukin-3 which mediate class II expression. Blood, in press

Spielholz C, Heaney ML, Morrison ME, Houghton AN, Vera JC, Golde DW: Granulocyte-macrophage colony-stimulating factor signals for increased glucose uptake in human melanoma cells. Blood 85: 973-980, 1995

Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314, 1994

Standiford TJ, Strieter RM, Chensue SW, Westwick J, Kasahara K, Kunkel SL: IL-4 inhibits the expression of IL-8 from stimulated human monocytes. J. Immunol. 145: 1435-1439, 1990

Tadmori W, Feingersh D, Clark SC, Choi YS: Human recombinant IL-3 stimulates B cell differentiation. J. Immunol. 142: 1950-1955, 1989

Taga T, Kishimoto T: Cytokine receptors and signal transduction. FASEB J. 6: 3387-3396, 1992

Takahashi GW, Andrews III DF, Lilly MB, Singer JW, Alderson MR: Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. Blood 81: 357-364, 1993

Tanabe O, Akira S, Kamiya T, Wong GG, Hirano T, Kishimoto T: Genomic structure of the murine IL-6 gene. J. Immunol. 141: 3875-3881, 1988

Tavassoli M: Hemopoietic endothelium, incognito. Exp. Hematol. 20: 386-387, 1992

Tavernier J, Devos R, Cornelis S, Tuypens T, Van der Heyden J, Fiers W, Plaetinck G: A human high affinity interleukin-5 receptor (IL5R) is composed of an IL-5-specific α chain and a β chain shared with the receptor for GM-CSF. Cell 66: 1175-1184, 1991

Thornhill MH, Kyan-Aung U, Haskard DO: IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils. J. Immunol. 144: 3060-3065, 1990

Towatari M, May GE, Marais R, Perkins GR, Marshall CJ, Cowley S, Enver T: Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. J. Biol. Chem. 270: 4101-4107, 1995

Vadas MA, Gamble JR, Smith WB: Adhesion, its role in inflammatory disease. Editors Harlan JM, Lin DY. San Fransisco, Freeman 1992. pp. 65-81.

Vadas MA, Butterworth DJR, Pisani NT, Siongok TA: A new method for the purification of human eosinophils and neutrophils, and a comparison of the ability of these cells to damage schistosomula of Schistosoma mansoni. J. Immunol. 122: 1228-1236, 1979

Wall RT, Harker LA, Quadracci LJ, Striker GE: Factors influencing endothelial cell proliferation in vitro. J. Cell. Physiol. 96: 203-213, 1978

Vanhoutte PM: A matter of life and breath. Nature 368: 693-694, 1994

Van Zee KJ, De Forge LE, Fischer E, Marano MA, Kenney JS, Remick DG, Lowry SF, Moldawer LL: IL-8 in septic shock, endotoxemia, and after IL-1 administration. J. Immunol. 146: 3478-3482, 1991

Warner SJC, Friedmann GB, Libby P: Immune interferon inhibits proliferation and induces 2'-5'-oligoadenylate synthase gene expression in human vascular smooth muscle cells. J. Clin. Invest. 83: 1174-1182, 1989

Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koendermann L: Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. J. Allergy Clin. Immunol. 91: 1198-1205, 1993

Warringa RA, Schweizer RC, Maikoe T, Kuijper PH, Bruijnzeel PL, Koendermann L: Modulation of eosinophil chemotaxis by interleukin-5. Am. J. Respir. Cell Mol. Biol. 7: 631-636, 1992

Watanabe S, Mui AL-F, Muto A, Chen JX, Hayashida K, Yokota T, Miyajima A, Arai K-I: Reconstituted human granulocyte-macrophage colony-stimulating factor receptor transduces growth-promoting signals in mouse NIH3T3 cells: Comparison with signalling BA/F3 Pro-B cells. Mol. Cell. Biol. 13: 1440-1448, 1993

Watanabe Y, Kitamura T, Hayashida K, Miyajima A: Monoclonal antibody against the common β subunit (β_C) of the human interleukin-3 (IL-3), IL-5 and granulocyte-macrophage colony-stimulating factor receptors shows upregulation of β_C by IL-1 and tumor necrosis factor-α. Blood 80:2215-2220, 1992

Weller PF, Rand TH, Goelz SE, Chi-Rosso G, Lobb RR: Human eosinophil adherence to vascular endothelium mediated by binding to vascular cell adhesion molecule 1 and endothelial leukocyte adhesion molecule 1. Proc. Natl. Acad. Sci. USA 88: 7430-7433, 1991

Welham MJ, Dechert U, Leslie KB, Jirik F, Schrader JW: Interleukin (IL)-3 and granulocyte-macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase. J. Biol. Chem. 269: 23764-23768, 1994

Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR: Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343: 76-79, 1990

Wodnar-Filipovicz A, Heusser CH, Moroni C: Production of the hemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. Nature 339: 150-152, 1989

Woodcock JM, Zacharakis B, Plaetinck G, Bagley CJ, Qiyu S, Hercus TR, Tavernier J, Lopez AF: Three residues in the common β chain of the human GM-CSF, IL-3 and IL-5 receptors are essential for GM-CSF and IL-5 but not IL-3 high affinity binding and interact with Glu21 of GM-CSF. EMBO J. 13: 5176-5185, 1994.

Xia X, Li L, Choi YS: Human recombinant IL-3 is a growth factor for normal B cells. J. Immunol. 148: 491-497, 1992

Yang Y-C, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC: Human IL-3 (multi-CSF): identification by expression cloning of a novel hemopoietic growth factor related to murine IL-3. Cell 47: 3-10, 1986

Yasumoto K, Okamoto SI, Mukaida N, Murakami S, Mai M, Matsushima K: Tumor necrosis factor α and interferon γ synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-κB-like binding sites of the interleukin 8 gene. J. Biol. Chem. 267: 22506-22511, 1992

Yi T, Mui ALF, Krystal G, Ihle JN: Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor β chain and down-regulates IL-3 -induced tyrosine phosphorylation and mitogenesis. Mol. Cell. Biol. 13: 7577-7586, 1993

Yokota T, Lee F, Rennick D, Hall C, Arai N, Mosmann T, Nabel G, Cantor H, Arai K-I: Isolation and characterization of a mouse cDNA clone that expresses mast cell growth factor activity in monkey cells. Proc. Natl. Acad. Sci. 81: 1070-1074, 1984

Yong K, Cohen H, Khwaja A, Jones HM, Linch DC: Lack of effect of granulocyte-macrophage and granulocyte colony-stimulating factors on cultured human endothelial cells. Blood 77:1675-1680, 1991

Zoellner H, Filonzi EL, Stanton HR, Layton JE, Hamilton JA: Human arterial smooth muscle cells synthesize granulocyte colony-stimulating factor in response to interleukin-1α and tumor necrosis factor-α. Blood 80: 2805-2810, 1992

Clarifications

Chapter 2

Human recombinant cytokines (page 16) were expressed in *E. coli.*

Chapter 3

Data on HUVEC expressing 2000 IL-3 receptors per cell is shown (fig. 3.3). Data on HUVEC not expressing IL-3 receptors is not shown. Variability in receptor number is not due to contaminating cells, as HUVEC used in binding assays were essentially pure after several passages *in vitro*. It may rather reflect the differences between donors, as suggested in discussion (page 38). Most HUVEC lines (19/20) tested were found to express IL-3 receptors.

Cyclohexamide on page 38 should read cycloheximide.

Chapter 4

The cytokines used in receptor regulation studies did not contain LPS as judged by *Limulus* amoebocyte assay (Materials and methods, page 16).

The induction of the IL-3R α and β chain mRNAs (Fig. 4.1) was observed in all HUVEC lines tested (5 separate experiments). The band between the IL-3R α and β chain signals appears consistently under the conditions of the protection assay, and is caused by incomplete digestion of vector sequences in the IL-3R α chain probe.

The conversion of units to milligrams for TNF-α and IFN-γ is given in Materials and methods (page 16).

All the RNase protection gels were quantitated using a phosphorimager as described in Materials and methods (page 21).

← BARCODE (OVER PAGE)

Chapter 5

IL-3 enhanced IL-8 production in TNF-α stimulated HUVEC but not in IFN-γ stimulated HUVEC (3 separate experiments). As the response in IFN-γ stimulated HUVEC could be delayed, longer (48-h) stimulations were also performed. However, no induction of IL-8 was observed. This is consistent with the role of IL-3 as an amplification factor (discussion, page 72): IL-3 does not induce IL-8 by itself, but rather enhances the induction caused by TNF-α. When IFN-γ (which does not itself induce IL-8) is used as a co-stimulant, IL-3 does not have any effect.

The experiment shown in fig. 5.2 is representative of 4.

The experiment shown in fig. 5.4 is representative of 3.

Chapter 6

In fig. 6.1 the IL-3Rα chain mRNA signal in TNF-α -stimulated HFF is faint, nevertheless it has been consistently observed (4/4 experiments).

While both TNF-α and IFN-γ induce the IL-3R α chain mRNA in SMC, only IFN-γ increases the protein expression on the cell surface (figures 6.1 and 6.2). Expression of the IL-3R α chain may thus be controlled also at the level of translation or membrane transport, as suggested in the discussion (page 94).