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UNIVERSITY OF ALBERTA

IN VIVO AND *IN VITRO* FUNCTIONS OF INTERLEUKIN 13

BY

YEW HON LAI ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

IN

MEDICAL SCIENCES-IMMUNOLOGY

Edmonton, Alberta

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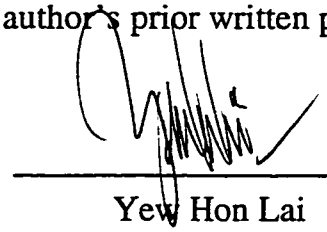

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***IN VIVO* AND *IN VITRO* FUNCTIONS OF INTERLEUKIN 13** submitted by **YEW HON LAI** in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MEDICAL SCIENCES -IMMUNOLOGY.

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ABSTRACT

Interleukin (IL)-13 shares some but not all of IL-4's functions, including inhibition of monocyte/macrophage activation, stimulation of human B cells, and induction of growth and differentiation of mouse bone marrow cells *in vitro*. As IL-13 is a relatively new cytokine, its biological functions are much less characterized *in vivo*. This study explores the *in vivo* functions of IL-13 by short term *in vivo* administration of purified IL-13 into mice and construction of IL-13 transgenic mice.

Purified mouse IL-13, expressed by stably-transfected cells, was administered in BALB/c mice via osmotic pumps. IL-13-treated mice exhibited splenomegaly, enhanced extramedullary hemopoiesis, and striking monocytosis. The strong Th2 response induced by *Nippostrongylus brasiliensis* infection was also accompanied by an increase in hemopoietic precursor frequencies in the spleen, suggesting that endogenous IL-13 may contribute to replenishment of effector cells during strong Th2 responses.

Both IL-4 and IL-13 stimulate Ig class switching in human B cells. Although IL-13 had been reported to have no effects on mouse B cells, this study showed that mouse B cells directly responded to IL-13. CRBC immunization and IL-13 treatment enhanced both the plasma IgG and CRBC-specific Ig levels. In the presence of anti-CD40, IL-13 enhanced antibody production *in vitro*, survival of B cells, and the numbers of antibody producing cells but induced neither B cell proliferation nor Ig class switching. Hence, IL-13 can enhance mouse B cell antibody production by increasing survival of the B cells.

Three transgenic founders were obtained which carried IL-13 cDNA under the control of the human cytomegalovirus regulatory region (hCMV), which should result in ubiquitous IL-13 expression. Two founders transmitted the transgenes to their offspring. No IL-13 transgene-derived IL-13 mRNA or protein was detected in these transgenic mice, although this transgene promoted high levels of IL-13 expression *in vitro*. As IL-13 protein was toxic at high doses and low frequencies of non-expressor hCMV-IL-13 transgenic mice were obtained, IL-13 may be lethal for embryogenesis.

This thesis is dedicated to my parents

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ABBREVIATIONS

• Ab	Antibody
• ACN	Acetonitrile
• AEC	3-amino-9-ethyl-carbazole
• APC	Antigen-presenting cell
• BFU-E	Erythroid burst colonies
• bp	Base pair
• BSA	Bovine serum albumin
• CFA	Complete Freund's adjuvant
• C	Control
• CD40L	CD40 Ligand
• cDNA	Complementary DNA
• CFSE	(5-(and 6)-carboxyfluorescein diacetate, succinimidylester
• CFU	Hemopoietic colony
• CFU-C	Myeloid and monocytic colonies
• CFU-E	Erythroid colonies
• CK	Chemokine
• CSF	Colony stimulating factor
• CSF-1/M-CSF	Colony stimulating factor-1
• CO_2	Carbon dioxide
• Con A	Concanavalin A
• CRBC	Chicken red blood cell
• CTL	Cytotoxic T lymphocyte
• DMEM	Dulbecco's modified Eagles' medium
• DNA	Deoxyribonuclease acid
• dNTP	Dinucleotide triphosphate
• DTH	Delayed-type hypersensitivity
• ELISA	Enzyme-linked immunosorbent assay
• ELISPOT	Enzyme-linked immunospot assay
• FACS	Fluorescence-activated cell sorter
• FCS	Fetal Calf Serum

• FITC	Fluorescein isothiocyanate
• G-CSF	Granulocyte-colony stimulating factor
• GM-CSF	Granulocyte-macrophage colony stimulating factor
• h	Human
• hCMV	human Cytomegalovirus
• H-E	Hematoxylin and Eosin staining
• HBSS	Hanks' balanced salt solution
• HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic
• IFN	Interferon
• IL	Interleukin
• Ig	Immunoglobulin
• ip	Intraperitoneal
• iv	Intravenous
• JAK	Janus-family kinases
• kb	Kilobase pair(s)
• KLH	Keyhole limpet hemocyanin
• LPS	Lipopolysaccharide
• LT	Lymphotoxin
• 2-ME	2-mercaptoethanol
• m	mouse
• mAb	Monoclonal antibody
• MHC	Major histocompatibility complex
• mRNA	Messenger RNA
• MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
• MTX	Methotrexate
• *Nb*	*Nippostrongylus brasiliensis*
• NK cells	Natural killer cells
• NO	Nitric oxide

• OD	Optical density
• PAGE	Polyacrylamide gel electrophoresis
• PBS	Phosphate buffered saline
• PCR	Polymerase chain reaction
• PFA	paraformaldehyde
• r	recombinant
• RNA	Ribonuclease acid
• mRNA	Messenger ribonuclease acid
• RPA	Ribonuclease protection assay
• RT	Reverse transcription
• sc	Subcutaneous
• SCF	Stem cell factor
• SDS	Sodium dodecyl sulfate
• STAT	signal transducers and activators of transcription
• Tc	Cytotoxic T cells
• TCR	T cell receptor
• TE	Tris-EDTA buffer
• TGF	Transforming growth factor
• TFA	Trifluoroacetic acid
• Th	T helper cells
• *T. muris*	*Trichuris muris*
• TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

A. Prologue

The immune system is a remarkable defense mechanism found in the simplest and most primitive forms of metazoa to the most complex and advanced forms of higher vertebrates. As metazoa emerge from their embryos, they are constantly struggling to express their individuality and to survive in their natural environment. To prevent invasion or destruction by parasites or even by members of their own species, metazoa have developed ways to defend themselves against external threats ranging from infections to colonization. Once activated, their effector mechanisms are able to eliminate external threats without damaging themselves. In addition to antigen-nonspecific immune responses such as phagocytosis and inflammation, vertebrates have also developed complex immune systems involving antigen-specific immune responses and specific memory responses.

Antigen-specific immune responses in mammals can generally be divided into cellular immunity and humoral immunity. The former is generally mediated by T lymphocytes and can be transferred only by antigen-specific T cells. This type of immune response is effective in controlling intracellular infections such as viral infections. Humoral immunity, however, is mediated by circulating antibodies (Ab). Protective humoral immunity is transferable to a naive recipient via protective antibodies from an immunized host in the absence of any immune cells. Thus, humoral immunity offers an advantage to the host in clearing

extracellular pathogens such as invading parasites and bacteria. These types of immune responses are often mutually exclusive during strong or chronic infections. Hence, mounting the appropriate immune response plays a critical role in controlling diseases, and extensive studies dissected the levels of regulation in mounting and controlling each immune response.

CD4 T helper (Th) cells are one of the major immune regulatory cells that help mount either cellular or humoral immune responses. For the last ten years, it has been well established that effector $CD4^+$ T helper cells can be divided into at least two subsets based on their functions and cytokine secretion profiles. Upon activation, T helper 1 (Th1) cells produce high levels of Interleukin (IL)-2, interferon (IFN)-γ, and Lymphotoxin (LT). Activated Th2 cells, however, produce high levels of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Nevertheless, other cytokines, including IL-3, TNF-α, GM-CSF, and members of the chemokine families, are produced by both subsets (Mosmann et al., 1986; Cherwinski et al., 1987; Mosmann and Coffman, 1989; Kagi et al., 1994; Minty et al., 1993; McKenzie et al., 1993a). Good correlation between Th1 and Th2 functions and their distinctive cytokines has been demonstrated. Th1 cells are predominantly involved in cell-mediated inflammatory reactions such as delayed-type hypersensitivity reactions (DTH) and IFN-γ is commonly detected at sites of DTH (Yamamura et al., 1991; Tsicopoulos et al., 1992). In contrast, Th2 cytokines are commonly associated with the induction of strong antibody and allergic responses (Mosmann and Coffman, 1989). Interestingly, some of the Th1 and Th2 cytokines are capable of mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. As Th1 or Th2 responses in mice and humans are associated with certain diseases and the outcome of the diseases depends on the proper immune responses, it is apparent that the

cross-regulation by Th1 and Th2 cytokines plays an important role in mediating host resistance and susceptibility to infections (Sher and Coffman, 1992). Thus, it is essential to investigate not only the *in vitro* but also the *in vivo* roles of each cytokine.

Studies reported here focus on the *in vivo* functions of IL-13, the most recently identified cytokine to join the umbrella of the Th2 family. A brief review of various T cell subsets, their cytokine profile and functions, and their physiological roles during immune responses will be included in the first part of the introduction. Then the genes, the physical properties, the biological functions, the receptors, and intracellular signaling events of IL-13 will be summarized.

B. T cell subsets cytokines and functions

Cytokines

Cytokine is a generic term used to describe small regulatory proteins (in the range of 70 to 190 amino acids) secreted by leukocytes and other nucleated cells (Cohen et al., 1979; Arai et al., 1997). Cytokines play important roles in regulating the development of immune effector cells. Like neurotransmitters, cytokines generally exhibit their potent biological functions over short distances in an autocrine or paracrine manner. In certain cases, similar to hormones which are carried by the blood stream throughout the whole body, cytokines can exert their effects over large distances. They can be preformed like neurotransmitters but they are generally synthesized *de novo* upon stimulation. Cytokines bind to specific receptors on the target cell membrane, triggering signal transduction

cascades. In general, the cytokines and their receptors exhibit very high affinity for each other, with the dissociation constants ranging from 10^{-10} to 10^{-12} M. Due to the high affinity, cytokines can mediate biological effects at low concentrations. Cytokines also exhibit pleiotropic, synergistic, and antagonistic effects to regulate cellular activity in a coordinated, interactive way.

The numbers of cytokine families and their members keep on expanding and now include the interleukins, interferons, colony stimulating factors (CSFs), transforming growth factors (TGFs), tumor necrosis factors (TNFs), and chemokines. These molecules are usually transiently secreted by activated cells to recruit other cells or control major physiological processes. Two striking features of this family of molecules are their extensive pleiotropy and their overlapping functions, as each cytokine demonstrates multiple functions and, remarkably, any one function can be mediated by more than one cytokine (Paul, 1989). Furthermore, two cytokines can exhibit synergistic or antagonistic properties, and one cytokine can inhibit or stimulate the synthesis of other cytokines. Taken altogether, cytokines are important regulatory mediators of an immune response.

The action of each released cytokine is mediated through high affinity binding to a multichain surface receptor (or single chain for chemokine receptors) expressed at low numbers (100-1000), resulting in changes in differentiation state, proliferation rate, or expression of cell surface molecules. In contrast to chemokine receptors which consist of several membrane-spanning domains, other cytokine receptors generally consist of two or more chains, each containing single transmembrane domains. In order to exhibit specificity in binding, a multichain surface receptor complex usually consists of at least a

cytokine-specific α subunit (Miyajima et al., 1992). The 150 kDa common β subunit (βc), which is essential for transducing growth signals, is a component of IL-3, IL-5, IL-11, and GM-CSF receptors. The common glycoprotein subunit, gp 130, is the essential component for signal transduction for another group of cytokines including IL-6, leukemia inhibitory factor (LIF), Oncostatin M, and G-CSF. In addition, a common gamma chain (γ_c) is shared by some members of the hemopoietin receptor superfamily including IL-2, IL-4, IL-7, and IL-15 (Kondo et al., 1994; Kondo et al., 1993; Noguchi et al., 1993; Russell et al., 1994; Giri et al., 1994). Upon receptor-mediated activation, Janus-family kinases (JAKs), which are associated with receptor chains, phosphorylate the STAT (signal transducers and activators of transcription) family members. For example, the IL-2 receptor is a multisubunit complex composed of IL-2Rα, IL-2β, and γ_c chains, whereas the IL-4 receptor complex consists of IL-4Rα and γ_c chains. The IL-2Rβ chain is associated with JAK1, while γ_c is found with JAK3 (Kondo et al., 1994; Kondo et al., 1993; Noguchi et al., 1993; Russell et al., 1994). On the other hand, the IL-4Rα chain is associated with JAK1 (Johnston et al., 1994; Darnell et al., 1994). IL-2 activates STAT5, whereas IL-4 induces the activation of STAT6 (Hou et al., 1994; Quelle et al., 1995). Subsequently, activated STAT factors dimerize and migrate to the nucleus where they bind to DNA motifs and induce transcription of cytokine response genes (Ivashkiv, 1995). The activation of these pathways leads to the transcription of different genes, resulting in the induction of distinctive biological activities. By utilizing different combinations of the receptor chains, cytokine specificity and down-stream signaling cascades involving JAK and STAT family members can be achieved.

Th1 and Th2 cells

CD4 T helper cells are essential for regulating all types of immune responses. Most of their immune regulation functions are mediated by an array of cytokines, which are critically involved in cell growth, inflammation, immunity, differentiation, and repair. These cytokines are not constitutively expressed but produced after T-cell activation via recognition of antigen presented by MHC on antigen presenting cells in the presence of costimulatory molecules. After activation, different cytokines exhibit different production kinetics lasting from a few hours to a few days. The importance of T-cell-derived cytokines in regulating *in vivo* immune responses is strongly supported by the observation that cyclosporin A, a potent immununosuppressive pharmacological agent used in various clinical situations such as transplantation, mediates its function primarily by inhibiting transcription of the cytokine genes.

The study of T-cell-derived cytokines has now spanned several decades and is well established. As a result of the analysis, cytokine profiles produced by various T cell clones, mouse $CD4^+$ T helper cells were originally categorized into at least two polar subtypes (Cherwinski et al., 1987; Mosmann et al., 1986). Similar observations were later reported among human T cells (Del Prete et al., 1991; Romagnani, 1991b; Romagnani, 1991a). However, the production of IL-2, IL-6, IL-10, and IL-13 by human T cells is not as restricted as their mouse counterparts.

Good correlation between their functions and their cytokine secretion profile has also been established for both Th1 and Th2 cells. The former are predominantly involved in cellular immune responses such as delayed type hypersensitivity

reactions, whereas the latter can efficiently induce allergic and humoral immune responses such as antibody production, particularly IgE responses, and also enhance eosinophil proliferation and function (Mosmann and Coffman, 1989). As the functions of Th1 and Th2 are generally mutually exclusive, it not surprising that the cytokines produced by each subset can be mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. For example, IFN-γ, a Th1 cytokine, can inhibit the differentiation and proliferation of Th2 cells (Mosmann and Coffman, 1989), and IL-10, a Th2 cytokine, inhibits cytokine synthesis and functions of Th1 cells (Fiorentino et al., 1991; Fiorentino et al., 1989). This cross-regulation may explain the strong biases in Th1 or Th2 phenotype during strong immune responses during numerous infections in humans and mice (Mosmann and Sad, 1996a). Consistent with this idea, manipulating the cytokine patterns using exogenous cytokines or anti-cytokine antibodies alters the susceptibility or resistance to infection (Sher and Coffman, 1992; Finkelman et al., 1997). Th2 immune response plays an important role in the expulsion of gastrointestinal nematode *Trichuris muris* infection. Treatment with a neutralizing anti-IFN-γ mAb leads to rapid expulsion of *T. muris* larvae (Else et al., 1994). Conversely, IL-12 or anti-IL-4 receptor mAb treatment in normally resistant mice increases host susceptibility to the parasites (Finkelman et al., 1997). Thus, the dichotomy of the immunoregulatory functions of Th1 and Th2 cells appears to play an important role in mediating resistance or susceptibility to infections.

Although the two major $CD4^+$ helper cell subsets have been well documented based on their cytokine secretion profiles, not all $CD4^+$ helper cells are encompassed within the Th1 and Th2 designations (Mosmann and Sad, 1996a). Other cytokine subsets have been identified both *in vitro* and *in vivo*. For

instance, T cells expressing both cytokine patterns are called Th0 (Mosmann and Coffman, 1989), and T cells expressing high amounts of TGF-β have been called Th3 (Chen et al., 1994). Based on single cell cytokine analyses, there appears to be heterogeneity within the Th1 and Th2 subsets. IL-4 and IL-5 are usually co-expressed, IL-4 and IFN-γ are mutually exclusive, but IFN-γ and IL-10 can be detected in some cells (Assenmacher et al., 1994; Elson et al., 1995; Bucy et al., 1995; Openshaw et al., 1995). Thus, it has been hotly debated whether T helper subsets can be categorized into simple Th1 and Th2 subsets or to the other extreme of non-discrete subsets with a continuum of different combinations of cytokine production where Th1 and Th2 cytokines are randomly distributed (Mosmann and Sad, 1996b). Although patterns of single-cell cytokine expression indicate that there may be more than the polar Th1/Th2 pattern, cytokine expression is argued to be nonrandom (Assenmacher et al., 1994; Elson et al., 1995; Bucy et al., 1995; Openshaw et al., 1995). In addition, allospecific clones from unimmunized mice or mice infected with *Nippostrongylus brasiliensis (Nb)* show strong positive correlation between IL-4 and IL-5 synthesis but an inverse correlation of IFN-γ and IL-4 synthesis (Street et al., 1990).

Despite the extensive studies on the mechanisms governing the differentiation of Th1 or Th2 cells, the mystery is still not fully resolved. Upon stimulation by antigen on antigen-presenting cells, naive $CD4^+$ T cells, which are called T helper precursor (Thp) cells, produce only IL-2 (Seder and Paul, 1994). These cells can then differentiate into other phenotypes that produce various cytokines. It appears that the pathway leading to Th1 and Th2 may proceed through an intermediate stage expressing multiple cytokines. Transgenic mice carrying a herpes-virus thymidine kinase gene under the control of the IL-4 promoter were

generated, and IL-4 promoter activity was detected during the differentiation to Th1 or Th2 phenotypes, suggesting that during their differentiation pathways both cell types expressed IL-4 (Kamogawa et al., 1993).

The factors that influence the differentiation of Th1 or Th2 cells both *in vitro* and *in vivo* are currently under intense investigation. Cytokines play a major role in regulating the early differentiation of effector cells. IFN-γ, IL-12, and TGF-β are involved in enhancing Th1 development, whereas IL-4, produced by mast cells, basophils, or certain NK1.1$^+$ T cells, stimulates the differentiation or growth of Th2 cells (Seder and Paul, 1994). In addition, costimulatory molecules such as B7.1 and B7.2 molecules on antigen presenting cells may selectively influence the choice of differentiation to Th1 or Th2 (Thompson, 1995). The genetics of the host, hormone environments (Seder and Paul, 1994) and certain products of pathogens such as *Listeria* (Hsieh et al., 1993b; Hsieh et al., 1993a) can control the differentiation of Th1 or Th2 cells. Last but not least, antigen dose is a major regulator of the choice of effector functions. Both high and low levels of certain antigens induce a DTH immune response, whereas moderate levels of antigens stimulate an antibody response (Parish, 1972; Bretscher et al., 1992). Thus, the crucial choice for the Th1 or Th2 is determined and regulated at multiple molecular and cellular levels. Mounting the correct immune response will definitely be advantageous for the host to combat sophisticated and rapidly evolving pathogens.

The roles of Th1 and Th2 cytokines

Upon stimulation, both subsets of T helper cells produce large amounts of distinct cytokines which often correlate with their functions. Th1 cytokines are

efficient in activating cytotoxic cells so that a cell-mediated immune response can be mounted. IL-2 is a major growth factor for CD4 and CD8 T cells, especially in the expansion of tumor specific cytolytic T cells (Smith, 1984; Erard et al., 1985; Rosenberg and Lotze, 1986). IL-2 is also the most important B cell helper factor made by Th1 cells (Coffman et al., 1988) as it can enhance Ig production by activated human and mouse B cells (Miyawaki et al., 1987; Callard and Smith, 1988; Kishi et al., 1985). A high dose of IL-2 stimulates cytokine production, proliferation, and cytolytic functions of NK cells (Ortaldo et al., 1984; Trinchieri et al., 1984) and other cytotoxic cells, which has led to the use of IL-2 in immunotherapy of cancer and infectious diseases (Grimm et al., 1982; Mazumder and Rosenberg, 1984).

Due to its role as a potent activator for macrophages (Le et al., 1983; Nathan et al., 1983) and neutrophils (Stevenhagen and van Furth, 1993), IFN-γ plays an important role in the non-antigen-specific effector functions of cellular immunity such as intracellular (viral and parasite) infections or delayed-type-hypersensitivity responses. IFN-γ activates macrophages to produce TNF-α and reactive oxygen intermediates which aid in tumoricidal and antimicrobial activities (Philip and Epstein, 1986). When macrophages are treated with IFN-γ, both MHC class I and class II are upregulated. With higher expression levels of both class I and II, these activated macrophages can process and present antigen more efficiently to T cells (Sztein et al., 1984; Kelley and Pitha, 1985). IFN-γ can also upregulate MHC class I on virus-infected cells and enhance cytotoxicity of CD8 T cells (Blackman and Morris, 1985; Bukowski and Welsh, 1985; Simon et al., 1986b; Simon et al., 1986a). Although Th2 cells are generally more potent in providing help for B cells, Th1 cells which produce IFN-γ have been shown to induce high levels of IgG2a that can kill target cells

by complement lysis (Stevens et al., 1988; Boom et al., 1988). Fc receptors for IgG2a antibodies on macrophages are also induced by IFN-γ (Warren and Vogel, 1985) and macrophages can then bind to the increased IgG2a levels produced in response to IFN-γ, leading to increased antibody-dependent macrophage cytotoxicity. Both LT and IFN-γ activate macrophages, resulting in increased killing of intracellular parasites (Nathan et al., 1983) and tumor cells (Murray et al., 1985; Pace et al., 1983; Lee et al., 1984). In short, Th1 cytokines provide optimal stimulations for macrophages, granulocytes, CTL, and NK cells, all of which are appropriate for dealing with infections that require cellular immunity.

Similar to Th1, Th2 cells produce many cytokines. However, Th2 cells secrete cytokines that enhance Ab production in B cells and induce proliferation and activation of eosinophils and mast cells. In addition to inducing proliferation and clonal expansion of B cells (Alderson et al., 1987), IL-4 also enhances the cognate T-B cell interaction (Stein et al., 1986) by upregulating the expression of MHC class II (Noelle et al., 1984; Rousset et al., 1988; Roehm et al., 1984a), CD40 (Gordon et al., 1988; Valle et al., 1989; Banchereau and Rousset, 1991; Moore et al., 1991), IgE Fcε receptors (Hudak et al., 1987; Kikutani et al., 1986; Defrance et al., 1987), and B7 (Valle et al., 1991) on B cells. IL-4 plays a major role in the production of Ig, mainly IgG1 (IgG4 in humans) and IgE (Vitetta et al., 1985; Rothman et al., 1988; Gauchat et al., 1992b; Gauchat et al., 1992a) both of which are involved in humoral and allergic immune responses. Another Th2 cytokine, IL-5, which stimulates proliferation of eosinophils during parasitic infections (Sanderson et al., 1986), is also another growth factor for activated mouse B cells (Swain et al., 1988; Coffman et al., 1988; Rasmussen et al., 1988; Herron et al., 1988). IL-5 mainly supports late stage mouse B cell differentiation and leads to the proliferation and synthesis of all antibody isotypes *in vitro* but it

appears to act as a growth factor for B1 cells *in vivo* (Bond et al., 1987; Yokota et al., 1987; Lebman and Coffman, 1988). IL-5 synergizes with IL-4 or TGF-β to promote IgE secretion (Coffman et al., 1987) or IgA production (Lebman et al., 1990a; Lebman et al., 1990b), respectively. IL-6 appears to stimulate late-stage B-cell maturation (Hirano et al., 1986; Yamasaki et al., 1988) and antibody production (Splawski et al., 1990). In the presence of TGF-β, IL-10 induces IgA production in anti-CD40-activated human B cells (Rousset et al., 1992). Similar to IL-4, human IL-13 demonstrates IgG4 and IgE class switching effects on human B cells (Punnonen et al., 1993).

In addition to providing B cell help, Th2 cytokines also play major roles in the differentiation, proliferation, and chemotaxis of mast cells and eosinophils (Lee et al., 1989). In the presence of IL-3 and IL-4, human and mouse hemopoietic progenitor cells differentiate to mast cells (Favre et al., 1990; Rennick et al., 1987b). IL-4 (Hamaguchi et al., 1987; Tsuji et al., 1990) and IL-10 (Thompson Snipes et al., 1991) stimulate proliferation of mast cell lines. IL-5 selectively promotes *in vivo* and *in vitro* differentiation of eosinophils from hemopoietic progenitor cells (Sanderson, 1992; Dent et al., 1990). In addition, IL-5 activates human eosinophils to express integrin receptors which may enhance the extravasation of these cells to inflamed sites (Lopez et al., 1986; Walsh et al., 1990). Both IL-4 and IL-5 can be chemotactic for eosinophils (Tepper et al., 1989; Tepper et al., 1992; Yamaguchi et al., 1988b; Yamaguchi et al., 1988a; Wang et al., 1989). In summary, Th2 cytokines secrete a large panel of cytokines to orchestrate humoral and allergic immune responses via different mechanisms.

In vivo functions of Th1 or Th2 cells and their cytokines during immune responses

Based on the *in vitro* observations that Th1 and Th2 cells mediate their biological functions through their cytokines, subsequent studies further extended the notion that cytokines produced by both phenotypes strongly influenced the host immune response and the outcome of the diseases. Th1 cells are often involved in cell-mediated inflammatory reactions, and they are efficient in clearing intracellular pathogens. Antigen specific Th1 cells can be isolated from individuals who have mounted cell-mediated immune responses (Heinzel et al., 1991; Del Prete et al., 1994; Del Prete et al., 1991; Haanen et al., 1991). In addition, Th1 cytokines (IL-2 and IFN-γ) can be detected at the site of DTH (Tsicopoulos et al., 1992; Yamamura et al., 1991). Whereas Th1 cells are not efficient in clearing extracellular pathogens and their secreted products in certain infections, Th2 are more effective in generating Ab responses to neutralize free pathogens and their secreted products (Else et al., 1994; Finkelman et al., 1994; Urban et al., 1995). Similarly, Th2 cells or their cytokines are often present at the sites of allergic responses (Del Prete et al., 1991; Kay et al., 1991; Van Reijsen et al., 1992; Robinson et al., 1992; Robinson et al., 1993). Thus, the generation of either a Th1 or Th2 immune response proves to be crucial in establishing either protective or deleterious immune response to the host in certain diseases.

The importance of immune regulation of Th1 and Th2 cells in disease progression can be supported by several lines of evidence in various disease models. *Leishmania major* infection in mice clearly demonstrates the crucial role of mounting a dominant cell-mediated immune response to cure the disease. It

appears that the genetic background of the mice plays an important role in mounting the appropriate immune response (Scott et al., 1988). Resistant C57BL/6 mice infected with *L. major* develop a strong DTH reaction and their macrophages are activated to effectively localize the infection. Ag-specific T cells from these mice produce high levels of IFN-γ but low levels of IL-4, suggesting a strong Th1 response (Locksley and Louis, 1992). In contrast, susceptible BALB/c mice mount a Th2 response consisting of very high levels of Ab in the serum, and their T cells express high levels of IL-4 but low levels of IFN-γ. These mice fail to contain the disease and eventually develop severe and progressive disease, which proves to be fatal (Locksley and Louis, 1992). Consistent with this, anti-IL-4 mAb treatment at the time of infection decreases Ab responses and provides protection to susceptible mice (Sadick et al., 1990). T cell clones derived from *L. major*-infected BALB/c mice treated with anti-IL-4 Ab preferentially produce IFN-γ (Heinzel et al., 1989). Conversely, anti-IFN-γ Ab treatment renders resistant mouse strains susceptible by inhibiting cell-mediated immune responses (Belosevic et al., 1989). Adoptive transfer experiments further support the regulatory functions of Th1 and Th2 on disease progression. When *L. major* specific Th1 or Th2 cell lines were adoptively transferred into *L. major*-infected mice, Th1 cells mounted a DTH response and provided protection for subsequent infection, but Th2 cells exacerbated the course of the disease (Scott et al., 1988; Holaday et al., 1991).

In contrast to *L. major* infection which requires Th1 dominated cell-mediated immunity, an intestinal Helminth infection is associated with IL-4-dominated responses to clear the worms (Locksley, 1994). When infected with *T. muris*, BALB/k mice expel adult worms and acquire immunity to subsequent challenge. However, AKR mice mount an inappropriate immune response and maintain a

chronic infection as they fail to expel the worms in the intestine (Else et al., 1994). Mesenteric lymph node cells exhibit a strong Th2 or Th1 cytokine profile in the resistant or susceptible mice, respectively (Finkelman et al., 1991). Resistant mice mount a Th2 response by inducing eosinophilia, intestinal mastocytosis, and IgE production to clear the helminth infection. Resistance or susceptibility in these mice can be manipulated using anti-IL-4 or anti-IFN-γ mAb to neutralize the appropriate cytokine (Urban et al., 1991; Urban et al., 1993; Finkelman et al., 1991).

Similar correlations of Th1 or Th2 cells with different disease outcomes can be observed in human systems. The Th1 phenotype is associated with contact dermatitis, autoimmunity and chronic inflammatory diseases. At the site of a tuberculin reaction or nickel-induced contact hypersensitivity, high levels of IL-2 and IFN-γ are detected (Tsicopoulos et al., 1992; Romagnani, 1991a; Kapsenberg et al., 1992). In *M. leprae* infection, IL-2 and IFN-γ are expressed in tuberculoid leprosy lesions, and the disease is self-limiting. However, when the individual has mounted a Th2 response with IL-4, IL-5, and IL-10 expressed in lepromatous lesions, a high antibody titer is detected, and eventually this leads to disseminated infection of the bone and cartilage with extensive nerve damage (Yamamura et al., 1991). T cell clones from skin lesions in patients suffering from the autoimmunity disease psoriasis vulgaris, synovial tissue from rheumatoid arthritis patients, or T cell clones from multiple sclerosis patients, produce high levels of IFN-γ, indicating a bias towards Th1 immune responses (Schlaak et al., 1994; Saed et al., 1994; Simon et al., 1994; Voskuhl et al., 1993). In contrast, Th2 cells are associated with allergic responses against common environmental allergens and allergic atopic disorders (Romagnani, 1994). Allergen-specific T clones from allergic patients show a strong bias towards the Th2 phenotype, and

IL-4 and IL-5, but not IFN-γ, can be detected in late-phase cutaneous reactions (Del Prete et al., 1991; Kay et al., 1991; van der Heijden et al., 1991; Van Reijsen et al., 1992). Eosinophilia and Th2 cells producing IL-4 and IL-5 can be detected in the bronchial lavage from atopic asthma patients (Robinson et al., 1992; Robinson et al., 1993; Robinson et al., 1993). In summary, the dichotomy of Th1 and Th2 cytokines in immunoregulation has been demonstrated in human and various animal models, and the resolution or the progression of the diseases largely depends on mounting the appropriate immune response.

C. Physical properties of Interleukin 13 (IL-13)

Isolation of IL-13 cDNA

Mouse IL-13 (mIL-13) cDNA, originally known as P600, was initially cloned from induction-specific mRNA derived from a Con A-activated mouse helper Th2 line, C1.Ly1^{+}2^{-}/9 (Brown et al., 1989). It is named P600 due to the presence of a 600 bp Pst1 restriction fragment in the cDNA. The mIL-13 mRNA is a relatively abundant (up to 0.3% of poly-A mRNA) gene transcript expressed by Th2 cells in response to stimulation (Cherwinski et al., 1987; Brown et al., 1989).

Subsequently, human cDNA homologous to P600 was cloned by three independent groups (Minty et al., 1993; McKenzie et al., 1993a; Morgan et al., 1992). A partial human IL-13 (hIL-13) cDNA clone was initially identified by cross hybridization of a Th0 CD4^{+} T-cell clone, B21. A full length hIL-13 cDNA clone was isolated after Con A activation of the A10 CD8^{+} T-cell clone (McKenzie et al., 1993a). Another group cloned hIL-13 cDNA through differential screening of a cDNA library from activated human T lymphocytes

(Minty et al., 1993). Lastly, a partial hIL-13 cDNA was obtained through hybridization of an activated peripheral blood lymphocyte (PBL) cDNA library (Morgan et al., 1992).

Using RT-PCR, a cDNA clone of rat IL-13 was isolated from renal cortex RNA following the induction of anti-glomerular basement membrane antibody-induced glomerulonephritis. The coding region of rat IL-13 cDNA reveals 74% and 87% sequence identity with the coding regions of hIL-13 and mIL-13 cDNA, respectively (Lakkis and Cruet, 1993).

Chromosomal location and structure of IL-13

Both human and mouse IL-13 genes contain 4 exons and 3 introns, occupying about 4.5-kb and mapping to human chromosome 5q23-31 or mouse chromosome 11, the latter of which has known conserved synteny with human 5q and 17p (McKenzie et al., 1993b; Smirnov et al., 1995; Dolganov et al., 1996). Exon 4 of the hIL-13 gene has an alternate 3'-splice/acceptor site at its 5' end which determines the presence or absence of the mRNA nucleotides encoding the Gln98 residue at this position (McKenzie et al., 1993b). In mIL-13, the 5'-untranslated region and the first 47 N-terminal amino acid residues are contained in exon 1 (up to position 934). 18 and 35 amino acid residues are encoded by exon 2 (position 2193-2245) and exon 3 (position 2823-2926), respectively. Lastly, exon 4 (position 3240-4073) encodes the C-terminal 31 amino acid residues and the 3'-untranslated region (McKenzie et al., 1993b).

Both human and mouse IL-13 genes consist of four exons and three introns and, thus, share a common intron-exon structure with IL-4, IL-5, and GM-CSF genes

(McKenzie et al., 1993b). Circular dicroism (CD) analysis predicts that IL-13 belongs to the same family as IL-2, IL-4, IL-5, GM-CSF, M-CSF, and growth hormone, all of which contain a four anti-parallel α-helical core (Zurawski and de Vries, 1994). In addition, IL-13 is mapped in close proximity to a gene cluster for hemopoietic and immunoregulatory growth factors which include IL-3, IL-4, IL-5, and GM-CSF, suggesting that they may have evolved by gene duplication events and that this linkage may provide a mechanism for controlling the coordinate expression of these genes (McKenzie et al., 1993b; Morgan et al., 1992; Zurawski and de Vries, 1994). The hIL-13 gene is 12 kb 5' to the IL-4 gene in a tail-to-head orientation (Smirnov et al., 1995; Dolganov et al., 1996). Despite low homology between IL-4 and IL-13 protein sequences (~30%) (Minty et al., 1993; Zurawski et al., 1993a), they exhibit overlapping biological properties.

Expression of IL-13 mRNA and protein

mIL-13 and hIL-13 are produced by activated Th2 cells (Brown et al., 1989; Minty et al., 1993; Ihle et al., 1983). In contrast to mouse T cells, activated human Th0, Th1, and $CD8^+$ T-cell clones also synthesize hIL-13 (Zurawski and de Vries, 1994), suggesting an important role for IL-13 during immune responses. Resting mast cells express IL-13 mRNA but only begin to secrete the protein upon activation (Burd et al., 1995). Activated Epstein-Barr virus-immortalized B cell lines or malignant B cell lines express IL-13 mRNA (de Waal Malefyt et al., 1995; Kindler et al., 1995; Fior et al., 1994) and secrete small amounts of IL-13 protein (de Waal Malefyt et al., 1995). hIL-13 has not been detected in normal human tissues including the heart, brain, placenta, lung, liver, and skeletal muscle.

Significant levels of the 1.3 kb hIL-13 mRNA synthesis or protein production can be readily detected 1 h or 2 h post-activation of T cells, respectively. hIL-13 mRNA expression levels peak 2 h after activation, and steady state levels of hIL-13 mRNA can be detected after 72 h. In contrast to the early yet sustained hIL-13 mRNA expression following activation, hIL-4 mRNA expression in T cells is transient and can only be detected 24 h after activation (Zurawski and de Vries, 1994; Jung et al., 1996). Thus, hIL-13 appears to be produced by activated T cells early and over prolonged time periods.

mIL-13 cDNA with an open reading frame that commences at the first available ATG codon encodes a 131 residue protein (approximately 14 kDa) containing an ~20 amino acid leader peptide, a hydrophobic N-terminus, and four cysteine residues. One potential *N*-linked and two potential *O*-linked glycosylation sites are also predicted for this protein (Brown et al., 1989). Human IL-13 (hIL-13) cDNA encodes 132 amino acids and is 66% homologous to mIL-13 (McKenzie et al., 1993a; Minty et al., 1993). Interestingly, there are different forms of hIL-13 with a Gly—›Asp substitution at position 61 (Minty et al., 1993) or with an additional Gln residue at position 98 resulting from alternative splicing at an intron-exon boundary (McKenzie et al., 1993a; McKenzie et al., 1993b). Gly at position 61 is the prevalent IL-13 mRNA, whereas Asp at position 61 appears to be either a rare allelic variant or an error during cDNA construction of the cDNA library (Minty et al., 1993). No alternative splicing has been identified in the mouse gene (McKenzie et al., 1993b). Although all three forms of hIL-13 are biologically active, the significance of these differences remains to be determined. Both hIL-13 and mIL-13 have 58% amino acid sequence identity over the coding region. Rat IL-13 displays 63% and 79% homology with its human and mouse counterparts respectively (Lakkis and Cruet, 1993).

Recombinant hIL-13 or mIL-13 proteins produced by *E. coli*, *S. cerevisiae*, COS 7 monkey kidney, BW5147 mouse thymoma, or CHO cells have been used to characterize various biological activities, including their effects on cell lines (Minty et al., 1993; McKenzie et al., 1993a; Lai et al., 1996). hIL-13 is secreted as a ~10 kDa mostly unglycosylated protein (McKenzie et al., 1993a), whereas mIL-13 is a ~14 kDa closely spaced doublet, possibly due to differences in protein glycosylation (Brown et al., 1989; Lai et al., 1996). Western blotting of rat IL-13 produced by the baculovirus system reveals a ~12 kDa secreted protein (Lakkis and Cruet, 1993).

Recombinant forms of hIL-13 (with or without Gln98), mIL-13, and rat IL-13 have similar specific activities on the human premyeloid cell line TF-1 (McKenzie et al., 1993a; Lakkis and Cruet, 1993). However, hIL-13 has a ~100-fold lower specific activity than mIL-13 on the mouse plasmocytoma cell line B9 (Zurawski and de Vries, 1994), indicating that hIL-13 and mIL-13 can equally stimulate human cells, but mIL-13 has higher affinity for the mouse IL-13R.

D. Biological functions of IL-13

IL-13's effects on monocyte/macrophage surface markers

IL-13 exhibits profound pleiotropic effects on human peripheral blood monocytes and mouse GM-CSF-derived bone marrow macrophages (Fig. 1.1). In particular, IL-13, in common with IL-4, induces morphological and surface antigen changes in human monocytes and mouse GM-CSF-derived bone marrow macrophages. In the presence of IL-13, human monocytes adhere strongly to the culture plates,

form homotypic aggregates and syncytia, and develop extensive processes and lamellar sheets (McKenzie et al., 1993a; DeFife et al., 1997). IL-13 prolongs the survival of human monocytes, as viable cells can be detected for up to 6 weeks (McKenzie et al., 1993a), which is consistent with the effects of IL-4 on monocytes. Furthermore, mIL-13 induces a macrophage-like population of cells from mouse bone marrow (J. -M. Heslan, L. J. Guilbert, and T. R. Mosmann, unpublished). In common with IL-4, IL-13 upregulates cell surface markers on human monocytes including MHC class II complex antigens, CD11b, CD11c, CD13, CD18, CD23 (FcεRII), CD29, and CD49e (VLA-5), but downregulates CD14, CD16 (FcγRIII), CD32 (FcγRII), and CD64 (FcγRI) (de Waal Malefyt et al., 1993; Viale and Vercelli, 1995; Cosentino et al., 1995).

IL-13 modulates monocyte/macrophage proinflammatory functions

The production of inflammatory cytokines (such as IL-1, IL-6, and TNF-α) is a crucial initiating event in a number of infectious and inflammatory pathologies. IL-13 inhibits the production of proinflammatory cytokines, chemokines, hemopoietic growth factors, and nitric oxides in activated human monocytes (Minty et al., 1993; McKenzie et al., 1993a; de Waal Malefyt et al., 1993; Doherty et al., 1993), while enhancing the secretion of IL-1 receptor antagonist (IL-1ra) and the production of the type II IL-1 receptor that is an endogenous decoy for bioactive IL-1 (Minty et al., 1993; de Waal Malefyt et al., 1993; Muzio et al., 1994; Vannier et al., 1996; Colotta et al., 1994; Muzio et al., 1994; Cash et al., 1994). Hence, IL-13 shares most of its activities on human monocytes (Fig. 1.1) with IL-4. In particular, IL-13 inhibits the production of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, MIP-1α, TNF-α, GM-CSF, and G-CSF by LPS-activated macrophages. Treatment of IFN-γ- or LPS-activated mouse

macrophages with IL-13 reduces the production of nitric oxide and monokines while sparing the phagocytic function and anti-microbial capacity (Doherty et al., 1993; Doyle et al., 1994; Yano et al., 1996). Unexpectedly, IL-4 or IL-13 pretreatment of human peripheral blood mononuclear cells (PBMC) or LPS-treated mouse peritoneal macrophages enhances the accumulation of inflammatory cytokines such as IL-6, IL-12 and TNF-α, while coculturing the above cells with LPS and IL-4 or IL-13 has reverse effects, revealing the complexity of the regulatory role of IL-4 and IL-13 in the immune response (D'Andrea et al., 1995; Kambayashi et al., 1996). Similar to IL-4 and IL-10, IL-13 is a potent down-modulator of the production of macrophage proinflammatory cytokines *in vitro*. The inhibitory role of IL-13 is supported by *in vivo* findings that IL-13 provides protection in LPS-induced lethal endotoxemia, an observation which is correlated with the downregulation of the production of proinflammatory mediators (TNF-α, IFN-γ, IL-1β, and IL-12) (Di Santo et al., 1997). It is also consistent with the observations that Th2 cytokines such as IL-4, IL-10, and IL-13 are effective in selectively inhibiting macrophage activities associated with inflammatory responses, while Th1-derived cytokines such as IFN-γ are strong stimulators of macrophage activation. Thus, IL-13 can be added to the list of cytokine immunomodulators that may be beneficial for septic shock treatment.

Activated macrophages produce many cytokines including IL-10, which acts in an autocrine manner to suppress cytokine production. IL-4 and IL-13 suppress cytokine production by activated macrophages independently of IL-10 as the modulating effects of IL-13 or IL-4 on monocytes are observed in the presence of neutralizing anti-IL-10 mAbs. In addition, IL-13 also suppresses proinflammatory mediator production by activated macrophages in the presence

of anti-IL-4, indicating that IL-13 acts independently of IL-4 and IL-10 (de Waal Malefyt et al., 1991).

In macrophages, nitric oxide (NO) plays a critical role in the killing of intracellular parasites. Both IL-4 and IL-10 are potent inhibitors of NO production in macrophages, and IL-13 demonstrates similar effects. The reduction of NO release correlates with the increased *in vitro* survival of *Leishmania major*, an intracellular parasite, in GM-CSF-derived mouse macrophages (Doherty et al., 1993). Hence, IL-13 decreases the cytotoxic potential of macrophages in parasiticidal activity.

Similar to IL-4, IL-13 inhibits the antibody-dependent cellular cytotoxicity (ADCC) activity of human monocytes (de Waal Malefyt et al., 1993). In particular, IL-13 significantly inhibits the enhancing effects of IFN-γ on ADCC activity (de Waal Malefyt et al., 1993). On the other hand, like IL-4, IL-13 does not appear to be inhibitory to all macrophage functions as the phagocytic function of activated macrophages is not affected by IL-13 (Doherty et al., 1993). IL-13 upregulates the capacity of monocytes to present antigens to T cells and T cell clones (Zurawski and de Vries, 1994) and even enhances anti-listerial resistance in mice (Flesch et al., 1997). Moreover, IL-4 and IL-13 appear to be chemotactic factors for human monocytes (Magazin et al., 1994). Consequently, both IL-4 and IL-13 do not appear to be general monocyte deactivators but rather exhibit inhibitory functions on selective macrophage functions.

IL-13 mechanism of regulation in monocytes/macrophages

The Lsk (leukocyte COOH-terminal Src-kinase) and Csk (COOH-terminal Src kinase) genes encode structurally related nonreceptor tyrosine kinases (Chow et al., 1994) which are believed to negatively regulate the enzymatic activity of Src-family members involved in leukocyte activation (Nada et al., 1991; McVicar et al., 1994). Interestingly, Lsk is not constitutively expressed in untreated human monocytes, but is induced by IL-4 or IL-13 treatment. IFN-γ, a potent monocyte activator, inhibits IL-4- or IL-13-induced Lsk upregulation. In contrast, Csk is constitutively expressed in human monocytes, refractory to IL-4 or IL-13 treatment, and upregulated slightly by IFN-γ (Musso et al., 1994). These data suggest that expression of Lsk could preferentially be involved in phosphorylating the negative regulatory sites on certain Src-family kinases in monocytes in the presence of a Th2 immune response.

Effects of IL-13 on viral replication in monocytes

Th2-derived cytokines appear to have anti-HIV-1 activities in cells of the macrophage lineage. IL-13 can act on macrophages 72 h before or 18 h after HIV-1 infection by blocking the completion of reverse transcription, reducing viral protein (p24) production, and decreasing the infectivity of the progeny virions (Montaner et al., 1993; Montaner et al., 1997; Mikovits et al., 1994). However, the proviral DNA remained detectable, indicating IL-13 does not eradicate HIV-1. Although continuous IL-13 treatment on monocytoid cell lines increases HIV production, IL-13 does not stimulate HIV expression in latently infected cells (Mikovits et al., 1994). The role of IL-4 on HIV replication in monocytes remains to be elucidated, as inhibitory (Schuitemaker et al., 1992) and

enhancing (Kazazi et al., 1992; Mikovits et al., 1994) effects have been reported. The state of maturation of monocytes into macrophages appears to influence the enhancing or inhibiting effects of both IL-4 and IL-13 on HIV replication (Naif et al., 1997). In contrast to its effect on macrophages, IL-13 does not appear to affect the replication of HIV-1 in cultures of phytohemagglutinin-stimulated T cells, consistent with the observations that IL-13 does not exhibit any detectable biological functions on T cells (Zurawski et al., 1993a; Sornasse et al., 1996).

IL-13 enhances the expression of cytomegalovirus in otherwise latently infected human alveolar macrophages. On the other hand, IL-13 strongly inhibits HIV-1 production in alveolar macrophages that are infected with HIV-1 alone (Denis and Ghadirian, 1994) or coinfected with cytomegalovirus (Hatch et al., 1997). Taken together, these data emphasize the potential clinical applications of IL-13 in HIV infections.

Roles of IL-13 in hemopoiesis

Recently, IL-13 and IL-4 were shown to have overlapping but different functions in the regulation of proliferation and differentiation of primitive mouse hemopoietic progenitor cells (Jacobsen et al., 1994) (Fig. 1.1). IL-13 synergizes with stem cell factor (SCF) to stimulate proliferation and colony formation of $Lin^-Sca\text{-}1^+$ bone marrow progenitor cells more efficiently than IL-4. However, IL-13 does not exhibit any synergistic effects with SCF on the proliferation of the more mature $Lin^-Sca\text{-}1^-$ progenitor cells as the cloning frequency in response to SCF and IL-13 was at least 20-fold higher in the $Lin^-Sca\text{-}1^+$ population. While both IL-13 and IL-4 synergistically enhanced colony formation of $Lin^-Sca\text{-}1^+$ progenitors in response to G-CSF, only IL-13 synergizes with GM-CSF to

increase colony formation frequencies of similar progenitor cells. In contrast to SCF + G-CSF cultures of $Lin^{-}Sca\text{-}1^{+}$ cells which result in the formation of 90% granulocytes, the addition of IL-13 to SCF + G-CSF cultures results in the exclusive differentiation of macrophages (Jacobsen et al., 1994). Interestingly, both IL-13 and IL-4 synergistically suppress the proliferation of the late stage of committed macrophage progenitors because IL-13 and/or IL-4 inhibits the proliferation of macrophage colonies that are formed in the presence of IL-3, GM-CSF, M-CSF, and erythropoietin (Sakamoto et al., 1995). Thus, it appears that IL-4 and IL-13 have some overlapping but unique biological activities on myelopoiesis.

In addition, IL-13 exerts a stimulatory effect on the growth of megakaryocyte progenitor cells obtained from non-adherent mononuclear cells or highly enriched $CD34^{+}$ cells of human cord blood (Xi et al., 1995). These reports of IL-13's ability to stimulate early hemopoietic precursors *in vitro* are consistent with the data presented in Chapter V of this thesis in which it is shown that IL-13 administration *in vivo* also induces extramedullary hemopoiesis in mice, including increased megakaryocytes in the spleen and monocytes in the peripheral blood (Lai et al., 1996).

Activation of human B cells by IL-13

Human IL-13 and IL-4 have similar effects on human B cells, although IL-4 is generally more potent. IL-13 promotes growth of activated human B cells; modulates their surface antigens (Punnonen et al., 1993; McKenzie et al., 1993a; Minty et al., 1993); and induces class switching to IgG4 and IgE synthesis in human B cells (Fig. 1.1) in combination with activated T cells, anti-Ig, anti-CD40,

or CD40L antibodies (Punnonen et al., 1993; Defrance et al., 1994; Cocks et al., 1993).

Modulation effects of IL-13 on B cell surface molecule expression

Like IL-4, IL-13 induces CD23 expression and also upregulates MHC class II, sIgM, CD71, and CD72 on purified human splenic B cells (Punnonen et al., 1993). Although IL-13 stimulates virtually all human splenic B cells, IL-13 exhibits biological functions on only a proportion of the anti-CD40-stimulated tonsillar B cells to express CD23, suggesting that IL-13 may act only on a subpopulation of B cells in this system (Zurawski and de Vries, 1994). Despite the stimulatory function of IL-13 on human B cells, IL-13 does not significantly change the expression of CD19, CD20, CD25, CD40, MHC class I, antigens, ICAM-1, LFA-1, or LFA-3 (Punnonen et al., 1993). IL-4 but not IL-13 or IL-10 promotes CD25 expression in human high-density quiescent tonsillar B cells (Burlinson et al., 1995). Conversely, both IL-4 and IL-13 down-regulate the expression of CD70 (a marker for mature B cells which have been primed by an antigen *in vivo*) on B cells activated *in vitro* (Lens et al., 1996).

IL-13 promotes growth of human B cells

Human IL-4 and IL-13 promote growth of human B cells under culture conditions involving preactivation by anti-IgM or ligation of surface CD40 by anti-CD40 antibodies (McKenzie et al., 1993a; Defrance et al., 1994; Cocks et al., 1993; Briere et al., 1993) (Fig. 1.1). These effects are independent of IL-2-induced B cell proliferation (Aversa et al., 1993).

IL-13 induces class switching and immunoglobulin production in human B cells

At least two cell-derived signals are necessary for the induction of immunoglobulin isotype switching in B cells. The first signal is provided by a physical interaction between B cells and activated T cells, basophils, or mast cells (Punnonen and de Vries, 1994; Gauchat et al., 1993; Punnonen et al., 1995). The second signal is given by either of the soluble cytokines, IL-4 or IL-13, both of which induce germ-line epsilon transcript expression, but this alone is insufficient to trigger secretion of IgE (Punnonen et al., 1993; McKenzie et al., 1993a; Minty et al., 1993). In the presence of activated $CD4^+$ T helper cells or fixed membranes of activated $CD4^+$ T helper cells or mast cells, both IL-4 and IL-13 induce class switching of IgG4 and IgE production in human B cells or immature B cells derived from fetal bone marrow (Punnonen et al., 1993; McKenzie et al., 1993a; Minty et al., 1993; Punnonen and de Vries, 1994; Gauchat et al., 1993; Punnonen et al., 1995) (Fig. 1.1). Activated T cells induce or enhance B cell antibody production by delivering both contact and cytokine-mediated signals, inducing B cell proliferation and differentiation.

The major cell-surface signaling molecule of activated T cells during B cell help appears to be CD40 ligand (CD40L) (Armitage et al., 1992a; Noelle et al., 1992; Hollenbaugh et al., 1992; Spriggs et al., 1992) which is also expressed on mast cells (Gauchat et al., 1993). CD40L interacts with CD40, a member of TNF receptor family, on the B cell surface (Stamenkovic et al., 1989; Smith et al., 1990; Smith et al., 1994; Johnson et al., 1986). This signal appears necessary for Ig class switching as mutations of the CD40L gene, as seen in hyper-immunoglobulin M1 (HIGM1) patients, lead to the absence of germinal centers in their lymph nodes

and greatly decreased levels of IgG, IgA, and IgE (Callard et al., 1993). Similar defects are seen in mice lacking CD40 or CD40L (Kawabe et al., 1994; Xu et al., 1994; Renshaw et al., 1994). Highly purified naive $sIgD^+$ splenic human B cells can be induced to produce IgG4 and IgE by IL-13 when costimulated with anti-CD40 mAbs or L-cell transfectants expressing CD40L. However, IL-13 does not operate via an IL-4-dependent mechanism (Punnonen et al., 1993; Cocks et al., 1993) as the induction of IgG4 and IgE by IL-13 is not affected by neutralizing anti-IL-4 mAbs. Instead IL-13 synergizes with IL-4, supporting the argument that IL-13 acts independently of IL-4. By using highly purified naive $sIgD^+$ splenic B cells in the anti-CD40 system, it is unlikely that the production of IgE is due to selective outgrowth of contaminating IgE-committed B cells (Cocks et al., 1993; Defrance et al., 1994). Moreover, the induction of germ-line ε mRNA precedes IgE-switching (Gauchat et al., 1990), and both IL-4 and IL-13 induce germ-line ε transcription in highly purified $sIgD^+$ naive B cells (Punnonen et al., 1993; Punnonen et al., 1995). However, IL-13 is generally two- to five-fold less potent than IL-4 (Punnonen et al., 1993; McKenzie et al., 1993a; Punnonen et al., 1995).

IL-4 or IL-13 alone induces germ-line ε RNA synthesis in fetal spleen or bone marrow immature human B cells, and anti-CD40 mAb strongly synergizes with either IL-4 or IL-13 in inducing germ-line ε transcription in these B cells. Unlike IL-4, IL-13 fails to induce germ-line ε RNA synthesis in $sIgM^-$ pre-B cells even in the presence of anti-CD40 mAbs, indicating that functional IL-13 receptors (IL-13R) are expressed at a later stage of B cell ontogeny than IL-4R (Punnonen et al., 1995). IL-10 and anti-CD40 mAbs induce plasma cell differentiation. Addition of IL-13 to this system permits limited cell proliferation but, most importantly, results in very high Ig production by the plasma cells (Briere et al., 1993; Billard et al., 1997).

Interestingly, a mutant IL-4 protein, hIL-4.Y124D (a tyrosine residue at position 124 is replaced by aspartic acid), specifically blocks IL-4- and IL-13-induced proliferation of B cells costimulated by anti-CD40 mAbs in a dose dependent manner (Aversa et al., 1993), indicating the existence of commonalities between the IL-4 and IL-13 receptors. In addition, this mutant protein, which acts as a potent IL-4 and IL-13 receptor antagonist, strongly inhibits ongoing human IgE synthesis in SCID-hu mice (Carballido et al., 1995). Hence, IL-13 is another identified cytokine produced by T cells that induces IgG4 and IgE class switching in naive human B cells.

Regulation of human B cell apoptosis by IL-13

Similar to IL-4 (Illera et al., 1993; Dancescu et al., 1992; Mainou-Fowler et al., 1994), IL-13 promotes human B cell survival by reducing the spontaneous apoptosis of normal peripheral blood and transformed B cells *in vitro*. In combination with CD40L, IL-13 can further increase the survival of B cells (Lomo et al., 1997) (Fig. 1.1). Consistent with this idea, IL-13, in combination with CD40L, augments the expression of the *Bcl-2* homologues, *Bcl-xL* and *Mcl-1*, while leaving the levels of *Bcl-2* and two other *Bcl-2* family members, *Bax* and *Bak*, unaltered. In addition, IL-13 increases the expression of CD40 on B cells from normal peripheral blood or non-Hodgkin's lymphoma patients (Lomo et al., 1997; Billard et al., 1997). In cases in which malignant B cells respond spontaneously to IL-13, the IL-13R can also be detected on the surface by binding experiments with radiolabeled IL-13. Conversely, non-responsive B cells from non-Hodgkin's lymphoma patients do not express surface IL-13R unless activated by CD40L (Billard et al., 1997). IL-13 at doses ranging from 10 to 200 ng/ml

protects B chronic lymphocytic leukemia (B-CLL) cells *in vitro* from spontaneous apoptosis. The effects of IL-13 on neoplastic B cells are slightly less than those of IL-4 and occur independently of IL-4 (Chaouchi et al., 1996; Fluckiger et al., 1994). In contrast to the anti-apoptotic effects of IL-13 on neoplastic B cells, IL-13 appears to inhibit proliferation of blasts or cell lines isolated from B-lineage acute lymphoblastic leukemia patients (Renard et al., 1994). Thus, IL-13 can exhibit positive or negative effects on malignant B cells at different maturation stages and may contribute to the pathogenesis of some neoplastic B cells.

IL-13 effects on mouse B cells

In contrast to hIL-13's effects on human B cells, mIL-13 has been reported not to have any effects on mouse B cells (Zurawski and de Vries, 1994). Although this is consistent with the observation that IL-4-deficient mice do not produce IgE in response to nematode infection (Kuhn et al., 1991), these mice produce IgE upon malaria infection (von der Weid et al., 1994). This suggests that an IL-4-independent mechanism for IgE synthesis also exists in mice and raises the possibility that mouse IL-13 may also induce IgE synthesis in some circumstances. However, data from Chapter VI provided no direct evidence for IL-13 effects on IgE class switching in mouse B cells but rather demonstrated that IL-13 directly promote mouse B cells survival.

IL-13 does not activate human or mouse T cells

IL-4 not only promotes the differentiation of Th2 cells but also promotes the growth of activated T cells and T cell clones (Mosmann and Sad, 1996b). Given the overlapping functions of IL-4 and IL-13 on monocytes and B cells, and IL-4

stimulates T cells, it is rather surprising that IL-13 does not exhibit any biological functions on mouse (J. -M. Heslan and T. R. Mosmann, unpublished) or human T cells (Zurawski et al., 1993a; Sornasse et al., 1996). In contrast to IL-4, no growth promoting activities can be detected on PHA-activated T cell blasts or activated $CD4^+$ or $CD8^+$ T cell clones by IL-13, supporting the notion that T cells lack functional IL-13R. In the IL-13-deficient mouse model, IL-13 appears to act earlier than IL-4 in Th2 cell development (Bancroft et al., 1998).

IL-13 acts on NK cells

Similar to IL-2, IL-13 is an inducer of IFN-γ production by NK cells (Fig. 1.1). Although IL-13 is less potent in the induction of IFN-γ synthesis by large granular lymphocytes isolated from peripheral blood, IL-2 and IL-13 have synergistic effects on IFN-γ production by these cells (Young and Ortaldo, 1987; Minty et al., 1993). In this respect, IL-13 seems to differ from IL-4, because IL-4 strongly inhibits IL-2-induced IFN-γ production by highly purified $CD3^-$, $CD16^+$, $CD56^+$ NK cells (Hsu et al., 1992).

Experimental listeriosis is a characteristic Th1 cell-controlled infection, and effective protection against it largely depends on the activation of antibacterial mechanisms in infected macrophages by IFN-γ (Kaufmann, 1993). At the onset of infection, macrophages phagocytose listeriae and rapidly release IL-12 and TNF-α, both of which stimulate NK cells to produce IFN-γ. Together with IL-12, IFN-γ induces the differentiation of Th1 cells, which produce more IFN-γ and ultimately cause sterile resolution of listeriosis (Hsieh et al., 1993b; Locksley, 1993; Tripp et al., 1993). Neutralization of IL-4 by mAb prior to or concomitant with *Listeria monocytogenes* infection increases protection, suggesting that IL-4 is

detrimental to the resolution of listeriosis (Haak-Frendscho et al., 1992; Teixeira and Kaufmann, 1994). Based on the overlapping *in vitro* functions with IL-4, one may, therefore, speculate that IL-13, a Th2 cytokine, may interfere with anti-listerial protection. Surprisingly, IL-13 treatment improves various aspects of resistance and favors protection against listeriosis. IL-13 diminishes IL-4 production at early time points and enhances IL-12 production and NK cell cytolytic activity, thus, indirectly providing resistance to listeriosis (Flesch et al., 1997). Together, these data emphasize that although both IL-4 and IL-13 are generally considered as Th2 cytokines, they can exhibit different functions *in vivo* in that IL-13 stimulates the production of the Th1-promoting cytokine IL-12 and provides effective Type I protection against *L. monocytogenes* infection.

IL-13 promotes differentiation and growth of dendritic cells

Dendritic cells are the most potent professional antigen-presenting cells specializing in acquiring, processing, and presenting antigens to induce specific MHC-restricted immune responses (Steinman, 1991; Shurin, 1996) or tolerance (Matzinger and Guerder, 1989). Human and mouse dendritic cells can be isolated from spleens or generated from hemopoietic precursor cells in the peripheral blood or bone marrow. In the presence of GM-CSF and IL-4, the $CD34^-$ precursor cells differentiate into the characteristic morphology, nonadherence, and functions of dendritic cells. Because IL-13 demonstrates overlapping functions with IL-4, it is not surprising that IL-13 can replace IL-4 in promoting the development of dendritic cells from $CD34^-$ precursors (Romani et al., 1996) (Fig. 1.1). In addition, IL-4, IL-13, GM-CSF, and CSF-1 are effective in stimulating the proliferation of long-term cell lines of dendritic cell lineage (Yokota et al., 1996). Hence, it is likely that IL-13 may play an important role not only in promoting the

generation of dendritic cells, but also regulating their functions during an immune response.

IL-13 regulates adhesion molecule expression

IL-4 upregulates surface expression of the vascular cell adhesion molecule-1 (VCAM-1) but not the E-selectin or intercellular adhesion molecule-1 (ICAM-1) on human umbilical vein endothelial cells (HUVEC) and, thus, selectively enhances the adherence of human eosinophils, T cells, or macrophages to HUVEC (Sironi et al., 1994; Ying et al., 1997; Jahnsen et al., 1997). Incubation of HUVEC with IL-13 induces surface expression of VCAM-1, and the adhesion of eosinophils to IL-13-treated HUVEC is completely inhibited by antibodies to VCAM-1 or alpha 4 integrin (Bochner et al., 1995) or a combination of a mutant IL-4-antagonist and mAb to the IL-4Rα chain (CD124) (Kotowicz et al., 1996). The expression of the IL-4Rα chain is essential for HUVEC responses to IL-4 and IL-13 as the common gamma chain (γ_c), which is a component of classical IL-2 or IL-4 receptors, is not detected on HUVEC by flow cytometry or immunogold histochemistry (Kotowicz et al., 1996). Furthermore, IL-13 mRNA and protein co-localize to $CD3^+$ T cells in cutaneous cryostat sections from atopic subjects taken after allergen challenge and correlate with IL-13 expression and VCAM-1 immunoreactivity on endothelial cells, infiltrating eosinophils, T cells, and macrophages (Ying et al., 1997). These results strongly suggest that IL-4 and IL-13 may act through IL-4Rα but not γ_c on HUVEC and also suggest a possible role of IL-4 and IL-13 in promoting VCAM-1/α4β1 integrin-dependent accumulation of eosinophils, T cells, and macrophages during allergic and other inflammatory reactions.

IL-13 activates human eosinophils and is involved in inflammatory responses

IL-13 upregulates the expression of CD69 on human eosinophils in a dose dependent manner, significantly prolongs eosinophil survival *in vitro*, and is a chemotactic factor for eosinophils (Luttmann et al., 1996; Horie et al., 1997) (Fig. 1.1). The enhanced survival effects of IL-13 on eosinophils can be inhibited by mAbs against IL-3 and GM-CSF, suggesting that IL-13 induces IL-3 and GM-CSF production from eosinophils and that an autocrine mechanism is responsible for the eosinophil survival. In contrast, IL-4 does not appear to be a chemotactic factor for eosinophils *in vitro* (Horie et al., 1997). Interestingly, localized expression of IL-4 *in vivo* can promote eosinophil accumulation (Tepper et al., 1989), and IL-4 transgenic mice exhibit striking inflammation of the eyelid lesion with an excessive numbers of eosinophils and tissue mast cells (Tepper et al., 1990). These discrepancies reflect that *in vitro* studies may not fully predict the *in vivo* functions of IL-4. As IL-13 upregulates VCAM-1 on endothelial cells and VCAM-1 mediates selective eosinophil transendothelial migration, IL-13 may contribute to the accumulation and selective transendothelial migration of eosinophils during allergic responses.

Allergic reactions are characterized by the selective recruitment of several cell types, predominantly eosinophils and lymphocytes, into the lesion sites. It is generally accepted that cytokines orchestrate this complex cellular reaction. Bronchial hyperactivity and inflammatory responses of the airway as seen in bronchial asthma are associated with the presence of eosinophils and the upregulation of Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13 in the mucosa. Bronchoalveolar lavage (BAL) cells in atopic asthma patients secrete IL-4 and IL-13 after allergen challenge (Huang et al., 1995), and increased levels

of mRNA for both cytokines can be detected in the bronchial mucosa of allergen-challenged atopic and nonatopic asthma patients (Kotsimbos et al., 1996; Humbert et al., 1997). Unlike the transient IL-4 production, IL-13 production after activation can be sustained up to the late asthmatic response, and the concentration of IL-13 secreted strongly correlates with the number of eosinophils in BAL and bronchial submucosa biopsy specimens (Kroegel et al., 1996; Humbert et al., 1997). These data are consistent with the hypothesis that in addition to IL-5, which is a potent eosinophil growth, differentiation, and chemotactic factor (Sanderson et al., 1988; Yamaguchi et al., 1988b; Yamaguchi et al., 1988a; Tominaga et al., 1991; Dent et al., 1990), IL-13 also plays a role in the pathogenesis of both atopic and nonatopic asthma, at least partly through the upregulation of adhesion proteins, thus, promoting the recruitment and selective transendothelial migration of eosinophils, basophils, and lymphocytes into bronchial mucosa.

Mast cells and basophils produce IL-13

Mast cells play important roles in allergic and inflammatory processes, as upon activation through their IgE receptors or pharmacological stimulants, they release a large panel of low molecular weight inflammatory mediators such as histamine, leukotrienes, and cytokines. After stimulation through IgE receptors on mast cells, IL-13 mRNA expression can be detected as early as 15 min and peaks between 1 and 2 h but falls substantially by 6 h (Burd et al., 1995). The induction kinetics of IL-4 mRNA on activated mast cells are similar to those of IL-13, but peak expression can be detected after 1 to 3 h (Burd et al., 1995). Interestingly, bone marrow-derived mucosal-like mast cells constitutively express IL-13 (Marietta et al., 1996). As mast cells express receptors for IL-4 and IL-13, both cytokines can

modulate mast cell functions in an autocrine fashion. Both cytokines induce expression of *c-fos* and CD54 (ICAM-1) but downregulate the proliferation of the human mast cell line, HMC-1. However, the actions of IL-4 on mast cells are more potent than those of IL-13 (Nilsson and Nilsson, 1995).

Like mast cells, activated human basophils can rapidly secrete inflammatory mediators such as histamines and leukotrienes. Although basophils are less commonly recognized at the sites of chronic inflammation, the belief that basophils play an active role in allergic disease has intensified with the evidence that these cells secrete immunomodulatory cytokines such as IL-4 and IL-13 (Ochensberger et al., 1996; Li et al., 1996; Gibbs et al., 1996). In contrast to the similar IL-4 and IL-13 production kinetics in mast cells, these two cytokines are regulated differently in basophils. Preformed IL-4 can be secreted shortly after activation and the production of IL-4 is transient, whereas IL-13 activation kinetics are characterized by rapid *de novo* and prolonged expression (Ochensberger et al., 1996; Li et al., 1996; Gibbs et al., 1996). This is consistent with differences in transductional mechanisms regulating the release of IL-4 and IL-13, as the production of the latter by basophils is less dependent upon cell activation through the FcεRI receptor. In addition, PMA, which activates the PKC pathway, effectively stimulates IL-13 production while inhibiting IL-4 production (Redrup et al., 1998).

Human HMC-1 (mast cell) and KU812 (basophilic) cell lines, as well as freshly isolated purified human lung mast cells and blood basophils, express the CD40L and thus can induce IgE production (Gauchat et al., 1993). IL-4 and IL-13 are produced by mast cells and basophils (Brown et al., 1987; Plaut et al., 1989; Burd et al., 1995), and both types of cells express CD40L which can induce Cε

switching and IgE production *in vitro* through their ability to mediate contact-dependent help for B cells (Gauchat et al., 1993). These findings raise the possibility that peripheral organs in which mast cells are concentrated, such as the skin and lung, may also be sites of Ig switching and IgE production. In addition, IL-4 and IL-13 are mast cell growth factors that are very likely to influence mast cell numbers (Fig. 1.1). IL-4 also promotes the development and growth of IL-4- and IL-13-producing T cell subsets which can further amplify the response. Since basophil secretion of IL-13 is prolonged compared to the transient expression of IL-4, this could indeed support the hypothesis that IL-13 induces and sustains the production of IgE after the disappearance of IL-4. Therefore, it is conceivable that mast cells and basophils, which express IL-4, IL-13, and CD40L, may play a key role in allergies not only by producing inflammatory mediators but also by directly regulating IgE production independently of T cells.

Roles of IL-13 in allergies

Th2 cytokines are generally associated with the induction of IgE class switching and allergic responses. This is not surprising because IL-4 and IL-13 are the only two known cytokines to induce T-dependent IgE synthesis, while IL-5 exhibits potent effects on eosinophils. IL-4 and IL-13 mRNA can be detected in the epithelial compartment of the nasal mucosa of patients with perennial allergic rhinitis to house dust mites (Pawankar et al., 1995) and in acute and chronic skin lesions from atopic dermatitis patients (Hamid et al., 1996). In the majority of cases, PBMC from atopic individuals readily secrete IgE, IL-4, and IL-13 without further activation, suggesting that endogenously secreted IL-4 and IL-13 correlate well with allergic reactions. Furthermore, the number of cells expressing IL-4 and IL-13 mRNA at the allergen-challenged sites can be reduced in allergic patients

receiving topical steroid therapy (Ghaffar et al., 1997). These findings support the hypothesis that IL-4 and IL-13 are involved in the recruitment of inflammatory cells to the site of allergic inflammatory reactions.

Although IL-4 and IL-13 exhibit overlapping functions, expression of the former is more restricted, whereas IL-13 mRNA can be found in human $CD4^+$ clones of Th0, Th1, Th2 phenotype, as well as $CD8^+$ clones. Therefore, IL-13 is not a classical Th2 cytokine. Due to the transient production of IL-4, it may preferentially be involved in the early phases of activation. As IL-13 production continues over an extended period of time, it is conceivable that IL-13 maintains immune responses and is involved in chronic allergic diseases.

IL-13 exhibits biological functions on osteoblasts and osteoclasts

Osteoblasts and osteoclasts play important roles in the regulation of bone formation, healing, and resorption (Parfitt, 1984; Riggs and Melton, 1986; Riggs and Melton, 1992). Both IL-4 and IL-13 mediate osteoblast recruitment and migration with maximum chemotaxis at concentrations of 100 ng/ml of either cytokine (Lind et al., 1995). On the other hand, IL-4 and IL-13 suppress the release of mediators and osteoclast recruitment associated with IL-1α–induced bone-resorption (Onoe et al., 1996). The inhibitory effects of IL-4 and IL-13 on bone-absorbing activity are rather specific for IL-1α–induced bone resorption as neither cytokine affects bone resorption induced by other mediators *in vitro* (Onoe et al., 1996). Thus, IL-4 and IL-13 can take part in the recruitment of osteoblasts and inhibition of osteoclast activities *in vitro*. However, two independently derived founders of IL-4 transgenic mice under the direction of the lymphocyte-specific proximal promoter for the *lck* gene develop severe

osteoporosis primarily due to a profound decrease in osteoblast activity (Lewis et al., 1993). These data indicate that *in vitro* observations do not necessary translate into *in vivo* functions. Nevertheless, both cytokines appear to be of importance in the cytokine regulation of bone resorption and formation.

Responses of neutrophils to IL-13

IL-4 and IL-13 share similar biological activities on neutrophils. Treatment with IL-13 augments both IL-1 decoy receptor (type II) and IL-1 receptor antagonist transcripts, while inhibiting cytokine synthesis by human polymorphonuclear cells (Colotta et al., 1994; Girard et al., 1996). In addition, IL-4 and IL-13 activate cellular morphological changes in neutrophils and increase *de novo* protein synthesis (Girard et al., 1997; Girard et al., 1996). In contrast to IL-4, IL-13 does not delay neutrophil apoptosis (Girard et al., 1997). Despite this difference, IL-4 and IL-13 can both modulate neutrophil functions.

E. IL-13 receptors (IL-13R)

Identification of IL-13R chains

In 1993, Zurawski et al (Zurawski et al., 1993a) suggested that receptors for IL-4 and IL-13 are structurally related. The type I IL-4R is composed of two chains, the IL-4Rα chain (130 kDa) and the common γ-chain (γ_c) of IL-2 receptors. The γ_c is a subunit of the receptors of other four-helix bundle cytokines, including IL-2, IL-4, IL-7, IL-9, and IL-15 (Kondo et al., 1993; Noguchi et al., 1993). The IL-4Rα chain alone is thought to form a tight complex with its ligand, whereas the γ_c is responsible for signal transduction. An IL-4 mutant protein, which is a

competitive antagonist of IL-4, inhibits the action of IL-13 on various cells, including B cells (Zurawski et al., 1993a; Aversa et al., 1993). Although IL-13 does not bind to the ligand binding protein of IL-4, it displaces [^{125}I]IL-4 binding in various cells (Aversa et al., 1993; Feng et al., 1995). These experiments suggested that IL-4 and IL-13 share a receptor component, and the γ_c of IL-2 receptor was the likely candidate (Kishimoto et al., 1994). Subsequently, various groups have raised questions over the proposed model. Overexpression of the recombinant γ-chain in COS cells does not prevent the displacement of [^{125}I]IL-4 binding by IL-13 (Vita et al., 1995), and anti-IL-4Rα antibody, which blocks the biological function and binding of IL-4, inhibits the binding and function of IL-13 (Zurawski et al., 1995). In addition, IL-4- and IL-13-induced responses can be observed in cells which naturally do not express γ_c, including B9 plasmacytoma cells (He and Malek, 1995; He et al., 1995), a human carcinoma cell line (RCC) (Obiri et al., 1995), human endothelial cells (Schnyder et al., 1996), and lymphocytes obtained from severe combined immunodeficiency (SCID) patients who are deficient in γ_c or who have mutated γ_c (Schnyder et al., 1996; Obiri et al., 1995; Lin et al., 1995; Matthews et al., 1995; Izuhara et al., 1996). Therefore, it is speculated that a second form of IL-4R (type II) exists (He and Malek, 1995; He et al., 1995; Zurawski et al., 1995) and that IL-4R and IL-13R share some other receptor component.

Recently, two different cDNA clones have been isolated that encode receptor proteins which are capable of binding IL-13. These two receptor subunits, IL-13Rα1 (NR4) (Hilton et al., 1996) and IL-13Rα2 (Caput et al., 1996), share high levels of sequence identity with known cytokine receptors. A partial genomic mouse IL-13Rα1 clone has been isolated from an embryonic stem cell library using oligonucleotides encoding the amino acid sequence WSXWS which is

common to many members of hemopoietin receptor family. Based on the partial genomic IL-13Rα1 sequence, IL-13Rα1 cDNAs have been cloned from WEHI-3B cells, peritoneal macrophage, bone marrow, skin, and kidney libraries. Mouse IL-13Rα1, a 55-65 kDa protein containing 434 amino acids, is a low affinity receptor capable of binding IL-13 but not IL-4 or IL-2, IL-7, IL-9, or IL-15 (Hilton et al., 1996). IL-13Rα1 is the primary low affinity binding subunit of the IL-13R (K_d ~ 2-10 nM), and upon complexing with IL-4Rα, a substantially higher binding affinity to IL-13 (K_d ~ 75 pM) can be achieved. Furthermore, IL-4 can compete for IL-13 binding in cells expressing IL-13Rα1 and IL-4Rα (Hilton et al., 1996).

Subsequently, the human homologue of mouse IL-13Rα1 cDNA was cloned from the human T cell lymphotropic virus-I-transformed MT-2 cells (Aman et al., 1996), human Caki-1 cells (Miloux et al., 1997), and tonsillar B cells (Gauchat et al., 1997). Human IL-13Rα1 cDNA encodes a 65 kDa protein consisting of 427 amino acids with two consensus patterns characteristic of the hemopoietic cytokine receptor family, and a short cytoplasmic tail (Aman et al., 1996; Miloux et al., 1997). The gene encoding IL-13Rα1 has been mapped to chromosome X and demonstrates 81% and 76% homology to mouse IL-13Rα1 cDNA and protein, respectively. Interestingly, 57 out of the 60 amino acids in the cytoplasmic domain (95%) are identical in human and mouse IL-13Rα1, underscoring the likely significance of this region in signal transduction. This is further supported by the observations that IL-13Rα1 can serve as an alternative accessory protein to γ_c for IL-4 signaling in certain cells by forming the type II IL-4R (Aman et al., 1996; Miloux et al., 1997). IL-4 and IL-13 upregulate MHC class II molecules and inhibit nitric oxide production from macrophages derived from γ_c-deficient mice in the absence of type I IL-4R (Andersson et al., 1997). Moreover, B cells

from X-linked SCID patients do not respond to IL-2 or IL-15, but respond well to IL-4 and IL-13 (Matthews et al., 1995). These results further support the idea that γ_c does not function as the shared component between IL-4R and IL-13R. Interestingly, X-SCID patients have normal numbers of circulating B cells which respond to IL-4 and IL-13, suggesting that abnormal humoral responses in X-SCID patients may be due to an absence of T cell help (Matthews et al., 1995).

Human IL-13Rα2, identified by expression cloning using cDNA derived from Caki-1, consists of 380 amino acids with a short cytoplasmic tail. This receptor, with only 26% sequence identity with mouse IL-13Rα1, consists of two consensus patterns characteristic of the hemopoietic cytokine receptor family and shows homology with IL-5Rα. Due to its short cytoplasmic tail, IL-13Rα2 may not be involved in transducing signals (Caput et al., 1996; Guo et al., 1997). Along with the gene for γ_c, which is shared by receptors of the other four-helix bundle cytokines including IL-2, IL-4, IL-7, IL-9, and IL-15 and is located in the region Xq13.1, the IL-13Rα2 gene has also been mapped to the chromosome Xq (Xq24) (Guo et al., 1997). Other cytokine-related genes mapped to the X chromosome include IL-9R (Xq28) (Kermouni et al., 1995) and CD40L (Xq26) (Pilia et al., 1994).

IL-13Rα1 and IL-13Rα2 are expressed by cells present in various organs tested. The highest IL-13Rα1 mRNA levels can be detected in lymph nodes, fetal liver, PBL, and the spleen, but lower IL-13Rα1 mRNA levels can be detected in bone marrow and the thymus. The IL-13Rα1 mRNA can also be detected in adult liver, skeletal muscle, the heart, placenta, lung, and pancreas but not in the kidney and brain. Expression of the IL-13Rα2 mRNA is more restricted but can be found in bone marrow, fetal liver, PBL, placenta, and liver. The steady-state levels of the

two mRNAs appear to be regulated independently, suggesting that the two receptors may have different functions (Gauchat et al., 1997).

As soluble forms of many members of the hemopoietin receptor family have been described, it is not surprising that a soluble IL-13 binding protein (IL-13BP) has been purified from mouse serum and urine. Based on the near identity of the first 27 amino acids of this protein and human IL-13Rα2, IL-13BP is almost certainly the mouse soluble analog of human IL-13Rα2. However, no soluble human IL-13Rα2 can be detected in human plasma and urine, indicating that soluble human IL-13Rα2 may preferentially be degraded or regulation of the mouse and human IL-13Rα2 may be different (Zhang et al., 1997).

Extensive studies have been conducted to address the complexity of IL-13R complexes. Transfection studies reveal that IL-13Rα2 binds IL-13 with 100- to 300-fold higher affinity compared to IL-13Rα1 (K_d ~ 20-90 pM vs. 3-10 nM), but fails to bind IL-4. Because IL-13Rα2 does not appear to interact with IL-4Rα, the binding of IL-13 to IL-13Rα2 cannot be inhibited by IL-4. IL-13Rα1 in combination with IL-4Rα, however, recognizes both IL-4 and IL-13 (Zhang et al., 1997). Further binding studies of IL-13 on various cell types raise more questions on the nature of IL-13R complexes as IL-13 competes for IL-4 while IL-4 does not compete for IL-13 binding on some cells. Thus, four structural models of IL-13R involving different combinations of IL-13Rα1, IL-13Rα2, or IL-4Rα have been proposed : type I IL-13R involves a combination of IL-13Rα1 and IL-13Rα2; type II IL-13R may be composed of IL-13Rα1 and IL-4Rα; type III IL-13R consists of IL-13Rα1, IL-4Rα, and γ_c; and type IV IL-13R contains IL-4Rα, γ_c and maybe IL-13Rα1 (Obiri et al., 1997) (Fig. 1.2). Collectively, these data indicate that IL-13Rα1, but not IL-13Rα2, is the common component shared between IL-13R

and IL-4R, whereas the γ_c may influence IL-13 and its receptor interaction under certain conditions. Further studies are required to define the structures of IL-13R complexes.

IL-13R signal transduction

The IL-4Rα is a crucial component for IL-4 binding and signal transduction. As IL-4Rα is also part of some IL-13R complexes, it is not surprising that IL-4 and IL-13 can trigger very similar signaling cascades. Both IL-4 and IL-13 induce phosphorylation of several cellular proteins, including the IL-4Rα itself and insulin receptor substrate 2 (IRS-2), a 170 kDa protein expressed predominantly in cells of hemopoietic origin (Welham et al., 1995; Sun et al., 1995; Wang et al., 1995). The kinases responsible for the phosphorylation of IL-4Rα or IRS-2 are still unknown. In some cell lines, IRS-1, the principal phosphorylation substrate of the insulin receptor, can substitute for IRS-2 in IL-13R signaling (Keegan et al., 1995). Phosphorylated IRS-2 is then associated with the regulatory subunit of phosphatidylinositol 3-kinase (p85) and leads to further downstream events (Wright et al., 1997), including the formation of phosphatidylinositol (3,4)-biphosphate and phosphatidylinositol (3,4,5)-triphosphate, mobilization of calcium ions, and an increase in intracellular cAMP in monocytes, human B cells, and the colonic epithelial cell line, HT-29 (Sozzani et al., 1995; Wright et al., 1997).

Unlike other cytokines, IL-4 and IL-13 do not activate components of the ras/mitogen-activated protein (MAP) kinase pathway, including tyrosine phosphorylation of the *src*-homology 2 (SH-2)-domain-containing adaptor protein (Shc) or its association with Grb2, Sos1, *ras*, and *erk-1* and *erk-2* MAP kinases

(Welham et al., 1995). However, JAKs and STATs play significant roles in IL-4 and IL-13 signaling. Biochemical studies in T cells and myeloid cells have demonstrated that JAK1 and JAK3 are activated after IL-4 stimulation (Johnston et al., 1994; Darnell et al., 1994). Indeed, IL-4Rα and γ_c are associated with JAK1 and JAK3, respectively. After JAK stimulation, STATs are believed to be phosphorylated on tyrosine residues in the cytosol and then translocated to the nucleus, where they bind to DNA or DNA-binding proteins and enhance the transcription of specific genes (Darnell et al., 1994). Activation of JAK1 and JAK3 leads to the tyrosine phosphorylation, activation, and translocation of the STAT6 transcription factor (Hou et al., 1994; Quelle et al., 1995). Although IL-4 and IL-13 have many overlapping biological functions, IL-13 activates the phosphorylation of IRS-2 and JAK1 without the participation of JAK3 (Welham et al., 1995; Wang et al., 1995; Sozzani et al., 1995; Izuhara et al., 1996). Electrophoretic mobility shift assays show that IL-13 and IL-4 are able to activate STAT6 in cells expressing both IL-4Rα and IL-13Rα1, while no activation can be observed in cells expressing either one alone (Leach et al., 1996; Miloux et al., 1997). In addition, STAT6 plays an important role in some of the IL-4- and IL-13-induced responses, as revealed by STAT6 deficient mice (Kaplan et al., 1996; Takeda et al., 1996). The activation of STAT6 by IL-4 in cells coexpressing IL-4Rα and IL-13Rα1 clearly shows that IL-13Rα1 can replace γ_c in the reconstitution of an active IL-4R.

Given that JAK3 is reported to associate with γ_c and IL-13 activates STAT6 in γ_c-deficient B cells derived from X-SCID patients, it appears that IL-13R does not utilize γ_c for STAT6 activation (Izuhara et al., 1996). However, BA/F3 cells which express IL-4Rα, IL-13Rα1, and γ_c can mediate the activation of JAK3 and STAT6 in response to IL-4 or IL-13 (Malabarba et al., 1996). Therefore, certain

cells may utilize type III or IV IL-13R, which both contain γ_c, to activate JAK3. In addition, IL-4 and IL-13 appear to activate JAK2 and STAT6 in human endothelial cells which do not express γ_c (Palmer-Crocker et al., 1996). Both cytokines can also activate JAK2, Tyk-2, and IRS-1 in the human colon carcinoma cell lines, HT-29 and WiDr (Murata et al., 1996). As the cytoplasmic domain of IL-13Rα1 is 26 amino acids shorter than that of γ_c (Nelson et al., 1996) and a proline-rich motif is located near the transmembrane domain of IL-13Rα1 (Miloux et al., 1997), it is conceivable that IL-13Rα1 may associate with some other JAK family members. Collectively, all available data show that the structure and signal transduction cascades of IL-13R are complex, and additional detailed studies are necessary to resolve these issues.

F. Involvement of IL-13 in pathogenesis and clinical applications

Th1 and Th2 cytokines are strongly associated with the induction and outcome of several immune responses concerning infections, allergy, and autoimmunity (Sher and Coffman, 1992). Generally considered a member of the Th2 family, IL-13 appears to play important roles in numerous diseases which Th2 immune response is essential in providing necessary protection.

Cytokines produced by Th subsets have distinct functions in providing the appropriate host defense against infections. The Th2 subset, more specifically IL-4, plays a central role in host defense against gastrointestinal nematode parasites (Urban et al., 1991; Else et al., 1994; Finkelman et al., 1997), whereas IFN-γ exacerbates disease (Urban et al., 1993; Else et al., 1994; Finkelman et al., 1994). IL-4 and IL-13 are critical but exhibit different roles in mediating immunity to intestinal helminths, *Trichuris muris* (Bancroft et al., 1998).

Interestingly, IL-4 does not appear to be essential for the expulsion of the mouse-adapted strain of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis* as expulsion occurs normally in BALB/c mice treated with anti-IL-4 or anti-IL-4R mAbs, as well as in IL-4-deficient mice (Madden et al., 1991; Kopf et al., 1993; Finkelman et al., 1997). However, IL-4Rα- or STAT6-deficient mice fail to expel the mouse-adapted strain of *N. brasiliensis*. In addition, a soluble IL-13Rα2-Fc fusion protein effectively prevents worm expulsion, suggesting that IL-13 may be more important than IL-4 as an inducer of the STAT6 signaling pathway that provides the necessary protection against this parasite (Urban et al., 1998). This finding is consistent with the data in Chapter V showing that supernatants from Con A-stimulated spleen cells of *N. brasiliensis*-infected mice exhibited enhanced bioactivity on TF-1 cells in which was likely due to IL-13.

Because Th2 cytokines are generally inhibitory to cell-mediated diseases, the potential applications of Th2 cytokines in suppressing cell-mediated responses have been intensely studied. Similar to IL-4, local administration of IL-13 at the site of transplanted cancer cells *in vivo* inhibits the growth of immunogenic and nonimmunogenic tumor cells (Lebel-Binay et al., 1995). By enhancing antigen-presenting activity which leads to enhanced cytolytic activity of lymphocytes, IL-13 induces *in vitro* tumor-specific cytolytic activity in spleen cells from tumor-bearing rats, thereby reversing the tumor-induced immunosuppression (Reisser et al., 1996). Anti-tumor effects of IL-13 *in vivo* are likely due to its pleiotropic effects, including recruitment of nonspecific cells such as neutrophils and monocytic cells and improvement in antigen presentation of tumor antigen to generate anti-tumor specific cells.

The Allergic encephalomyelitis (EAE) is associated with upregulation of Th1 cytokines but downregulation of Th2 cytokines; IL-4 and IL-13 mRNA levels are decreased as the disease progresses (Stumbles and Mason, 1995; Saoudi et al., 1995). Similar to IL-4, IL-13 exerts a protective effect on the development of EAE in rats as assessed by a reduction of the mean duration, severity, and incidence of disease. This Th1-initiated autoimmune disease can be efficiently attenuated through the administration of IL-13, which inhibits macrophages/monocytes, without having undesirable general suppressive effects on either T cells or B cells (Cash et al., 1994).

Systemic sclerosis (SSc) is a connective tissue disease characterized by vascular alterations and fibrosis. Elevated serum IL-4, IL-10, and IL-13 levels can be detected in a majority of SSc patients (Famularo et al., 1990; Hasegawa et al., 1997), indicating that these three Th2 cytokines may contribute to the disease process. Due to the cross-regulatory nature of Th1 and Th2 cytokines, these Th2 cytokines may be upregulated to suppress a Th1 immune response.

T cells isolated from nephrotic syndrome (NS), which is a renal disease characterized by proteinuria and hypoalbuminemia, spontaneously secrete IL-13, and their B cells constitutively express IL-13R but not IL-4R. Thus, IL-13 may play an important role in the production of IgG4 and IgE in these patients (Kimata et al., 1995). On the other hand, IL-4 appears to be correlated well with IgE and IgG4 production in atopic dermatitis patients as their T and B cells spontaneously secrete IL-4 and constitutively express IL-4R but not IL-13R, respectively (Kimata et al., 1995).

IL-10 is a strongly immunosuppressive cytokine (Howard et al., 1993) that is undergoing clinical trials for the treatment of various diseases including inflammatory bowel disease (IBD) (Schreiber et al., 1995). IL-10 is effective in the downregulation of IL-1β and TNF-α in IBD patients. Due to the anti-inflammatory functions of IL-4 and IL-13, numerous studies have focused on the possible applications of both cytokines in treating inflammatory diseases. IL-4 and IL-13, though less potent than IL-10, are effective in downregulating selective proinflammatory cytokines in macrophages derived from IBD patients (Kucharzik et al., 1996). The inhibition of monocyte functions by IL-4 and IL-13 is a general mechanism and does not appear to be specific for IBD as both cytokines diminish synovial fluid-derived monocyte responses from patients with acute inflammatory arthritis (Hart et al., 1993; Hart et al., 1995). IL-13 is consistently present in rheumatoid synovium, and IL-13 message can be detected in synovial fluid mononuclear cells, while IL-4 levels can not be detected (Isomaki et al., 1996). *In vivo* administration of IL-4 or IL-13 significantly reduces the severity and incidence of collagen-induced arthritis in mice. IL-13-induced suppression of this disease coincides with decreased TNF-α mRNA expression (Bessis et al., 1996). These evaluations of IL-13 suggest that it may be upregulated to inhibit inflammation associated with the disease, and may have anti-inflammatory effects *in vivo*. Though the sole use of IL-13 in regulating inflammation may not lead to the desired clinical outcome, possibly limited by its inability to downregulate T cell activity, IL-13 may be an effective therapeutic agent in combination with other anti-inflammatory mediators.

Figure 1.1. *In vitro* effects of IL-13 on cells of hemopoietic origin.

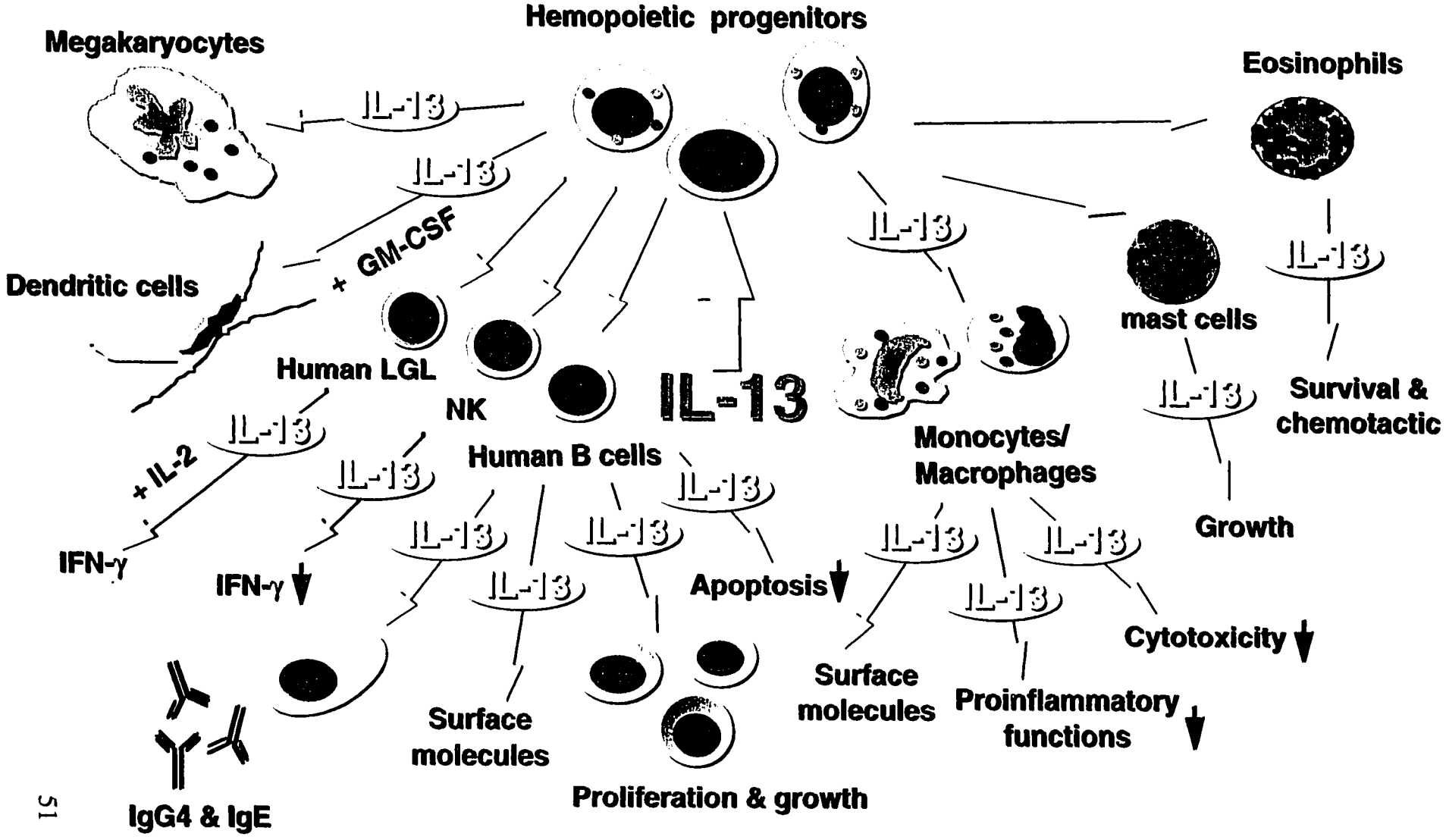
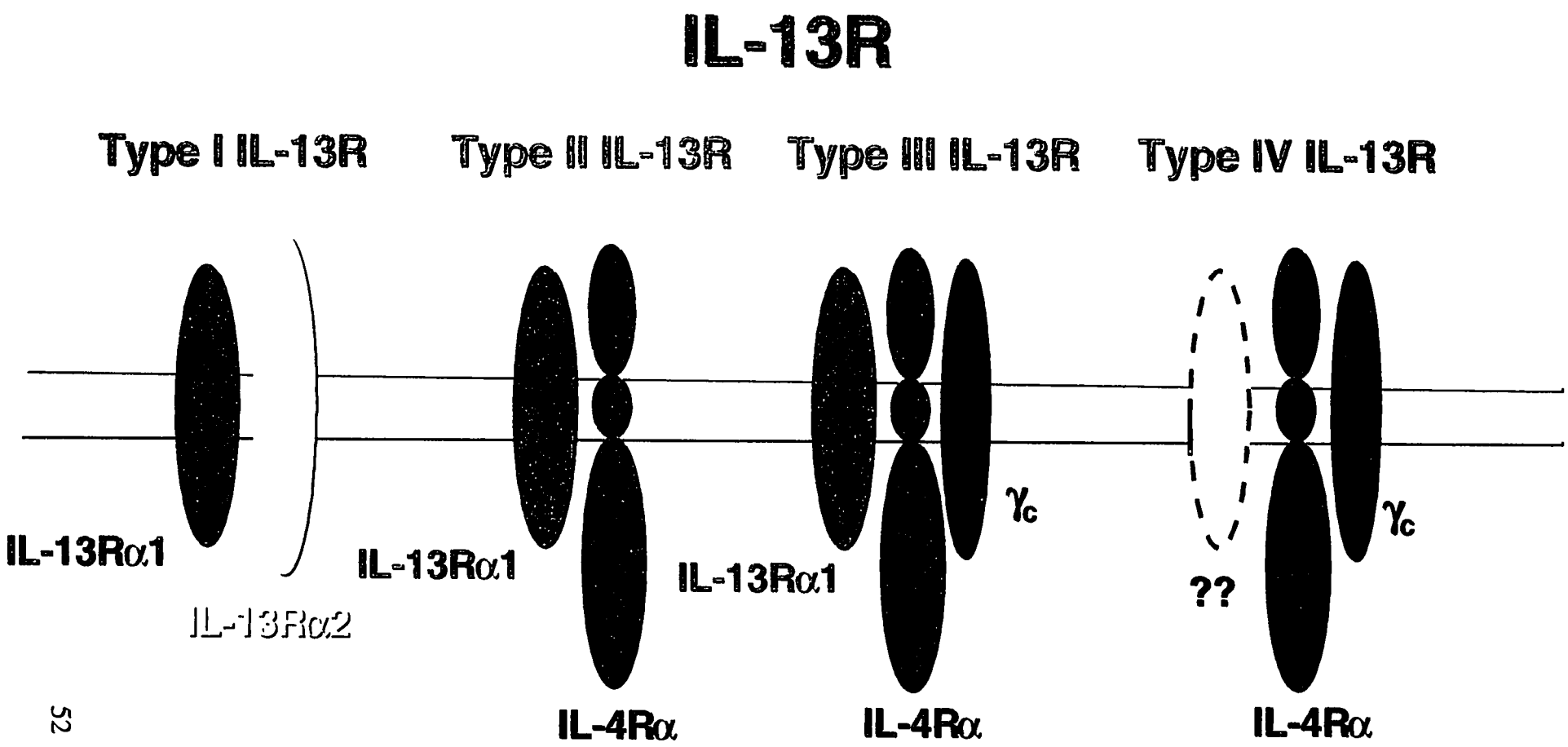


Figure 1.2. Proposed models for IL-13 Receptor. Adapted from N. I. Obiri et al. 1997. *J. Immunol.* 158:756.



CHAPTER II

RATIONALE AND OBJECTIVES

A. Rationale

Although *in vitro* culture systems continue to provide most of the information available on the role of cytokines both individually and in combination, whole animal models still represent a vital research model. It is unclear how closely *in vitro* models correspond to *in vivo* functions. Based on the available *in vitro* evidence, G-CSF is a potent late-stage factor in the generation of granulocytes but not other hemopoietic cell types. Interestingly, daily administration of G-CSF in mice results in a rise in CFU-GM and the number of functional circulating neutrophils. Moreover, G-CSF increases splenic CFU-S, CFU-Meg, and BFU-E, providing further evidence that G-CSF acts on several lineage-committed progenitors *in vivo* (Tamura et al., 1991). Although *in vitro* studies suggest that IL-5 is responsible for the differentiation of later stages of eosinophil differentiation (Sanderson et al., 1985; Yamaguchi et al., 1988b; Warren and Moore, 1988), IL-5 transgenic mice exhibit eosinophilia (Dent et al., 1990; Tominaga et al., 1991) and, unexpectedly, enhanced numbers of eosinophil progenitors in the bone marrow (Dent et al., 1990), suggesting that the action of IL-5 may therefore not be restricted to the later stages of eosinophil differentiation. IL-4, an eosinophil growth factor (Favre et al., 1990), does not appear to be an *in vitro* chemotactic factor for eosinophils (Horie et al., 1997). However, localized expression of IL-4 *in vivo* promotes eosinophil accumulation (Tepper et al., 1989), and IL-4 transgenic mice exhibit striking inflammation of the eyelid with excessive numbers of eosinophils and tissue mast cells (Tepper et al., 1990),

indicating that *in vitro* studies may not fully predict the *in vivo* functions of IL-4. IL-4 and IL-13 can take part in the recruitment of osteoblasts and inhibition of osteoclast activities *in vitro* (Lind et al., 1995; Onoe et al., 1996). However, two independently derived founders of IL-4 transgenic mice under the direction of the lymphocyte-specific proximal for the *lck* gene, develop severe osteoporosis primarily due to a profound decrease in osteoblast activity (Lewis et al., 1993). Collectively, these data indicate that *in vitro* observations do not necessarily predict *in vivo* functions.

Due to the overlapping nature of cytokine functions, designing appropriate strategies to dissect biological functions of each cytokine proves rather difficult. Overlapping functions within the cytokine family are more apparent with the generation of cytokine deficient mice using homologous recombination techniques to disrupt individual cytokine genes. While certain cytokine deficient mice demonstrate defects that can be predicted from *in vitro* studies, in certain cases there appear to be compensatory mechanisms utilizing an alternate cytokine with related biological properties. IL-13 deficient mice were generated but no phenotype was observed in the initial experiments (A. N. J. McKenzie and G. Zurawski, unpublished), strongly supporting the notion of compensation in the cytokine network.

When IL-13 was identified as the latest cytokine of the mouse Th2 pattern, both *in vitro* and *in vivo* functions of IL-13 were much less characterized compared to other well studied Th2 cytokines. In general both human IL-4 and IL-13 have *in vitro* biological effects on B cells, monocytes/macrophages, mast cells, endothelial cells, and hemopoietic precursor cells (Poulsen et al., 1991).

Although IL-13 shares some, but not all, of the *in vitro* functions of IL-4, the biological functions of IL-13 *in vivo* are not well understood.

In order to unravel the *in vivo* functions of IL-13 that may not be predicted by *in vitro* determinations, two alternate but complementary approaches were designed. Purified recombinant mouse IL-13 was administered to mice for short periods in the absence (Chapter V) or presence of an antigen (Chapter VI). *In vivo* studies with recombinant cytokines have shown that their half-lives in the circulation were short, generally less than 30 min. In addition, bolus administration of excessive cytokine doses to sustain long term efficacious serum concentration can be associated with fatality due to severe toxicity. In this study, IL-13 was administered to mice with osmotic pumps which continuously release low doses of purified recombinant IL-13. This delivery system not only reduced frequent handling of the mice but also prevented the fluctuation of cytokine levels in the mice.

As a parallel and complementing approach, IL-13 transgenic mice were generated in which IL-13 was under the control of the hCMV regulatory region which should result in high quantity of IL-13 expression in all tissues during embryogenesis, ontogeny, and throughout their lives. If high levels of IL-13 expression induced by the hCMV-IL-13 construct interfered with embryogenesis and therefore, no IL-13-expressing transgenic mice would be obtained, lower IL-13 expression levels could be regulated by tissue-specific transgenes. A tissue-specific IL-13 transgenic construct driven by CD3δ plus TCR Vβ regulatory regions was constructed. It is conceivable that even low levels of IL-13 is lethal for embryogenesis. Nevertheless, the short term studies of *in vivo*

administration of IL-13 would provide important information on the biological functions of IL-13.

B. Objectives

1. Construct the hCMV-IL-13 transgene that promotes strong and general expression in all cell types, test the functionality of the transgene transient transfectants, generate IL-13 transgenic mice with this transgene, test the transmission and expression of the transgene, and investigate the hemopoiesis and immune systems of these transgenic mice. In addition, construct and test the T cell specific transgene which should induce IL-13 expression only in T cells.

2. Purify a large amount of biologically active mammalian recombinant mouse IL-13, test the hemopoiesis and general immune systems of the mice treated with purified IL-13.

3. Investigate the impact of short term IL-13 administration in mice undergoing a strong immune response and examine the biological functions of IL-13 on mouse B cells.

CHAPTER III

MATERIALS AND METHODS

A. General Materials and Methods

Animals. BALB/c female mice (6 to 8 weeks old) were obtained from the Health Sciences Laboratory Animal Services (HSLAS, Edmonton, Alberta). 6 to 8 week old C57BL/6 female mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). All mice were maintained in a HSLAS conventional unit in accordance with the guidelines of the Canadian Council on Animal care. All transgenic mice or their litter-mates were backcrossed to C57BL/6 female mice and maintained in the HSLAS virus antigen free (VAF) unit.

T cell clones and cell lines. Stably transfected BW5147 cells expressing mammalian IL-13 were generated and kindly provided by Dr. J. F. Elliott (for detail see chapter IV). M264-15 is an H-2^d MHC allospecific Th1 clone, derived from A.TL (Cherwinski et al., 1987; Cher and Mosmann, 1987). HDK-1, a BALB/c-derived Th1 clone, is specific for soluble keyhole limpet hemocyanin (KLH) antigen (Cherwinski et al., 1987; Cher and Mosmann, 1987). D10.G4.1, derived from AKR/J mice, is a Th2 clone that recognizes H-2^b or conalbumin in the presence of H-2^k (CBA/J) (Cherwinski et al., 1987). WEHI 164.13 is a TNF-/LT-sensitive cell line (Torres et al., 1982). M12.4.1, a B lymphoma expressing H-2^d, was obtained from Dr. L. Glimcher (Glimcher et al., 1983). Sheep red blood cells (SRBC) and Chicken red blood cells (CRBC) were purchased from Triage Microbiologicals (Ardrossan, Alberta, Canada).

Cytokines. Recombinant mouse IL-2, expressed in *Escherichia coli* (Zurawski et al., 1986), was used as crude extract. Recombinant forms of IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF, IL-12, and IL-13 were prepared as supernatants of COS 7 cells transfected with the appropriate plasmid (Lee et al., 1986), standardized according to the laboratory standards, and used in cultures. Purified recombinant mouse IL-4 and human CSF-1 was kindly provided by Schering-Plough and Dr. L. J. Guilbert, respectively. In most studies, purified IL-13 or culture supernatant produced by IL-13 stably-transfected BW5147 mouse thymoma cell line (Lai et al., 1996) was used.

Antibodies. Anti-IFN-γ and anti-IL-4 antibodies were purified from the supernatant of XMG1.2 (Cherwinski et al., 1987) and 11B11 (Ohara and Paul, 1985) hybridomas, respectively. Rat-anti-mouse Gr-1 (IgG2b), CD4 (IgG1), Ly-2-FITC (anti-CD8α, clone YTS 169.4) and L3T4-PE (clone YTS 191.1) monoclonal antibodies were purchased from Cedarlane Laboratories Limited (Hornby, Ontario). Rat-anti-mouse Mac-3 (IgG1), Ly3-PE (anti-CD8β, clone 53-5.8), NK-1.1-PE (clone PK136), CD44-FITC (clone IM7), anti-B220 FITC, anti-IgD FITC, anti-IgM PE, anti-MHC class I ($H\text{-}2^d$), anti-MHC class II ($I\text{-}A^d$), anti-CD23 PE, and hamster anti-CD40 (IgM) monoclonal antibodies were purchased from PharMingen (San Diego, CA).

Cytokine ELISA. Cytokine levels were quantitated by double mAb sandwich enzyme-linked immunosorbent assays (ELISA) (Sad et al., 1995). 50 µl of 1-2 µg/ml of purified capture antibody in PBS was used to coat Falcon 3911 microtiter plates overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBST) (Sigma Chemical Co. St. Louis, MO) in between steps, and all subsequent incubation steps were performed for at least 30 min at room

temperature. After washing the plates, dilutions of plasma or culture supernatants (25 µl) were added to each well. 50 µl of 1-2 µg/ml of biotinylated antibody in 1% BSA-PBST were added to each well before 50 µl of strepavidin-peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in 1% BSA- PBST was added. 100 µl of either ABTS (2,2'-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co. Cat. A-1888) or TMB (3,3'5,5'-tetramethylbenzidine, free base) (Sigma Chemical Co. Cat. T-2885) were used as substrate. A yellow color solution was obtained when TMB color development was stopped with 25 µl of sulfuric acid (1:20). Color development for ABTS or TMB was measured by a spectrophotometer (Molecular Devices, Menlo Park, CA) using wavelengths of 405-490 nm or 450-570 nm, respectively.

The following capture and biotinylated-detection antibodies for each cytokine were used: JES6-1A12 and JES6-5H4-biotin for IL-2 (PharMingen); 8F8 and 43D11-biotin (Yokota et al., 1988) for IL-3; BVD4-1D11 or 11B11 (Ohara and Paul, 1985) and BVD6-24G2-biotin (PharMingen) for IL-4; TRFK5 and TRFK4-biotin (PharMingen) for IL-5; MP5-20F3 and MP5-32C11-biotin (PharMingen) for IL-6; SXC1 and SXC2-biotin (Mosmann et al., 1990) for IL-10; R4-6A2 (American Type Culture Collection; ATCC HB170) and XMG1.2-biotin (Cherwinski et al., 1987) for IFN-γ; F33 (35E10)and F34-biotin (22e9) for GM-CSF.

ELISA for immunoglobulins. The cytokine ELISA protocol as described above was used to assay mouse Ig levels in the supernatant or plasma. All coating antibodies were used at 1:1000 dilution unless stated otherwise. Affinity-purified goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM antibodies (Sigma Chemical Co.) in PBS were used to coat Falcon 3911 (Becton Dickinson

Labware, Oxnard, CA) microtiter plates at 4°C overnight. Each coating antibody exhibited minimal cross-reactivity with other Ig subclasses. After the plates were washed, dilutions of plasma or culture supernatants were added to the wells and incubated for at least 30 min at room temperature. Peroxidase conjugated anti-mouse polyvalent immunoglobulin was used for detection (Sigma Chemical Co.). Purified mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (Sigma Chemical Co.) were used as standards. Serum IgE levels were quantitated in comparison to purified mouse IgEκ by two-site sandwich ELISA (PharMingen) as previously described (Lai et al., 1996) and according to the manufacturer's recommendations. Rat anti-IgE clones R35-92 (2 μg/ml) and R35-72 (1 μg/ml) were used as coating or secondary antibodies, respectively.

IL-13 bioassay. Mouse IL-13 bioactivity was initially assayed on mouse bone marrow cells. Bone marrow cells from both hind legs of BALB/c or C57BL/6 mice were flushed with RPMI 1640, and a single cell suspension was obtained. 0.5 to 1 x 10^5 bone marrow cells were added to each well. 5 to 7 days later, the proliferation or activation response was assessed by the MTT colorimetric assay (Mosmann, 1983). Mouse IL-13 bioactivity was also assayed on human premyeloid TF-1 cells as described (McKenzie et al., 1993a). Briefly, TF-1 cells (10^3/well) were incubated in the presence of different concentrations of IL-13 for 2 or 3 days, and the proliferative or activation response was assessed by the MTT (Sigma Chemical Co.) colorimetric assay (Mosmann, 1983). Units were defined by comparison with an arbitrary laboratory standard. In comparison to purified human IL-13 (R&D Systems, Minneapolis, MN), the final purified mouse IL-13 (2.56 unit/ng) had approximately 1.4-fold higher specific activity (Lai et al., 1996), and it was used in the experiments reported in Chapter V. This purified IL-13 contained less than 0.1 ng/ml of endotoxin as determined by Limulus assay

(Sigma Chemical Co.). Subsequent experiments in Chapter VI were performed with the purified IL-13 from anion exchange and reverse phase chromatography. This purified IL-13 demonstrated a specific activity of 3.6 unit/ng and contained <0.1 ng of endotoxin per 400 μg of IL-13, as determined by the *Limulus* assay (Sigma Chemical Co.).

Statistical analyses. The unpaired Student's two-tail *t*-test was used to calculate *p*-values. Alternatively, the Kruskal-Wallis Test was used to calculate *p*-values for serum CRBC agglutination titers.

B. Materials and Methods specific for Chapter IV

Generation of stably transfected BW5147 cells expressing mammalian recombinant mouse IL-13 (IL-13). In order to modify the mouse IL-13 gene so that the sequence 5' to the ATG was more similar to the consensus sequence for translation initiation in eukaryotes (Kozak, 1987a; Kozak, 1987b) and also to facilitate subcloning, the polymerase chain reaction (PCR) was used to add the sequence TCTAGACCACC ATG GCG CTC.... to the 5' end of the coding region, and the sequence ..... CCC TTC TAA GCGGCCGC to the 3' end. The ~420 bp amplicon was digested with XbaI and NotI and ligated into the XbaI- and NotI-digested pJFE14 vector (Elliott et al., 1990). The resulting pJFEΔ123 plasmid was transfected by electroporation into the BW5147 mouse thymoma cell line along with a second plasmid containing the dihydrofolate reductase gene. Stable transfectants were selected in the presence of methotrexate (Sigma Chemical Co.) and cloned by limiting dilution. A number of stable transfectant cell lines (including 5F8) producing high levels of IL-13 were isolated.

Production and purification of mammalian IL-13. In order to collect IL-13 in a serum free medium, 5F8 cells were initially cultured in RPMI 1640 medium (GIBCO BRL), including 8% FCS (Hy-Clone), 1 μm Methotrexate, and 50 μm 2-ME (Sigma Chemical Co.), and then collected and washed before resuspending at $5x10^5$ cells/ml in RPMI 1640 with ITS^{TM} premix (Collaborative Biomedical Products, Bedford, MA.) and 50 μm 2-ME. After 5 to 7 days the supernatant containing IL-13 was collected, concentrated using Amicon YM3 ultrafiltration (Amicon Inc., Beverly, MA), and equilibrated in appropriate buffers.

Size exclusion chromatography. For size exclusion chromatography, concentrated supernatant was equilibrated in 0.5 M NaCl 10 mM HEPES pH 7.5 buffer before loading onto an FPLC (Fast Protein Liquid Chromatography) HiLoad 16/60 Superdex^{TM} 75 prep grade column (Pharmacia Biotech., Uppsala, Sweden). 1 ml fractions were collected at a flow rate of 1 ml/min. A mixture of purified standard proteins -- Ribonuclease A (1.48 mg), Chymotrypsinogen A (0.44 mg), Ovalbumin (1.04 mg), and Bovine serum albumin (1.04 mg) (Pharmacia Biotech.) -- was dissolved in the buffer and used to calibrate the column.

Cation exchange chromatography. 20 mM MES (Aldrich. Milwaukee, WI) pH 5.7 buffer was used to equilibrate the concentrated supernatant and loaded onto an FPLC Mono S^{TM} 5/5 cation exchange column (Pharmacia Biotech.). Fractions were eluted with a NaCl gradient from 0 to 1 M (in 20 mM MES pH 5.7 buffer).

Anti-IL-13 mAb (RAMP1) affinity chromatography. Either purified isotype control antibody, GL117, or purified anti-IL-13 monoclonal antibody, RAMP1

(IgG2a isotype) dissolved in PBS was bound to a Protein G column (Pharmacia Biotech.). Supernatant from 5F8 containing IL-13 was passed through this column. Fractions were collected, and the column was washed with three column volumes of PBS. Subsequently, antibody and any bound IL-13 was eluted with 0.1 M glycine pH 2.5 for at least three column volumes. Fractions were neutralized with 1 M Tris-HCl pH 9.0 and assayed for IL-13 bioactivity by TF-1 and rat Ig by ELISA.

Anion exchange chromatography. Concentrated supernatant equilibrated in 20 mM Tris-HCl pH 8.66 buffer was loaded onto an FPLC Mono Q^{TM} 16/10 anion exchange column (Pharmacia Biotech.) and proteins were eluted with a NaCl gradient from 0 to 1 M (in 20 mM Tris-HCl pH 8.66 buffer). Fractions were collected and screened for IL-13 bioactivity on TF-1 cells (see above). Positive fractions were pooled, concentrated by Centricon 3 (Amicon Inc.), and equilibrated in PBS.

Reverse phase chromatography. The concentrated supernatant or pooled positive fractions from a Mono Q^{TM} column was equilibrated in 0.1% Trifluoroacetic acid (TFA)-dH_2O. A RESOURCE RPC column (Pharmacia Biotech.) was used as a second step column to purify IL-13, and the bound proteins were eluted with 0 to 70% Acetonitrile (ACN) with 0.1% TFA at a flow rate of 2 ml/min. Fractions exhibiting bioactivity on TF-1 cells were pooled, concentrated by an Ultrafree-15 centrifugal filter device (Millipore Corp. Bedford, MA), and equilibrated in PBS. In some earlier experiments a ProRPC HR 5/10 column (Pharmacia Biotech.), and similar buffers were used except at a flow rate of 0.3 ml/min.

All fractions collected above were screened for IL-13 bioactivity in TF-1 cells or mouse bone marrow cells (see Chapter III). Positive column fractions were pooled, concentrated by Centricon 3 (Amicon Inc.), and equilibrated in PBS. Total protein was quantitated using the Bradford method (Bio-Rad Laboratories, Mississauga, Ontario), and normalized against BSA (Sigma Chemical Co.).

D10.G4.1 cells (2 x 10^6 cell/ml) were stimulated with 2 µg/ml of Con A in serum-free medium for 24 h. Supernatant was concentrated, equilibrated with 0.1% TFA-dH_2O, and filter sterilized before loading into a ProRPC 5/10 reverse phase column. This column was eluted with 0.1% TFA-ACN at 0.3 ml/min. Column fractions were collected, screened for IL-3, IL-4, IL-5, and GM-CSF using ELISA, or IL-13 bioactivity using TF-1 cells.

Electrophoresis and Western blotting. The purity of the IL-13 was assessed by 4M Urea-15% SDS-PAGE. IL-13 was also compared to natural IL-13 secreted by the Th2 clone D10.G4.1 using two Th1 clones (HDK1, M264-15) as negative controls. T cell lines were maintained as described previously (Cher and Mosmann, 1987) and serum-free supernatants were obtained by stimulating $5x10^6$ cells/ml for 48 h in RPMI 1640 containing 5 µg/ml Con A. After SDS-PAGE of recombinant or natural IL-13, proteins on gels were either stained with Coomassie Brilliant Blue R (Sigma Chemical Co.) or were transferred to Immobilon-P (Millipore Corp., Bedford, MA) in 20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3, for 2 hours at 50 volts with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Subsequently, Western blotting was performed. Briefly, the membranes were blocked for at least 2 h with 4% BSA (Sigma Chemical Co.) in washing buffer (20 mM Tris, 136 mM NaCl, and 0.1% Tween 20, pH 7.6) (Sigma Chemical Co.) and incubated with

0.1 μg/ml of RAMP1 mAb purified on a protein G column (Pharmacia Biotech.) followed by horseradish peroxidase-conjugated rabbit anti-rat IgG second antibody (Jackson ImmunoResearch Laboratories Inc.) After extensive washing, blots were incubated with Renaissance Chemiluminescence reagent (DuPont NEN Research Products, Boston, MA) and exposed to Xomat AR film (Eastman Kodak Company, Rochester, NY) or REFLECTION™ autoradiography film (E. I. DU PONT De Nemours & Co., Wilmington, DE).

IL-13 in vivo clearance rate. One ml of either PBS or IL-13 was injected intraperitoneally into three (PBS) or six (IL-13) three 6-8 week old BALB/c female mice. Approximately 100 μl of blood was collected from the tail vein of each PBS-treated mouse every hour up to 24 h. A similar amount of blood was collected from alternating groups of three IL-13-treated mice every 30 min up to 24 h. Serum was collected and assayed for IL-13 bioactivity on TF-1 cells.

Stability of purified IL-13 at 37°C. IL-13 was incubated in small aliquots at 37°C for 0 to 7 days. Each day one small aliquot was pulled from the incubator and stored at -70°C. The bioactivities of these aliquots were assessed by TF-1 cells.

C. Materials and Methods specific for Chapter V

In vivo IL-13 treatment. PBS controls or IL-13 were coded to allow blind evaluation of results and administered by Alzet micro-osmotic pumps (Alza Corporation, Palo Alto, CA). BALB/c female mice (6 to 8 weeks old) obtained from the Health Sciences Laboratory Animal Services (HSLAS) were anesthetized with Metofane, and Lacri-Lube (Allergan Inc., Markham, Ontario)

was applied to both eyes. The back of the mouse was shaved and cleaned with Clinidine solution (Clinipad Corporation, Guilford, CT). The coded osmotic pump containing PBS or IL-13 was then implanted into the peritoneal cavity through a small dorsal incision. Lastly, the peritoneal cavity wall was sutured and skin was closed with a wound clip. Three or four mice were implanted with pumps for PBS or each dose of IL-13. The mice were maintained in the HSLAS animal facility according to the guidelines of the Canadian Council on Animal Care and monitored daily for any abnormality. On day 7, blood, spleens, livers, and both hind legs were collected from the mice.

Histological and hematological analyses. Liver and spleen weights were recorded, and then small pieces of spleen from each mouse were fixed in formalin. Histological sectioning and Hemotoxylin-eosin staining were performed by S. Poppema. Whole blood was collected by cardiac puncture in a heparin-coated syringe, and a complete blood count was performed in a Coulter-ZF counter. Differential counts were performed on 100 white blood cells from a Wright-stained peripheral blood smear under a light microscope.

Immunohistochemical staining. Mac-1 (MI/70.15.1, Serotec Ltd., Kidlington, Oxford, England), Mac-3, or isotype control antibodies (PharMingen) staining was modified from Rabinovitch et al., (Rabinovitch et al., 1996). Cytospin preparations of peripheral blood nucleated cells were fixed with 1% (for Mac-1) or 4% (for Mac-3) paraformaldehyde in PBS for 10 min and washed three times with cold PBS for a total of 20 min. Cells were treated with 0.3% H_2O_2, washed with PBS, treated with 1% H_2O_2, washed and incubated with appropriate antibodies for 2 h on ice at 4°C. Subsequent steps were carried out in a humidified chamber at room temperature, with PBS washes between steps. The

cells were incubated with biotin-rabbit anti-rat immunoglobulin (Dimension Laboratories, Mississauga, Ontario) for 30 min and then with strepavidin-peroxidase conjugate (Zymed Laboratories, Inc., So. San Francisco, CA) for 30 min. Slides were developed with AEC substrate solution (Zymed) until maximum color developed and then counterstained with Hematoxylin (Sigma Chemical Co., cat # GHS-2-16). One hundred nucleated cells were analyzed using light microscopy.

Flow cytometry of spleen cells. Spleens cells were incubated with 10% normal goat serum (Jackson ImmunoResearch Laboratories) on ice for 30 minutes, stained on ice with PE-anti-CD11b (Mac-1) (Serotec Ltd.) or isotype control antibody, washed, and fixed as described previously (Sad et al., 1995). At least 15,000 cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

Spleen cell responses to hemopoietic cytokines. Spleens were homogenized with frosted glass slides in RPMI 1640 medium. After at least two washes, 1 x 10^5 spleen cells/well were cultured for 6 days in the presence of various concentrations of recombinant mIL-3, mIL-4, mGM-CSF (all collected from transfected COS 7 cells) (Yokota et al., 1984), purified IL-13 (obtained as described above), or human CSF-1. Stimulation or proliferation was assessed by the MTT assay. Spleen cells did not show any response to mock transfected COS supernatants.

Hemopoietic colony (CFU) assays. Spleen cells were obtained as described above, and bone marrow cells were obtained by flushing femurs and tibias with RPMI 1640 medium. Spleen or bone marrow cells were resuspended in

Methocult^{TM} media (Stemcell Technology Inc., Vancouver, British Columbia, Canada) according to the manufacturer's recommendations. Briefly, 3 x 10^6 spleen cells, or 3 x 10^5 bone marrow cells, or 1.5 x 10^6 Ficoll-purified peripheral blood nucleated cells were resuspended in 3 ml of methylcellulose medium, and 1 ml aliquots were plated in duplicate on 35 mm tissue culture dishes (Lux. Nunc, Inc. Napperville, IL). Erythroid (CFU-E) and myeloid (CFU-C) colonies were enumerated after 2 or 5-7 days, respectively. Erythroid burst colonies (BFU-E) were quantitated after 7 to 9 days of culture.

IL-6 and IL-10 production from spleen cells in response to LPS. Spleen cells from PBS or IL-13-treated mice were cultured in the presence or absence of 10 μg/ml of *E. coli* O26:B6 Lipopolysaccharide (LPS) (Sigma Chemical Co.) for 48 h. IL-6 and IL-10 levels in the supernatants were quantitated by two-site sandwich ELISA (Sad et al., 1995).

Nippostrongylus brasiliensis infection. Each 6-8 week old BALB/c female mouse was intradermally injected with either water or an aqueous suspension of 500 infective L_3 larvae of *Nb* (kindly provided by Dean Befus). Eight days later, the mice were anesthetized and blood was collected by cardiac puncture. Spleen cells were cultured with or without 2 μg/ml of Con A (Pharmacia Biotech.) in RPMI 1640, and the 48 hour supernatants were tested for the presence of IL-4, IL-5, and IFN-γ by two-site sandwich ELISA as described above. The frequencies of hemopoietic progenitors in the spleens were measured by CFU assays as described above. Mouse serum IgE levels were quantitated as described above.

D. Materials and Methods specific for Chapter VI

In vivo IL-13 treatment and immunization. BALB/c female mice (6 to 8 weeks old) were obtained from HSLAS. PBS controls or IL-13 were coded and loaded into Alzet micro-osmotic pumps (Alza Corporation), which were then implanted into the peritoneal cavity through a small dorsal incision as described above. Three or four mice were implanted with pumps containing PBS or each dose of IL-13. Four hours later, each mouse was immunized intraperitoneally with 0.2 ml of 50% CRBC resuspended in saline. The mice were maintained in the HSLAS animal facility according to the guidelines of the Canadian Council on Animal Care and monitored daily for any abnormality. On day 7 the mice were anesthetized, blood was collected by cardiac puncture in uncoated or heparin coated syringes, the mice were sacrificed, and their spleens were collected and weighed.

Hemagglutination assay. Direct and indirect hemagglutination assays were performed as described (Mosmann et al., 1980; Longenecker et al., 1979). Briefly, wells of round-bottom 96-well plates containing 100 µl of doubling dilutions of plasma in PNS buffer (0.05 M phosphate, 0.1 M NaCl, 1% FBS) were incubated with 100 µl of 0.5% CRBC for a minimum of 45 min, after which the wells were scored for direct hemagglutination titer. To obtain indirect hemagglutination titers, the cells were washed three times with PNS and 200 µl of a 1:1000 dilution of anti-mouse IgG1, IgG2a, IgG2b, IgG3 (Sigma Chemical Co.), or Ig pan-specific (PharMingen) antibodies were added. Titers were expressed as the reciprocal of the last dilution that gave positive CRBC agglutination.

Cytokine and antibody production from spleen cells in response to Con A or CRBC. Spleen cells from CRBC-immunized and PBS- or IL-13-treated mice were cultured in the presence or absence of 5 μg/ml of Con A (Sigma Chemical Co.) or 0.4% CRBC for 48 h or 6 days, respectively. IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, and GM-CSF levels in the supernatants were quantitated using two-site sandwich ELISAs (Sad et al., 1995). The mouse immunoglobulin ELISA was performed as described above.

Flow cytometry of spleen cells. Spleens cells were incubated with Fc blocker (2-4G.2, anti-Fc mAb) on ice for 30 minutes, stained on ice with FITC-anti-B220, or PE- or FITC- conjugated anti-CD4 or anti-CD8, or isotype control antibodies (PharMingen), washed, and fixed as described previously (Sad et al., 1995). At least 15,000 cells per sample were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

B cell purification. $B220^+$ or $sIgD^+$ B cells were obtained as previously described (Sad et al., 1995). Briefly, non-adherent spleen cells were obtained from 8-12 week old female BALB/c mice (HSLAS), stained with PE-conjugated anti-CD4 and anti-CD8 and FITC-conjugated anti-B220 or anti-IgD (PharMingen) on ice, washed, and their lymphocytes purified by Lympholyte-M CL5030 (Cedarlane Laboratories Limited) before sorting for $B220^+$ or $sIgD^+$ cells using a Coulter EPICS Elite ESP cell sorter. The purity of the sorted cells was verified to be >98.0 to 99.5% through FACScan analysis (Becton Dickinson).

B cell stimulation by fixed T cells. T cell lines were maintained as described previously (Cher and Mosmann, 1987). After being coated overnight at 4°C or

room temperature for 4 h with purified anti-CD3 (145-2C11, provided by J. Bluestone, University of Chicago, Chicago, IL) in PBS, polystyrene plates were rinsed with PBS, and T cells were added at a density of $1x10^6$ cell/ml for 24 h activation. Anti-CD3 activated T cells were collected, washed three times with PBS, and fixed with 0.4% paraformaldehyde for 5 min (Noelle et al., 1989; Kawakami and Parker, 1993). The fixed anti-CD3 activated T cells were washed with PBS and resuspended in RPMI 1640 (GIBCO BRL) before being added to cultures. $3\text{-}5x10^4$ sorted B cells were cultured for 7 days with different ratios of 0.4% PFA-fixed anti-CD3-activated M264-15 or D10.G4.1 cells. Antibody levels were measured by ELISA as described below.

Parental and CD40L stable transfectants. 2R50.20 parental mouse T hybridoma cells and the 40LDS clone expressing high levels of transfected-CD40L was provided by D. Parker. The latter cell line was maintained in G418. IL-2, IL-4, IL-5, or IL-6, and IL-13 levels were not detected in non-activated 40LDS and 2R50.20 cells.

B cell stimulation by CD40L-transfected cell line. 40LDS expressing high levels of CD40L or wildtype parental (2R50.20) cell lines growing at log phase were irradiated with 3000 rad or fixed with 0.4% PFA. RPMI (GIBCO BRL) medium containing 8% FCS (Hy-Clone), 5 μg/ml of gentamicin sulfate (GIBCO BRL), and 50 μM 2-ME (Sigma Chemical Co.) was used in all cultures. $5x10^4$ sorted $B220^+$ B cells were cultured with different numbers of 40LDS or 2R50.20 cells in the presence or absence of purified recombinant mouse IL-4 (a generous gift from Schering-Plough, NJ) or IL-13. Supernatants were harvested after 6 to 7 days of culturing, and immunoglobulin levels were quantitated as described above.

B cell stimulation by anti-CD40 antibody. $5x10^4$ sorted $sIgD^+$ B cells with at least 99.5% purity were cultured with various concentrations of anti-CD40 antibody (PharMingen) with or without purified recombinant mouse IL-4 or IL-13 or anti-IL-4 mAb (11B11). IMDM (GIBCO BRL) medium containing 8% FCS (Hy-Clone), 5 μg/ml of gentamicin sulfate (GIBCO BRL), and 50 μM 2-ME (Sigma Chemical Co.) was used in all anti-CD40 cultures. Supernatants were harvested after 6 to 7 days of culturing, and immunoglobulin levels quantitated as described below. For microcultures, the bulk cultures were serially diluted, and 10 μl of the cultures with various concentrations of anti-CD40 antibody with or without purified IL-4 or IL-13, or anti-IL-4 mAb (11B11), containing 90 to 100 sorted $sIgD^+$ B cells, were cultured in Terasaki 96 well trays (Robbins Scientific Corp., Sunnyvale, CA), which were incubated in moist containers. B cells in each well were enumerated after culturing for 2 days under a Nikon inverted microscope.

Determination of cell surface molecules by FACS. $5x10^4$ c/ml of sorted $sIgD^+$ B cells were cultured with 1 or 3 ng/ml of anti-CD40 antibody with or without purified mouse IL-4 or IL-13 for 2 days. Cells were harvested, enumerated, and stained with antibodies to MHC class II, MHC class I, CD23, and IgM (all from PharMingen). 25,000 cells/population were analyzed by a FACScan and CellQuest software (Becton Dickinson).

ELISPOT assay for mouse IgM production. B cells from each Terasaki well were transferred to MultiScreen 96-well plates (Millipore Corporation) which were coated with affinity-purified goat anti-mouse IgM antibody at 1:1000 dilution (Sigma Chemical Co.). After overnight incubation, the plates were

developed for antibody spots. After each step, the incubation buffers were filtered and washed with 0.05% Tween 20 (Sigma Chemical Co.) in PBS. Each well was incubated with biotinylated affinity-purified goat anti-mouse Ig dissolved in PBS buffer containing 1% FCS and 1% Tween 20 for 1 h. After being incubated with peroxidase-conjugated strepavidin (Jackson ImmunoResearch Laboratories Inc.), the plates were washed and developed with AEC (Aminoethyl carbazole) substrate (Vector Laboratories, Inc. Burlingame, CA) according to the manufacturer's instructions. The plates were dried, and antibody spots were enumerated.

B cell proliferation assay. Sorted $sIgD^+$ B cells ($5x10^4$ or $1x10^4$/well) were cultured with various concentrations of anti-CD40 antibody with or without purified IL-13 or IL-4 for three days. One μCi/well of ^3H thymidine (Amersham Life Science Inc.) was added during the final 18 h of culture. Cells were harvested using a MicroMate 196 cell harvester (Canberra Packard, Meriden, CT), and radioactive incorporation was determined on a Matrix 96 (Canberra Packard).

Determination of B cell division. Sorted $sIgD^+$ B cells were stained with CFSE (5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester, Molecular Probes, Eugene, OR) as described (Lyons and Parish, 1994). Briefly, the cells were washed with IMDM and stained with 0.1 to 1 μM CFSE for 10 min. At the end of the incubation, the cells were washed three times with ice cold IMDM containing 8% FCS. The stained cells (at a density of $1.25x10^5$ c/ml) were cultured in a 24-well Falcon plate (Becton Dickinson Labware) in the presence of 3 ng/ml of anti-CD40 with or without 5 to 20 ng/ml of IL-13 or 10 ng/ml of IL-4. On day three, the cells were harvested and washed, and viable cells were counted

using Trypan Blue. Cell division was assessed by flow cytometry analysis of 25,000 cells/population using a FACScan and CellQuest software (Becton Dickinson). Dead cells were excluded based on their light-scattering properties.

E. Materials and Methods specific for Chapter VII

Generation of pCRβTP600 plasmid (strong and general regulatory region). The pCRβT plasmid containing an ampicillin resistance gene, hCMV promoter/ enhancer, rabbit β-globin splice site, and rabbit β-globin poly-A fragments was kindly provided by Dr. J. Marth (UBC, Vancouver, British Columbia). This vector was digested with XhoI and treated with Calf intestinal alkaline phosphatase (CIP) (Promega, Madison WI) according to the manufacturer's instructions. After separation by electrophoresis in 1% agarose in TBE buffer, the linearized 4.5 kb vector was excised and purified using a GENECLEAN II kit (BIO 101 Inc., La Jolla, CA).

The mouse IL-13 cDNA fragment (427 bp) was excised from pJFEΔ123 plasmid (see Materials and Methods specific for Chapter IV) using XbaI and EcoRI (Boehringer Mannheim, Germany) for at least 4 h at 37°C. 5 mM DTT, 250 μm dNTP's, and Klenow enzyme (labeling grade) (Boehringer Mannheim) were added to fill the overhangs in the digested fragments. DNA fragments were separated by electrophoresis on 1% agarose in TBE buffer. The blunt-ended 427 bp-mouse IL-13 cDNA was excised from agarose and purified using a GENECLEAN II kit. Next, the purified cDNA was ligated overnight with XhoI linker, d(pCCCTCGAGGG), in the presence of T4 DNA ligase (Boehringer Mannheim) at 14-16°C, before the modified cDNA was digested with XhoI. This modified IL-13 cDNA with XhoI overhangs was purified using a GENECLEAN II kit and

quantitated by a Fluorimeter (Tyler Research Instruments Corporation, Edmonton, Alberta). An aliquot of the purified cDNA was electrophoresed on a 1% agarose/TBE gel before ligating to the CIP-treated vector in the presence of T4-DNA-ligase (Boehringer Mannheim) at 14-16°C.

The ligation mixture was transformed into DH5α competent cells (GIBCO BRL, Burlington, Ontario) according to the manufacturer's instructions. Transformed cells were inoculated and cultured on ampicillin (50 μg/ml) agar plates overnight. Ampicillin resistant colonies were screened for the insert using one of the following IL-13 oligonucleotide probes, which overlap different exons.

P600-3

(dGGCCTTGCGGTTACAGAGGCCATGCAATATCCTCTGGGTCCTGTAGA TGGCATTGCAATT) or

P600-4

(dGGAGATGTTGGTCAGGGAATCCAGGGCTACACAGAACCCGCCAGCG GCCAGGTCCACACT),

Briefly, the plates were refrigerated for at least 30 min. Bacteria colonies were transferred to Colony/Plaque screen (NEN Research Products, Boston, MA). In between the following steps, membranes were air dried on paper towels for 2-3 min. Bacteria were lysed twice with 0.5 M NaOH for 2-3 min each time. The membranes were neutralized with 1 M Tris-HCl pH 7.5 for 2 min and 0.5 M Tris-HCl pH 7.5 for 2 min. Lastly, the membranes were washed in 3X SSC, air dried, and baked at 80°C for 2 h.

Generation of pML179P600 plasmid (T cell specific transgene). The pML179 vector was kindly provided by Dr. J. Marth. pML179P600 plasmid was generated as described in the section above with some modifications. The mouse IL-13

cDNA fragment (427 bp) was excised from the pJFEΔ123 plasmid (see Materials and Methods specific for Chapter IV) using XbaI and NotI (Boehringer Mannheim) for at least 4 h at 37°C. 5 mM DTT, 250 μm dNTP's, and Klenow enzyme (labeling grade) (Boehringer Mannheim) were added to the blunt end of the digested fragments. The blunt-ended 413 bp-mouse IL-13 cDNA was purified and ligated overnight with BamHI linker, d(pCGGGATCCCG), in the presence of T4 DNA ligase (Boehringer Mannheim) at 14-16°C, before digesting with BamHI. This modified IL-13 cDNA with BamHI overhangs was purified and ligated to the BamHI-digested and CIP-treated pML179 vector in the presence of T4 DNA ligase (Boehringer Mannheim) at 14-16°C. Positive colonies were identified using an IL-13 oligonucleotide probe as described above.

Oligonucleotide or DNA labeling. Oligonucleotides were labeled using T4 polynucleotide kinase (GIBCO BRL) in a forward labeling reaction to add a radioactive-phosphate group to the nucleic acid residues with 5' hydroxyl groups, according to the manufacturer's recommendation. Briefly, 5 pmol of oligonucleotide was incubated with forward reaction buffer (350 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 500 mM KCl, and 5 mM 2-ME), 10 units of T4 polynucleotide kinase, [γ-^{32}P]ATP (10 μCi/ml, 2000 Ci/mmol) (Amersham Life Science Inc. Oakville, Ontario), and autoclaved water for at least 10 min. at 37°C. Oligonucleotides were separated from free [γ-^{32}P]ATP with a G-50 Sephadex column which was equilibrated in TE pH 7.6 buffer. Radioactive oligonucleotide fractions were identified by a gamma counter (LKB, Pharmacia Biotech), pooled, and stored at -70°C.

The pCRβT vector was digested with SalI (Boehringer Mannheim), and the hCMV promoter/enhancer (632 bp) fragment was purified and quantitated as described

above. This fragment was labeled with [α^{32}P]dCTP (3000 Ci/mmol) (Amersham Life Science Inc.), using a random primed DNA labeling kit (Boehringer Mannheim). Similar to the labeled oligonucleotides, DNA fragments were purified using G-50 Sephadex. DNA fragments were boiled for 5 min before being added to the hybridization solution.

Hybridization and screening with ^{32}P-labeled oligonucleotide or DNA. Membranes were prehybridized with prehybridization/hybridization solution containing 50% formamide (GIBCO BRL) at 42°C for at least 2 h in a Hybridization incubator HI-12000 (Tyler Research Instruments Corporation). Then ^{32}P-labeled-oligonucleotide or boiled random-primed ^{32}P-labeled-DNA was added for overnight hybridization at 42°C. The membranes were washed twice for 15 min each with 2X SSC, 0.1% SDS at room temperature and 0.1X SSC, 0.1% SDS at 68°C. After being rinsed briefly with 0.1X SSC, the membranes were exposed to autoradiography film (Reflection NEF-496, DU PONT, Wilmington, DE) at -70°C.

Positive colonies were selected and expanded in miniprep cultures. Plasmids from these colonies were purified (Magic minipreps, Promega), and restriction sites were verified with restriction digest mapping. Finally, the inserted IL-13 cDNA was sequenced.

In vitro expression of the transgenes. Monkey kidney COS 7 cells were propagated in D-MEM containing 8% FCS. COS 7 cells were transfected either with no plasmid (MOCK), pSP6KIL-13, pCDSRαIL-13, pJFEΔ123, pCRβTP600, or pML179P600 plasmid using the DEAE dextran/chloroquine method (Moore et

al., 1990). Seventy-two hours later, COS 7 supernatants were collected, and IL-13 bioactivity was tested with TF-1 or mouse bone marrow cells as described above.

Alternatively, pJFEΔ123, pCRβTP600, or pML179P600 plasmid was transfected into the EL4-IL-2 cell line using the DEAE-dextran procedure (Gorman et al., 1982). Briefly, EL4-IL-2 cells were grown in DMEM containing 7% FCS. While growing in log phase, the cells were washed three times with DMEM-High glucose followed by Tris Buffered Saline (TBS: 25 mM Tris pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$,, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). $2x10^7$ cells in 1 ml of TBS were incubated with 15-20 μg of DNA resuspended in 1 ml of 500 μg/ml of DEAE-dextran at 37°C for 30 to 120 min. Subsequently, the cells were washed once with TBS and 2 times with DMEM-High glucose and plated in DMEM-High glucose with 7% FCS. Cell supernatants were harvested after 7 days and tested for IL-13 bioactivity.
(Mosmann et al., 1990)

Transgenic mice production. The 2.2 kb transgene was purified from SphI-digested pCRβTP600 plasmid and reconstituted at a concentration of 5 ng/μl before microinjection into embryos. The 349-1 founder carrying the pCRβTP600 transgene was generated in collaboration with Dr. J. Marth at UBC (Vancouver, B. C.), whereas the 150 founder was generated at UA HSLAS according to their guidelines and transgenic techniques S.O.P. Briefly, superovulation was induced in 6-8 week old female (C57BL/6XCBA/J)F1 or BALB/c mice by intraperitoneal injection of 0.1 ml of PMSG (pregnant mares serum gonadotropin) and 0.1 ml of hCG (human chorionic gonadotropin) 47 h later. The female mice were then mated immediately with (C57BL/6XCBA/J)F1 or BALB/c mice. Approximately 18 h later, the embryos in the oviduct were collected in M2 medium containing

hyaluronidase (Sigma Chemical Co.) and stored in CZB or M16 medium (Sigma Chemical Co.) at 37°C with 5% CO_2. Pronuclears of the embryos were injected with 5 ng/µl of the diluted transgene under a Leica microscope. After the pronuclei microinjection, the embryos were incubated in CZB or M16 medium at 37°C with 5% CO_2 overnight or until implantation on the same day. FvB pseudo-pregnant mice (mated with vasectomized FvB mice) were used as surrogate mothers. The surrogate mothers were anesthetized with 0.2 ml of a mixture of hypnorm, midazolam, and sterile water (1:1:2 ratio). Lacri-Lube (Allergan Inc.) was applied to both eyes. The back of the mouse was shaved and cleaned with Clinidine (Clinipad Corp.). An incision was made on the dorsal of the mouse. The ovary, oviduct, and ovarian fat pad were located, and the fat pad was anchored with a serafine clamp. Under a dissecting microscope, ten surviving embryos at the two-cell stage (after overnight incubation) were implanted into the infundibulum using a thinly-drawn glass pipette. After the implantation, the peritoneal cavity wall was sutured with 5/0 Dexon absorbable suture (Ethicon, Inc.), and the skin was closed with a wound clip. The mouse was kept on a heating pad overnight during the recovery. The wound clips were removed after 7 days. All mice were kept and monitored in the VAF animal facility (HSLAS). Tail biopsy was performed on the mice one week after weaning. Founders were backcrossed to C57BL/6 mice and maintained in the VAF animal facility according to the guidelines of the Canadian Council on Animal Care.

Transgenic mice screening by dot blot. Possible founders and their offspring were screened using the dot blot method. Briefly, 1 to 2 cm of tail biopsy samples were digested with 1 mg/ml of Proteinase K (Boehringer Mannheim) in 300 µl of TE (50 mM Tris pH 8.0, 50 mM EDTA, and 1% SDS) overnight at 55°C. After adding 300 µl of TE (10 mM Tris pH 8.0, and 1 mM EDTA) to the digested tail

samples, 600 µl Phenol/Chloroform was added and mixed well. Samples were centrifuged for 5 min before removing the aqueous layer. 600 µl of Chloroform was mixed with the aqueous solution containing genomic DNA and centrifuged for another 5 min before transferring the aqueous layer to a clean eppendorf tube. Genomic DNA was precipitated with 2 to 3 volumes of cold ethanol and pelleted by centrifugation. Lastly, genomic DNA was dissolved in TE (20 mM Tris pH 8.0, and 1 mM EDTA) and quantitated using a Fluorimeter (Tyler Research Instruments Corporation).

Approximately 5 µg of each tail DNA sample was spotted on a nitrocellulose membrane and air dried. DNA samples were denatured twice for 1 min each with 0.5 M NaOH, 1.5 M NaCl and neutralized twice for at least 1 min each with 0.5 M Tris pH 7.0, 1.5 M NaCl. Finally, the membranes were baked at 80°C for at least 2 h. Membranes were prehybridized, hybridized, and washed as described above. Autoradiography films (Reflection NEF-496) were exposed for at least 2 days at -70°C before being developed.

hCMV-IL-13 transgene detection by PCR. Genomic mouse tail DNA was extracted using the QIAamp Tissue Kit (QIAGEN) and quantitated with a Fluorimeter. 1-2 µg of purified genomic DNA was used in each PCR reaction with either set of primers.

<u>5'P600 (266 bp amplicon)</u>

5' linker primer 5'-GTCTAGACCACCATG-3'

3' primer 5'-GTCCTGTAGATGGCATTG-3'

<u>3'P600 (260 bp amplicon)</u>

5' primer 5'-ATGGTATGGAGTGTGGAC-3'

3' linker primer 5'-GAGGGGAATTCGGCC-3'

Total RNA purification. Total RNA from tissue samples or cells in suspension was extracted using TRIzol reagent (GIBCO BRL) based on the RNA isolation procedure developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly, homogenized tissue samples or cells were incubated with the TRIzol reagent. Chloroform was added to the samples. After centrifugation at 4°C, the aqueous phase containing RNA was isolated, and isopropyl alcohol was used to precipitate the total RNA. The total RNA was pelleted by centrifugation and washed with 75% ethanol. The RNA pellet was dissolved in RNase-free water and stored at -70°C. Total RNA was quantitated by spectrophotometer and A260/280 values were calculated.

First strand cDNA synthesis. Reverse transcription was carried out according to the manufacturer's recommendation. In a 20 µl reaction, 2 µg of total RNA was incubated with $1x10^{-4}$ OD unit of oligo-dT (dT_{24}) at 70°C for 10 min before incubating with First strand buffer (GIBCO BRL), 10 mM DTT, 0.5 mM dNTP (500 mM each dATP, dGTP, dCTP, and dTTP), and RNase-free water for 2 min at 37°C. Subsequently, the mixture was incubated with 200 units of SuperScript RNase H^- for 1 h at 37°C before the reaction was terminated with 10 min incubation at 70°C.

General PCR assay. A 50 µl reaction contained 50 µM dNTP, 0.01 OD unit of each of the primers, Taq buffer, and DNase- and RNase-free water. Five µl of the reverse transcribed sample, standard, or 1-2 µg of genomic DNA was added along with mineral oil. Samples were incubated at 95°C for 2 min, and at least 2.5 units of Taq DNA polymerase were added while the samples were incubating at 72°C for 4-5 min. PCR was performed for 35 cycles of 1 min at 95°C, 2 min at 72°C,

and 1 min at 42°C, in RoboCycler 40 (Strategene La Jolla, CA). Samples were analyzed by 2% agarose-TBE.

Primers and the expected amplicons were as follows :

Mouse β–actin (540 bp amplicon)

5' primer 5'-GTGGGCCGCTCTAGGCACCAA-3'

3' primer 5'-CTCTTTGATGTCACGCACGATTTC-3'

IL-13-1 (266 bp amplicon)

5' primer 5'-GTCTAGACCACCATG-3' (5'P600 linker)

3' primer 5'-GTCCTGTAGATGGCATTG-3' (3'P600)

IL-13-2 (257 bp amplicon)

5' primer 5'-ATGGTATGGAGTGTGGAC-3' (5'P600)

3' primer 5'-GAGGGGAATTCGGCC-3' (3'P600 linker)

IL-13-3 (889 bp amplicon)

5' primer 5'-GTCTAGACCACCATG-3' (5'P600 linker)

3' primer 3'-CACTACACATCACCTTGAGTGTAACAGGCC-3' (TM20 NC 1092-1121)

Generation of the in vitro transcription template for Ribonuclease protection assay (RPA). Both pCDSRαP600 and pBluescript II SK+ (Strategene) were digested with PstI (Boehringer Mannheim) and EcoO109I (New England Biolabs Inc., Beverly, MA). After purification by agarose and a GENECLEAN II kit (BIO 101 Inc.), the 307 bp fragment from pCDSRαP600 was subcloned into the 2910 bp linearized vector without the PstI-EcoO109I 51 bp fragment. Ampicillin resistant colonies were isolated and expanded for miniprep cultures. Positive colonies, L121.1 and L121.4, were identified by restriction mapping using PvuII

(Boehringer Mannheim). The 503 bp multi-cloning site flanked by T3 and T7 RNA promoters containing the PstI-EcoO109I P600 cDNA fragment was digested with BssHI (New England Biolabs, Inc.) and gel purified.

In vitro transcription with T3 RNA polymerase. 1 μg of BssHI-digested L121.1 cassette or pSP6/β-actin control template was transcribed with MAXIscript (Ambion Inc., Austin, TX) using 10 units of T3 RNA polymerase. The transcription reaction contained 200 mM DTT, 10 mM ATP, 10 mM CTP, 10 mM GTP, RNase inhibitor, 2 μl of 10X transcription buffer, and 5 μl of [α-^{32}P]UTP, ≥ 400 Ci/mmol. This mixture was incubated at room temperature for at least 30 min before the DNA template was digested with RNase-free DNaseI for 15 min at 37°C. The probes were separated by 8 M urea-5% acrylamide gel in TBE buffer for 20 min to 1 h at 250 volts. The gel was exposed to film, and the full length probes were excised and eluted overnight at 37°C with elution buffer provided in the RPA II kit (Ambion Inc.). The labeled RNA was stored at -70°C.

Ribonuclease protection assay. RPA II (Ambion Inc.) was used according to the manufacturer's instructions. Briefly, 1-2 μg of total RNA from D10.G4.1 stimulated with Con A or tissue samples were heated at 95°C for 5 min with the *in vitro* transcribed ^{32}P-labeled probes in Solution A and then incubated overnight at 42°C. RNase was added to digest the unprotected RNA for 30 min at 37°C. The protected RNA fragments were precipitated with Solution D and ethanol for 15 min at -20°C. RNA pellets were centrifuged and analyzed by 8 M urea-5% acrylamide gel in TBE buffer. The gels were exposed to films for 2 days at -70°C.

CHAPTER IV

RECOMBINANT IL-13 EXPRESSION AND PURIFICATION

(Some of the data presented in this chapter have been published in an article entitled "Continuous Administration of IL-13 to Mice Induces Extramedullary Hemopoiesis and Monocytosis" by Yew Hon Lai, Jean-Marie Heslan, Sibrand Poppema, John F. Elliott and Tim R. Mosmann in *J. Immunol.* 156:3166-3173.)

A. Introduction

The first part of this project, involved short term *in vivo* administration of IL-13 in mice. Despite the relative abundance of the mIL-13 mRNA which can be up to 0.3% of poly-A mRNA expressed by stimulated-Th2 cells (Brown et al., 1989), it is not feasible to purify natural mIL-13 from Th2 cells when a large quantity of mIL-13 is required for *in vivo* studies. In addition, the majority of cytokine studies are performed with various forms of recombinant cytokines. Recombinant cytokines are generally expressed in prokaryotic cells as the methodologies for their expressions have been well established. However, eukaryotic forms of recombinant cytokines not only offer alternative sources of recombinant cytokines but also may provide the additional advantages that the protein folding and post-translational modification are more likely to be similar to the natural forms of cytokines. For the *in vivo* studies described in this thesis, recombinant mammalian rather than prokaryotic, IL-13 was produced. Recombinant mouse IL-13 was stably-expressed in the BW5147 mouse thymoma cell line. Subsequently, various strategies were utilized to purify recombinant mouse IL-13 from serum-free

supernatants of IL-13-transfected BW5147 cells. Lastly, the *in vitro* and *in vivo* stability of purified IL-13 will be described.

B. Results

Generation of BW5147-IL-13 stable transfectant. In order to obtain large amounts of purified mammalian recombinant IL-13, collaboration was initiated with Dr. J. F. Elliott's laboratory to produce the stable mouse IL-13 transfectant. Due to the high frequency of co-insertion of Dihydrofolate reductase (DHFR) and IL-13 cDNAs, DHFR and IL-13 cDNA (pJFEΔ123) constructs were co-transfected into the BW5147 mouse thymoma cell line. Methotrexate was added to the culture medium, which allowed selection of cells that had acquired methotrexate resistance by amplifying the copy numbers of the transfected DHFR cDNA. This usually resulted in co-amplification of the IL-13 cDNA copy numbers. Methotrexate-resistant transfectants were screened for IL-13 production by Dr. Jean-Marie Heslan, and high IL-13 producing clones, 5F8 and 4F11, were identified by mouse bone marrow assay.

To minimize protein contamination from FCS for subsequent purification, I optimized the serum-free culture conditions for IL-13 production by the 5F8 clone. Cells from the 5F8 clone growing at log phase in FCS were harvested and washed before being resuspended in ITS serum-free medium at various cell densities. Supernatants were collected, and IL-13 levels were assayed by TF-1 bioassay. After at least four days in the serum-free culture medium at an initial seeding density of $5x10^5$ c/ml, this clone can produce up to 1 μg/ml of IL-13 in the supernatant. Due to its high production level, this clone was used as a source for IL-13 purification. Purified IL-13 was subsequently used for *in vitro* and *in vivo* studies.

Purification of mammalian recombinant mouse IL-13. Various chromatography methods were utilized to purify IL-13.

Size exclusion chromatography. A size exclusion column, HiLoad 16/60 Superdex 75, was calibrated with a known protein standard consisting of a mixture of Ribonuclease A, Chymotrypsinogen A, Ovalbumin, and Bovine serum albumin. This protein mixture was separated into four peaks based on their sizes with BSA (67 kDa) eluted first followed by Ovalbumin (43 kDa), Chymotrypsinogen A (25 kDa), and Ribonuclease A (13.7 kDa) (Fig. 4.1). When the log of the molecular weight of the four proteins was plotted against the fraction number of the maximum absorbance for each of the four peaks, a relative straight curve was obtained, indicating that the rates of migration through the column are proportional to the molecular weight of the proteins (Fig. 4.2). Thus, this column gives excellent resolving power for proteins in the tested molecular weight ranges.

Next, attempts were made to separate IL-13 from other major proteins in the concentrated serum-free supernatant of IL-13-transfected-BW5147 cells using this size exclusion column. Column fractions were collected at the rate of 1 ml/min. Based on the bioactivity on mouse bone marrow cells, IL-13 bioactivity was detected in a majority of the column fractions (Fig. 4.3), suggesting that IL-13 from BW5147-transfected cells was aggregated or heterogeneous in nature. Similar results were obtained when Triton X-100 was added to the chromatography buffer (data not shown).

In order to determine whether IL-13 was secreted as aggregates due to over production by BW5147 transfectants, the secreted forms of IL-13 by COS 7 cells

were investigated. COS 7 cells were transiently transfected with IL-13 plasmids and cultured in 5% FCS-DMEM medium. Concentrated and pooled day 3 and 6 supernatants from IL-13-transfected COS 7 cells were applied to the size exclusion column. Biologically active IL-13 was eluted either as monomeric or dimeric forms as revealed by the mouse bone marrow cell bioassay (Fig. 4.4). Based on the absorbance profile, IL-13 was separated from other proteins produced by COS 7 cells, and IL-13 was secreted as a minor component. It is possible that other protein molecules in the COS 7 supernatant may have affected the migration rate of IL-13 in the size exclusion column. Consequently, the mouse bone marrow cell bioassay positive fractions from Fig. 4.4 were pooled and chromatographed over the size column in order to determine the majority of the biologically active forms of COS 7-IL-13. Interestingly, IL-13 was further separated from other proteins, and it appeared to migrate as monomer (Fig. 4.5).

Cation exchange chromatography (Mono S). Analysis of IL-13 amino acid composition indicate that it is a basic protein with a predicted pI of 8.15. As a result, a cation exchange column, Mono S, was used to purify IL-13 from 5F8 supernatant. The majority of the proteins were eluted at low concentrations of Na^+, and IL-13 was poorly separated from other proteins in the supernatant (Fig. 4.6). Despite the fact that acetate and MES buffers with pH's ranging from 5.0 to 6.0 were used, IL-13 could not be separated from other proteins in the serum-free supernatants (data not shown).

Affinity chromatography using RAMP1 anti-IL-13 mAb. In the meantime, Dr. J. -M. Heslan (in our laboratory) had generated a Sprague-Dawley rat anti-mouse-IL-13 mAb (IgG2a), RAMP1, using a GST-IL-13 fusion protein as an antigen (J. -M. Heslan, R. Marcotte, and T. R, Mosmann, unpublished). I have

attempted to purify IL-13 by affinity chromatography. Either the isotype control mAb, GL117, or RAMP1 was bound to a Protein G column in PBS buffer, and supernatant of BW5147-IL-13 cells was incubated in the column. PBS was used to wash off unbound BW5147-IL-13, and fractions were collected. Lastly, GL117 or RAMP1 was eluted with 0.1 M glycine pH 2.7 along with any bound IL-13. Column fractions were then assayed for IL-13 bioactivity or Ig levels by mouse bone marrow cells or ELISA, respectively. Neither GL117 or RAMP1 depleted biologically active IL-13 from the supernatant (Fig. 4.7). In addition, RAMP1 did not appear to block biological activity of IL-13 (J. -M. Heslan and T. R. Mosmann unpublished). However, RAMP1 was an excellent mAb for Western blotting (see below) (Lai et al., 1996). Thus, RAMP1 recognized denatured IL-13 but not biologically active IL-13 molecules.

Anion exchange chromatography (Mono Q). Next, mammalian IL-13 was purified from the concentrated supernatants of the 5F8 cell line by a Mono Q^{TM} anion exchange chromatography. The protein elution profile was assessed by the Bradford reaction (Fig. 4.8a), and the bioactivity profile was assayed on TF-1 cells (Fig. 4.8b). IL-13 bioactivity was detected in three regions, and the relative amounts of these three peaks varied in different experiments. In order to assess the protein composition of each fraction, column fractions were electrophoresed under denaturing conditions and stained with Coomassie blue. The first two peaks (peaks 1 and 2) of biologically active IL-13 were separated from the majority of the other proteins in the supernatant with a shallow NaCl gradient, whereas the third peak eluted along with other proteins at high ionic concentrations. Fractions from both peak 1 (fractions 20-24) and peak 2 (fractions 30-34) contained prominent ~14 kDa protein, and lesser amounts of heterogeneous material at higher molecular weights (Fig. 4.9a). The ~14 kDa

band of peak 1 migrated similarly to the upper band of the ~14 kDa closely spaced doublet in peak 2. This was further confirmed by Western blotting as the anti-mIL-13 mAb, RAMP1, strongly recognized the prominent ~14 kDa bands from peaks 1 and 2 (Fig. 4.9b). Thus, these peaks appeared to contain different isoforms of IL-13. Interestingly, Western blotting (Fig. 4.9b) and immunoprecipitation (J.-M. Heslan, and T. R. Mosmann unpublished) revealed the binding of RAMP1 to small amounts of heterogeneous material at higher molecular weights, which might be due to carbohydrate heterogeneity on IL-13 produced by 5F8.

Comparison of mammalian recombinant and native IL-13. In order to compare the IL-13 to the native IL-13 secreted by a mouse Th2 cell line, supernatant from the 5F8 stable transfectant, the wild type non-transfected wild type parental cell line (BWWT), and Con A-stimulated D10 (Th2), HDK-1 (Th1), and M264-15 (Th1) cells were analyzed by Western blotting with RAMP1 (Fig. 4.9c). RAMP1 recognized a ~14 kDa doublet of native IL-13 secreted by the Th2 cell line D10, but not by either of the Th1 cell lines HDK-1 or M264-15. The majority of IL-13 from the 5F8 stable transfectant migrated as a doublet of ~14 kDa and a band of ~10 kDa (Fig. 4.9c), whereas all these bands were absent in the supernatant of the parental BWWT cell line. The ~14 kDa components in peaks 1 and 2 were used for the *in vivo* experiments because they represented the major IL-13 components in the 5F8 supernatant and migrated similarly to Th2-derived IL-13 in SDS-PAGE. Peak 1 (fractions 20-24) of IL-13, which were slightly more homogeneous and had slightly higher specific activity, were pooled, concentrated, and equilibrated with PBS before being administered to the mice. In comparison to commercially available purified human IL-13, this purified mouse IL-13 preparation (2.56 units/ng) had approximately 1.4-fold higher

specific activity. Results shown in Chapter V were obtained with peak 1 IL-13. In other experiments, similar results were observed when a combination of peaks 1 and 2 was administered.

Reverse phase chromatography (RESOURCE RPC). Reverse phase chromatography was used as a second step column in purifying IL-13 from peak 2 of the Mono Q^{TM} column. Further purification was achieved with reverse phase chromatography as biologically active IL-13 was eluted relatively late compared to other proteins (Fig. 4.10a & b). Fractions 52 to 65 were pooled, concentrated, and equilibrated in PBS. The protein composition of this preparation was assessed using 4 M urea-15% SDS-PAGE electrophoresis, Coomassie blue staining, and Western blotting with RAMP1. The majority of the protein migrated as ~14 kDa doublet with small amounts of heterogeneous material at higher molecular weights (Fig. 4.9d). After anion exchange and reverse phase chromatography, purified IL-13 exhibited a specific activity of 3.6 units/ng with <0.1 ng of endotoxin (as determined by the *Limulus* assay) per 400 μg of IL-13. This material was used in the studies described in Chapter VI. Overall, the recovery of biologically active of IL-13 after anion exchange and reverse phase chromatography were 60-80% and 110-135%, respectively.

Evaluation of the specificity of human TF-1 cells in response to mouse cytokines. To determine the specificity of TF-1 cells to mouse cytokines, serum-free supernatant from Con A-stimulated Th2 cells, D10.G4.1, was chromatographed over a ProRPC reverse phase column. Proteins were eluted with a gradual gradient of Acetonitrile. Fractions were collected and assayed for different cytokines using cytokine sandwich ELISA or TF-1 bioassay. All cytokines tested were separated from other major proteins in the Con

A-stimulated D10.G4.1 supernatant (Fig. 4.11). In addition, this column separated mouse IL-3, IL-4, IL-5, and GM-CSF from IL-13 as identified by TF-1 bioassay (Fig. 4.11). Thus, human TF-1 cells appear to respond to natural mouse IL-13 or other unidentified cytokines, but not to mouse IL-3, IL-4, IL-5, or GM-CSF.

In vivo clearance rate of IL-13. In order to estimate the IL-13 *in vivo* clearance rate, IL-13 was injected intraperitoneally into BALB/c mice, and approximately 100 µl of blood was collected from the tail vein every hour for up to 24 h. IL-13 appeared in the circulation as early as 5 min and reached the maximum level in 90 min (Fig. 4.12). The exact *in vivo* half life of IL-13 could not be determined due to the slow release of IL-13 from the peritoneal cavity into the circulation. However, half of the IL-13 in the circulation was cleared in less than 34 min, and it was cleared to below detection limit (<20 units/ml) by 180 min. Thus, it could be deduced that the *in vivo* clearance rate of IL-13 was less than 34 min. The exact *in vivo* half-life of IL-13 was not studied in more detailed because, based on this experiment, IL-13 exhibited relatively short *in vivo* half-life. Moreover, bolus iv injection of IL-13 did not appear to be a feasible strategy to achieve sustained level of IL-13 in the circulation.

Knowing that IL-13 had a relatively short half life *in vivo* and that the experimental design in this study required the administration of purified IL-13 over a period of seven days, the ALZET osmotic pump with a delivery rate of 0.5 µl/h, was chosen to administer purified IL-13 in the peritoneal cavity of a mouse. This also prevented frequent handling of the animals during the experimental period.

Stability of purified IL-13 at 37°C. In order to study the stability of purified IL-13 at 37°C for the entire duration of the experiments, IL-13 was incubated in small aliquots at 37°C in 8% CO_2 for 0 to 7 days. Bioactivity of these aliquots was assessed by TF-1 cells. It appeared that purified IL-13 bioactivity diminished rapidly after 2 days of 37°C incubation (Fig. 4.13). However, residual biologically active IL-13 could be detected for up to 7 days.

C. Discussion

Prior to *in vivo* administration of IL-13, large amounts of IL-13 are required. Generally, a large quantity of cytokine can be obtained by expressing the cytokine recombinantly either in mammalian or prokaryotic cells. For this study, IL-13 was produced in mammalian BW5147 mouse thymoma cells, rather than prokaryotic cells, because protein folding and post-translational modification were more likely to be similar to natural IL-13. Although no obvious differences between the bioactivities of mammalian and *E. coli* IL-13 *in vitro* have been observed (J.-M. Heslan, Y. H. Lai and T. R. Mosmann, unpublished), it is possible that post-translational modifications (e.g. carbohydrate addition) may affect *in vivo* properties such as the half-life or tissue distribution.

After expressing IL-13 in mammalian cells, attempts were made to purify this cytokine using various chromatography methods. Due to the aggregated or heterogeneous nature of the IL-13, it migrated at a wide range of speed in a size exclusion column. A similar chromatography profile was obtained when recombinant IL-13 migrated over this size exclusion column in the presence of detergent, indicating that IL-13 aggregates could not be solubilized in the presence of Triton X-100. Alternatively, different isoforms or heavily glycosylated IL-13 was produced by 5F8. This is consistent with the observations with Western blotting (Fig. 4.8d) and immunoprecipitation (J.-M. Heslan, and T. R. Mosmann unpublished) which revealed the binding of RAMP1 to small amounts of heterogeneous material at higher molecular weights that may be due to carbohydrate heterogeneity on IL-13 produced by 5F8. In comparison, IL-13-transfected COS 7 cells secreted biologically active IL-13 as monomers, a result which is consistent with recombinant hIL-13 (Minty et al., 1993; McKenzie

et al., 1993a; Zurawski and de Vries, 1994). Nevertheless, IL-13 produced by COS 7 and BW5147 cells were biologically active.

Attempts to use anion exchange or antibody affinity columns to purify IL-13 were not successful. The anion exchange column did not separate IL-13 from other proteins in the supernatant of the BW5147-IL-13 cell line. RAMP1, the only anti-IL-13 mAb generated in this laboratory, did not appear to recognize biologically active IL-13. However, it proved to be an excellent Western blotting antibody for IL-13 (Lai et al., 1996).

By using a shallow NaCl gradient, various isoforms of IL-13 were isolated from other major proteins in the supernatant. As IL-13 from peak 1 was more homogenous compared to that of peak 2, and both Coomassie blue staining and Western blotting exhibited similar profile on the electrophoresed gels or blots, peak 1 IL-13 was administered in mice for the studies described in Chapter V. Due to its high loading capacity, a Mono Q^{TM} column was subsequently used as the first column step to purify IL-13.

In order to purify IL-13 in peak 2, reverse phase chromatography was used as a second step column purification. IL-13 was separated from other minor contaminating protein bands as seen on Coomassie blue stained gels, and higher specific activity was obtained compared to peak 1 IL-13 (2.56 vs. 3.6 units/ng). These data suggest that IL-13 purified from reverse phase chromatography may be slightly more homogenous or biologically active than IL-13 from peak 1. After SDS-PAGE and Western blotting with the anti-mIL-13 mAb, mammalian IL-13 showed multiple bands at ~14 kDa, a result which was also obtained from

the natural IL-13 secreted by the mouse Th2 clone, D10. This recombinant material was subsequently used in the *in vivo* and *in vitro* studies in Chapter VI.

TF-1 is a human premyeloid erythroleukemic cell line which shows a growth response to various human cytokines such as GM-CSF, IL-3, IL-4, IL-6 (Kitamura et al., 1989), and both mouse and human IL-13 (McKenzie et al., 1993a). However, TF-1 cells did not appear to respond to mouse cytokines such as IL-3, IL-4, IL-5, and GM-CSF, secreted by D10, a Th2 cell line. Nevertheless, it remains to be determined whether TF-1 cells respond to other mouse cytokines.

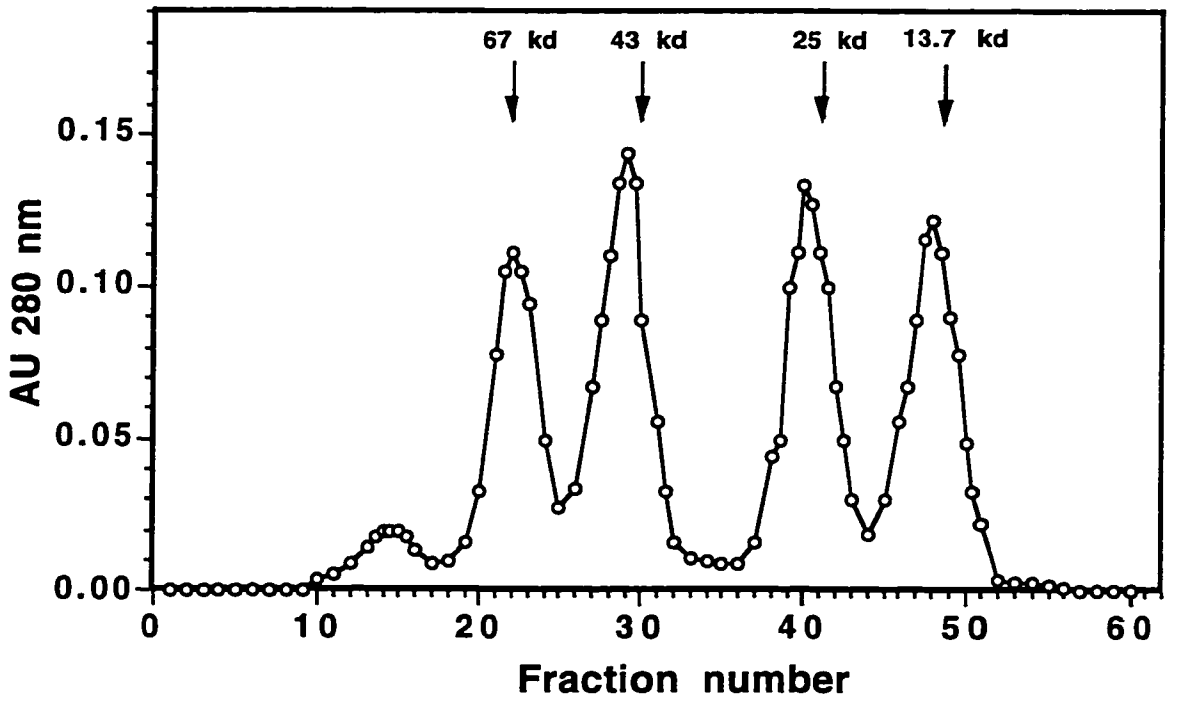


Figure 4.1. Elution profile of standard proteins on FPLC HiLoad 16/60 Superdex 75 prep column. 1.48 mg of Ribonuclease A, 0.44 mg of Chymotrysinogen A, 1.0 mg of Ovalbumin, and 1.0 mg of Albumin were resuspended in 0.5 M NaCl 10 mM HEPES pH 7.5 buffer and loaded onto a HiLoad 16/60 Superdex 75 size exclusion column with a flow rate of 1 ml/min. Absorbance of each column fraction was recorded (open circles).

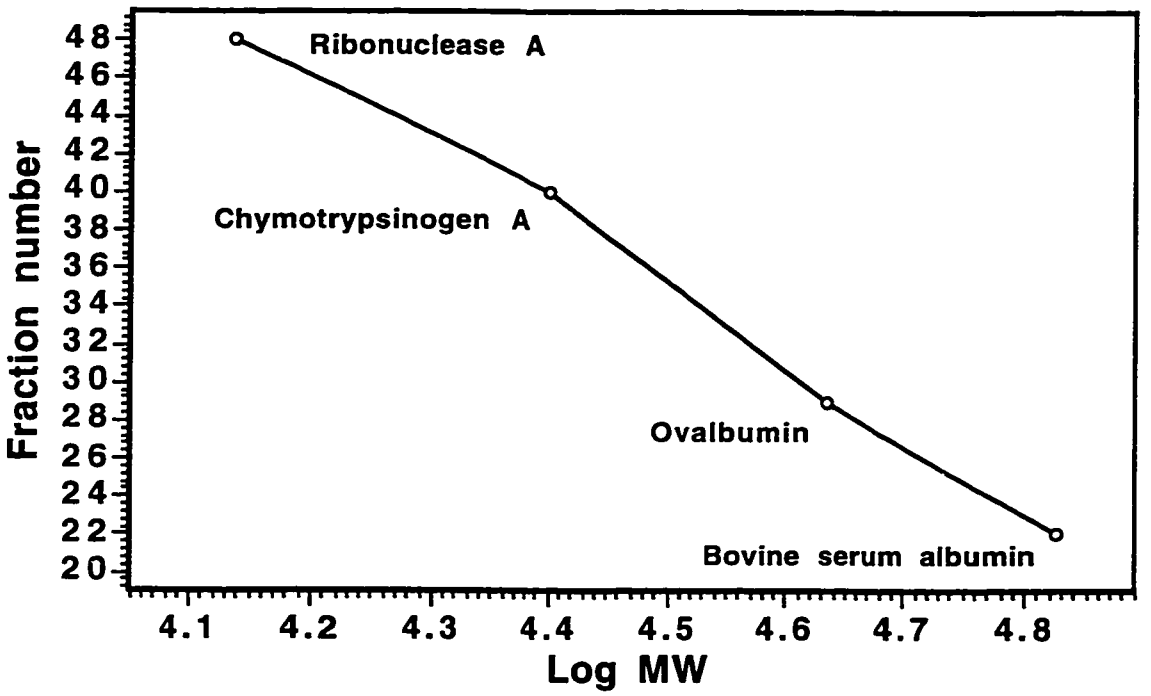


Figure 4.2. Migration rate through the size exclusion column was proportional to the molecular weight of the protein. Log MW of standard proteins were plotted against column fraction numbers obtained from Fig. 4.1.

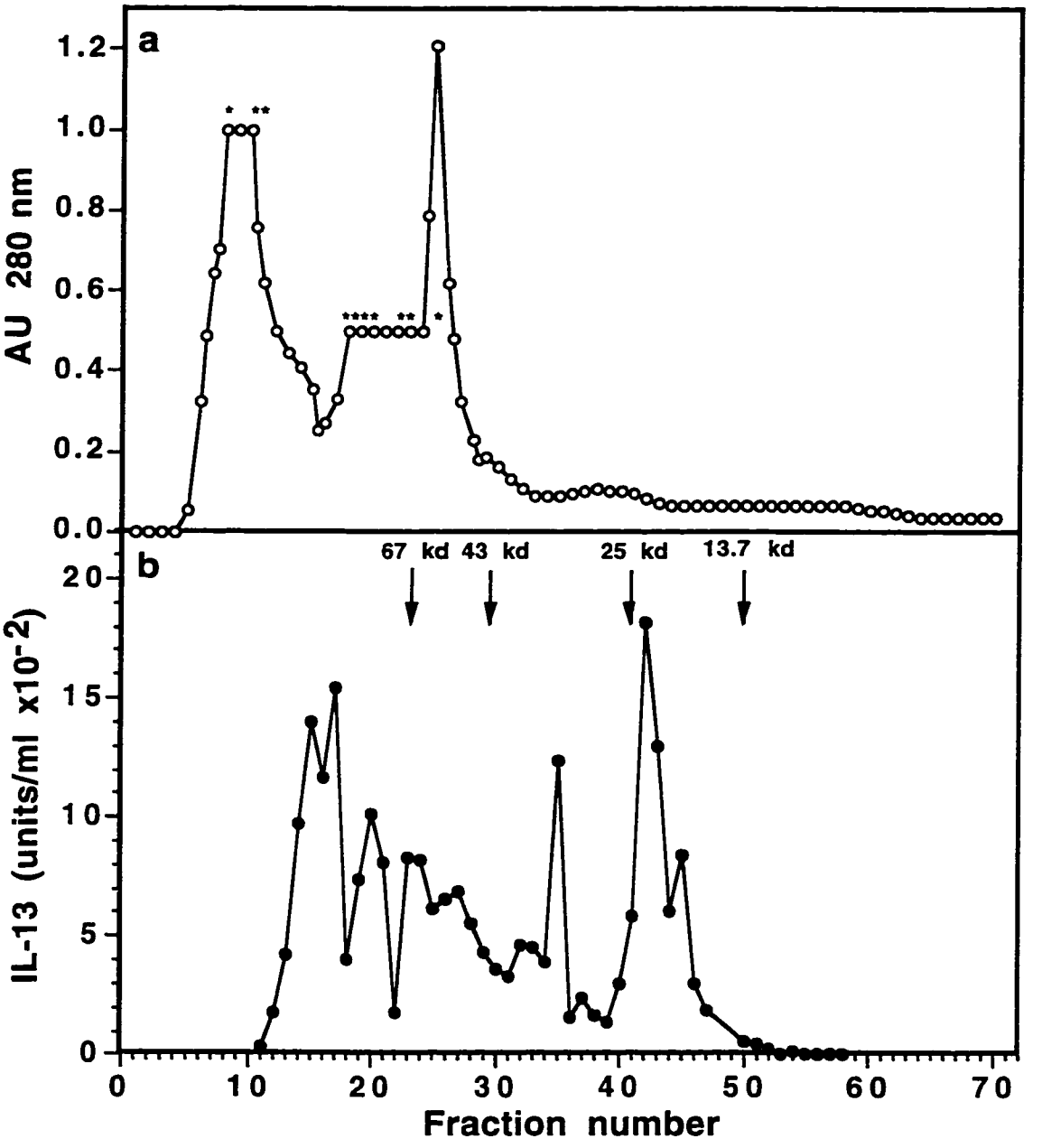


Figure 4.3. IL-13 from IL-13-transfected BW5147 cells eluted in multiple fractions from the size exclusion column. Serum-free concentrated BW5147-IL-13 supernatant was loaded onto a HiLoad 16/60 Superdex 75 size exclusion column, and absorbance values of all column fractions were recorded (a). Column fractions were collected and assayed for IL-13 bioactivity on TF-1 cells using MTT reagent (b). * indicates off-scale absorbance value.

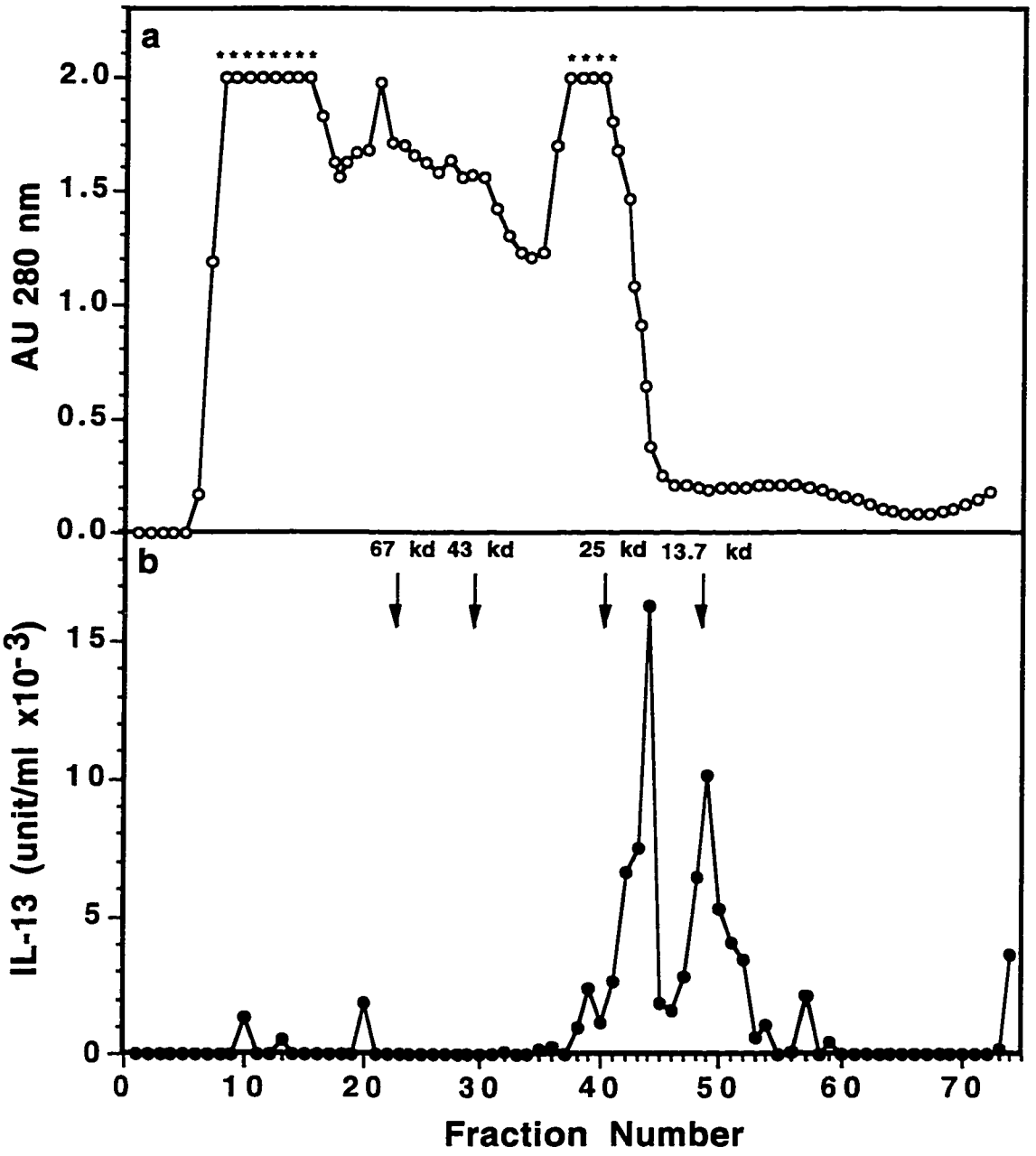


Figure 4.4. COS 7-IL-13 could be separated from other major proteins in the supernatant by the size exclusion column. Concentrated COS 7-IL-13 was loaded onto a HiLoad 16/60 Superdex 75 size exclusion column, and absorbance of each column fraction was recorded (a). Fractions were assayed for IL-13 bioactivity on TF-1 cells (b). * indicates off-scale absorbance value.

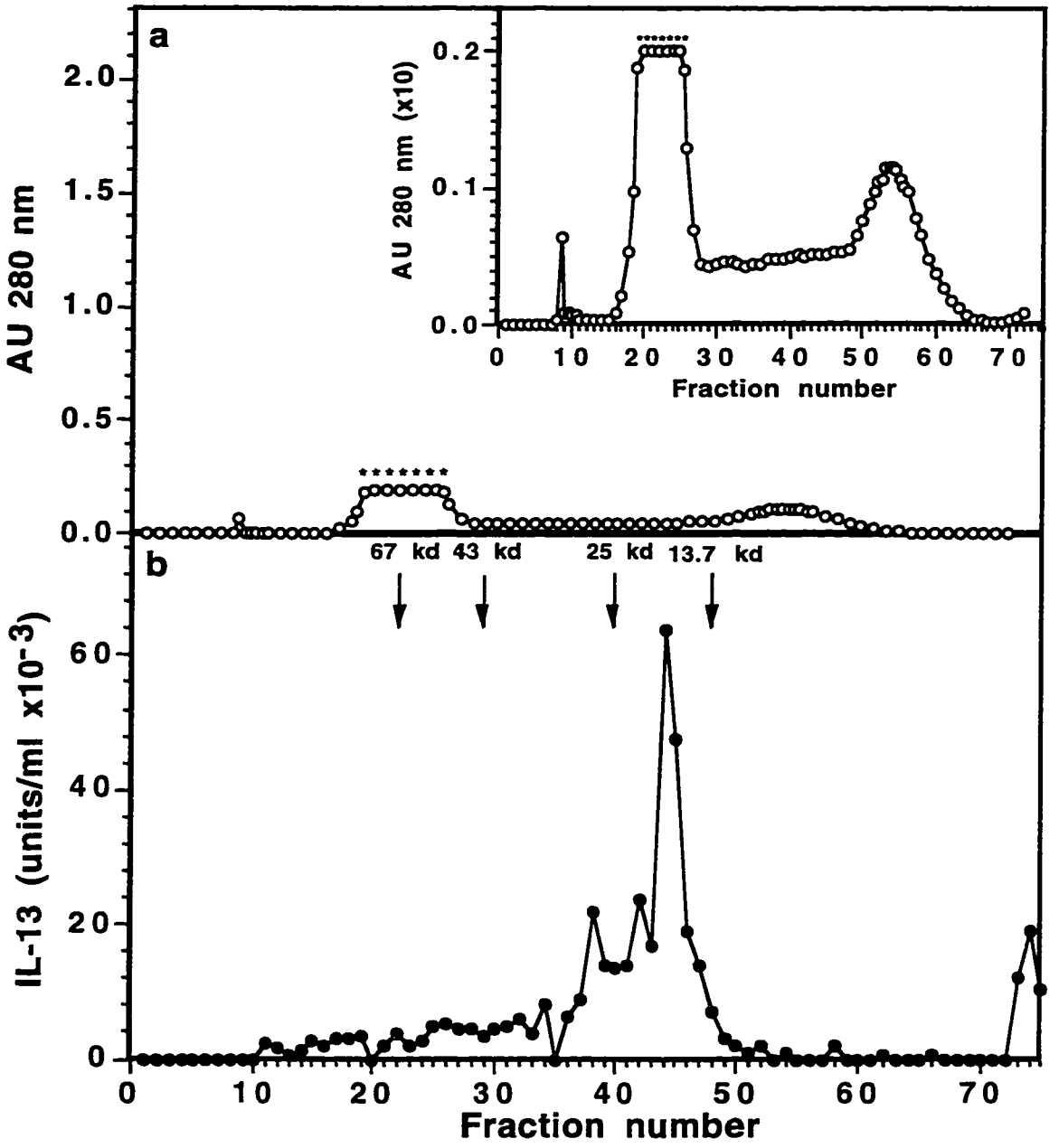


Figure 4.5. COS 7-IL-13 eluted as a monomer in the size exclusion column. Positive column fractions for IL-13 bioactivity from Fig. 4.4 were pooled and concentrated, before loading onto a HiLoad 16/60 Superdex 75 size exclusion column again. Absorbance of each column fraction was recorded (a). Bioactivity of IL-13 in each column fraction was screened by TF-1 cells (b). The insert is an expanded version of the absorbance graph. * indicates off-scale absorbance value.

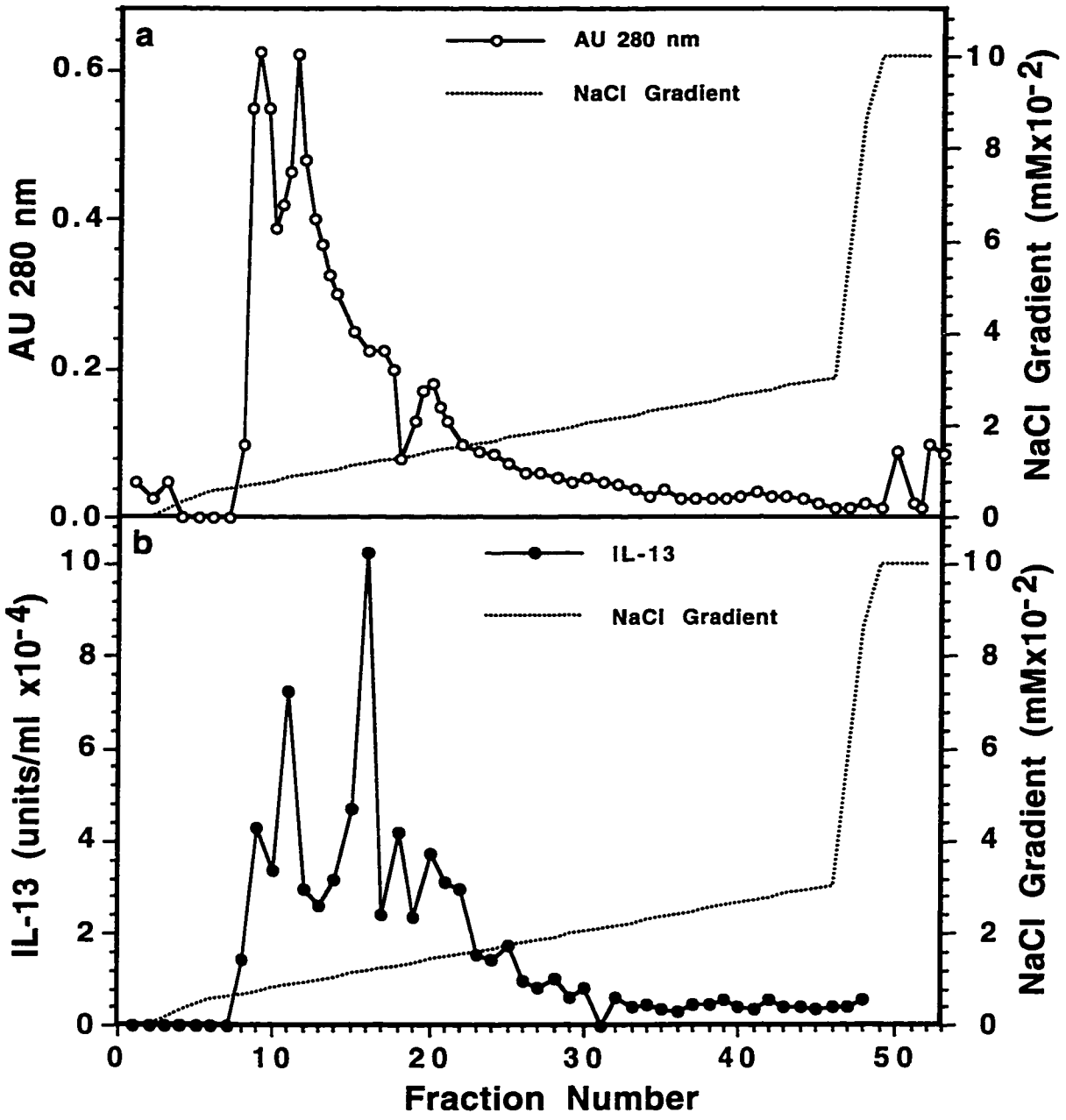


Figure 4.6. Cation exchange chromatography did not separate IL-13 from other BW5147 proteins in the supernatant. Concentrated serum-free supernatant from BW5147-IL-13 was equilibrated in 20 mM MES pH 5.7 buffer, chromatographed over a Mono S column, and eluted with a NaCl gradient (dotted line). Absorbance of each fraction was recorded (a). Column fractions were assayed for IL-13 bioactivity on TF-1 cells (closed circles, b).

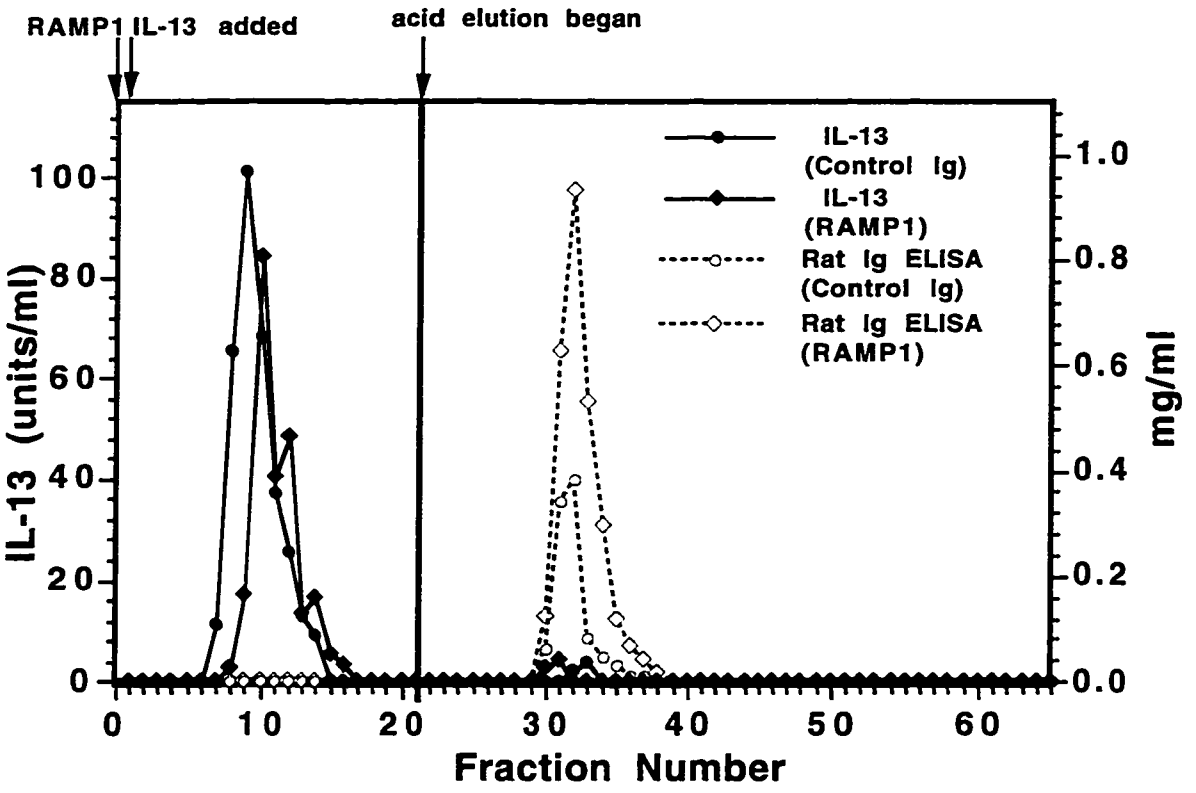


Figure 4.7. RAMP1 did not deplete biologically active IL-13 from the supernatant. BW5147-IL-13 was incubated with either RAMP1 (open and closed diamonds) or control Ig (open and closed circles) bound to a Protein G column. The column was washed with PBS. Column fractions were collected before and after 0.1 M glycine pH 2.7 buffer was added to elute all the bound antibody or IL-13. Column fractions were assayed for IL-13 bioactivity or rat Ig levels by mouse bone marrow cells (closed symbols and solid lines) or ELISA (open symbols and broken lines), respectively.

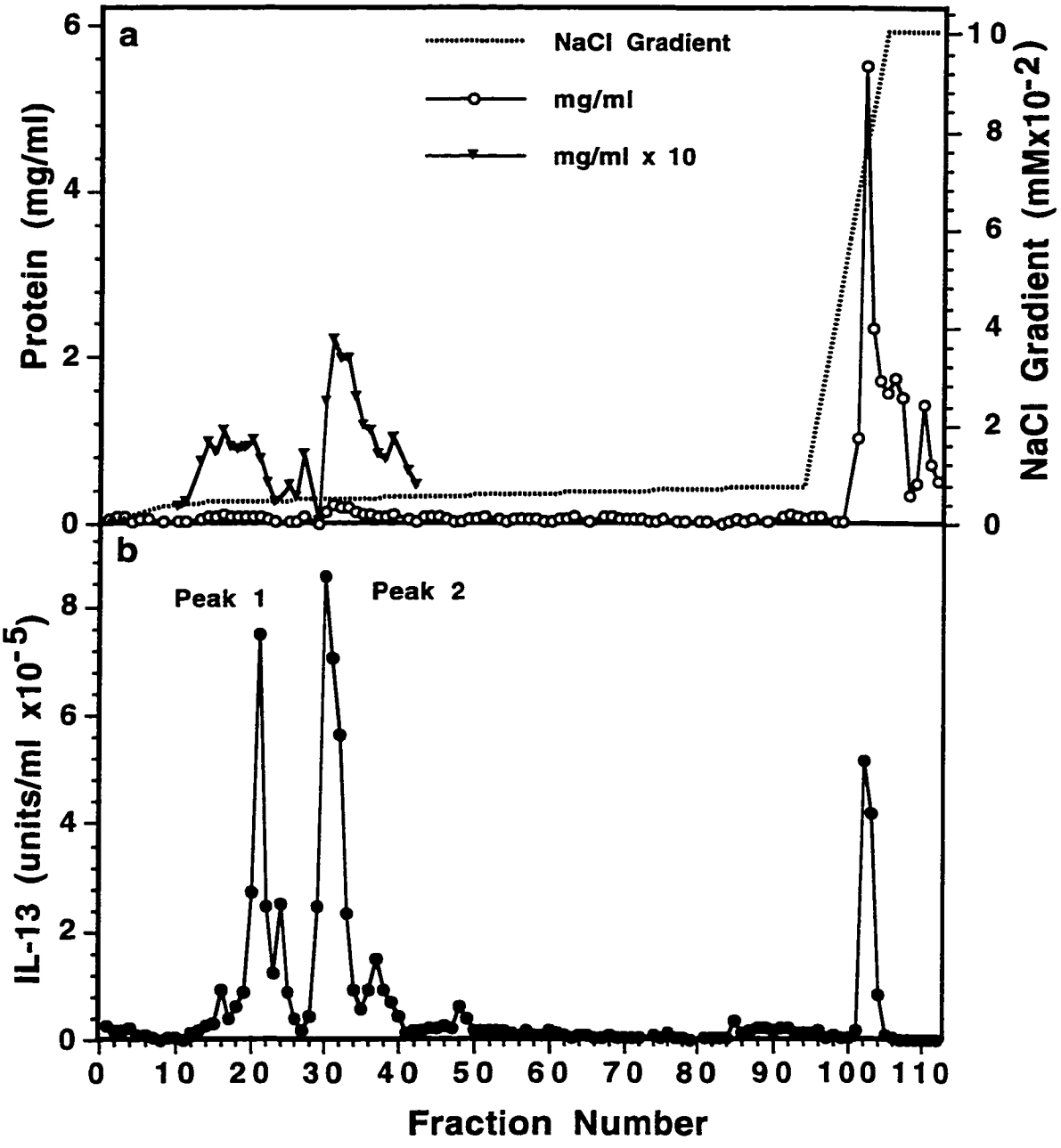


Figure 4.8. Purification of IL-13 by anion exchange chromatography. Concentrated serum-free BW5147-IL-13 supernatant was equilibrated with 20 mm Tris-HCl pH 8.66 buffer and chromatographed over a Mono Q anion exchange column and eluted with a NaCl gradient. Column fractions were assayed for protein concentrations (a) and IL-13 bioactivity on TF-1 cells (b).

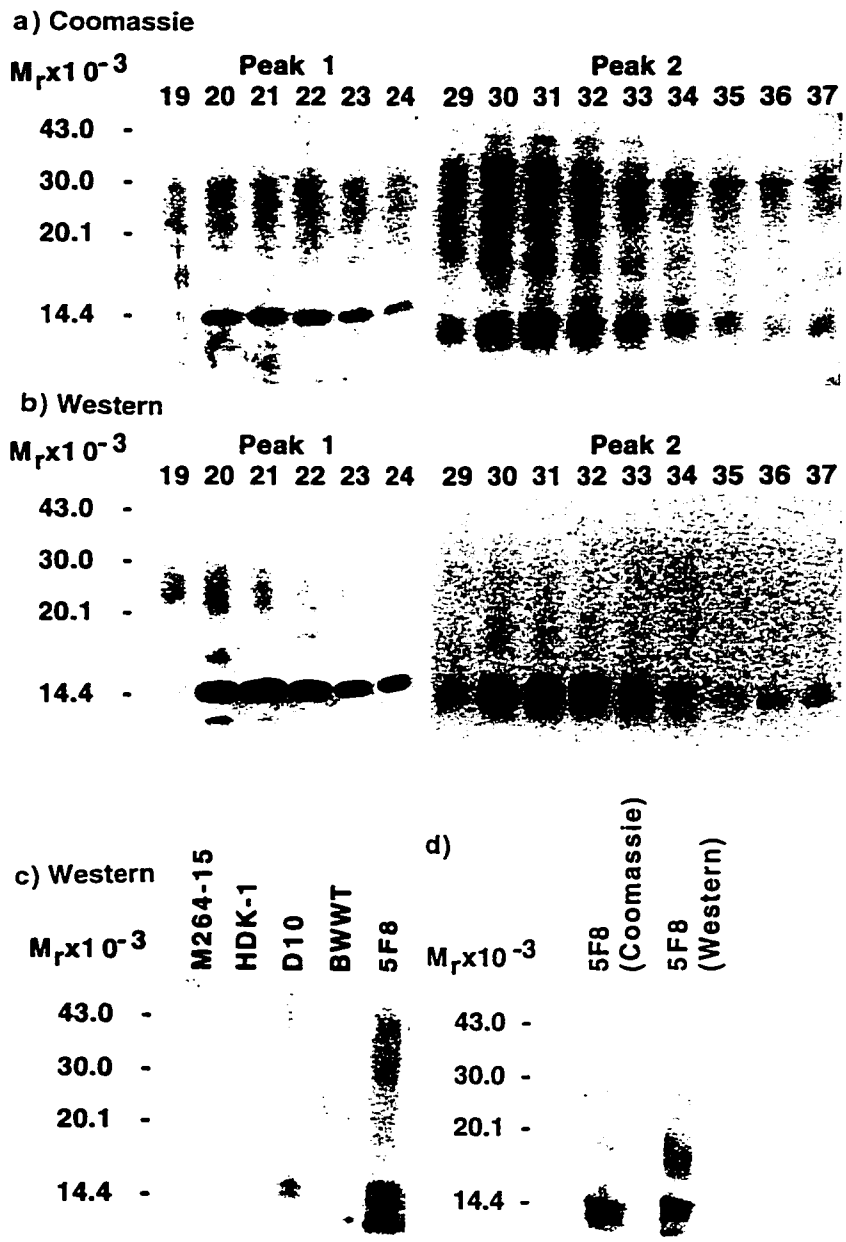


Figure 4.9. Coomassie blue staining and Western blotting of purified IL-13. The purities of the selected fractions surrounding peak 1(20-24) and peak 2 (30-34) from Fig. 4.8 were assessed by 4 M urea-15% SDS-PAGE and Coomassie blue staining (a) and Western blotting using anti-mIL-13 mAb, RAMP1 (b). Concentrated serum-free supernatants of BWWT (2 μg protein), 5F8 (0.5 μg), and Con A-stimulated M264-15, HDK-1, and D10 (4 μg each) were analyzed by 4 M urea-15% SDS-PAGE and Western blotting (c). Fractions 52 to 65 from Fig. 4.10 were pooled, concentrated, and equilibrated in PBS. The protein composition of this preparation was assessed using 4 M urea-15% SDS-PAGE electrophoresis, Coomassie blue staining, and Western blotting with RAMP1 (d).

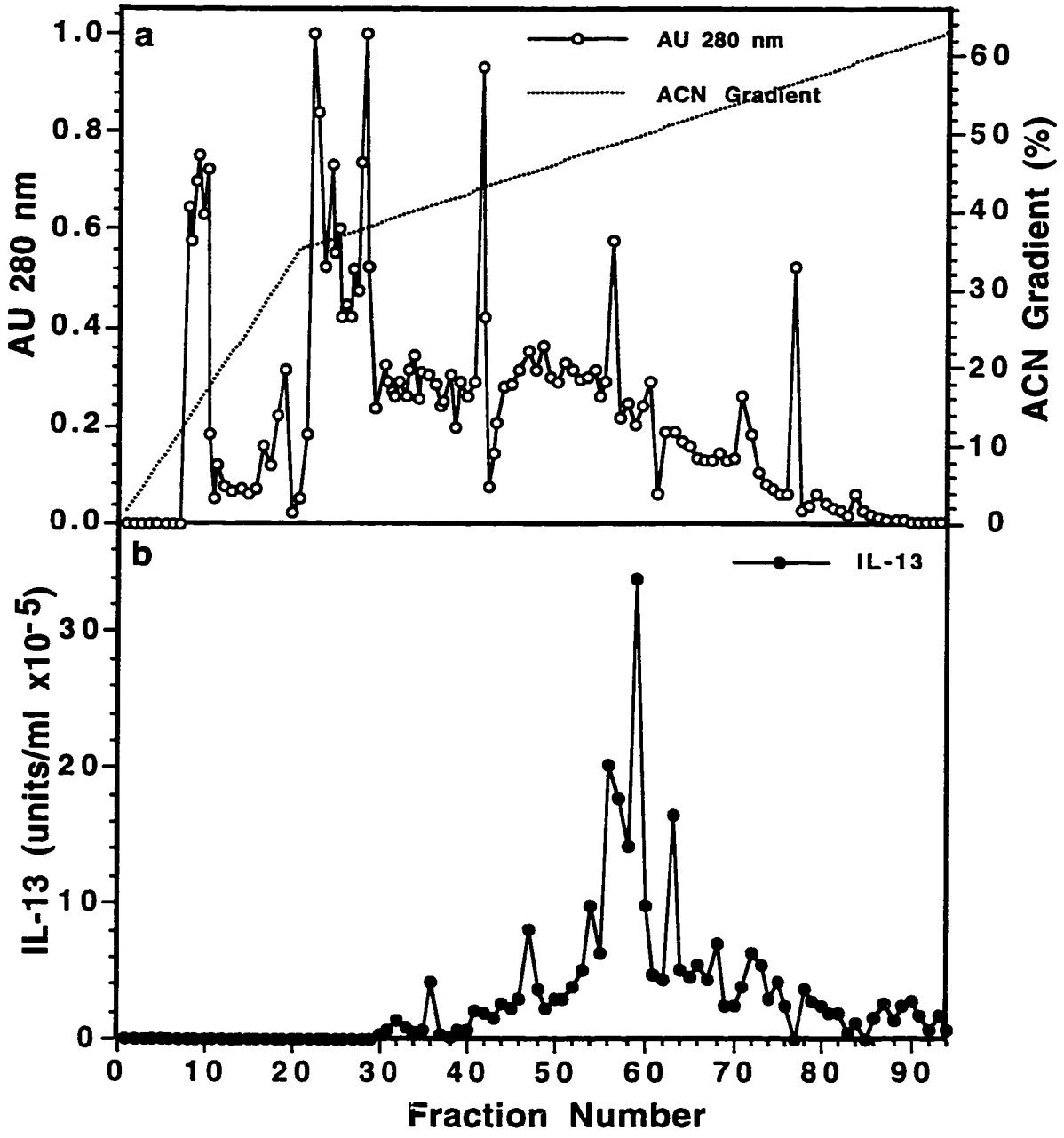


Figure 4.10. Reverse phase chromatography was used as a second step column purification for IL-13. Column fractions from peak 2 after anion exchange chromatography were pooled, concentrated, equilibrated in 0.1% TFA-deionized water, loaded onto a RESOURCE RPC reverse phase column, and eluted with an acetonitrile gradient. Absorbance of each column fraction was recorded (a). Bioactivity of IL-13 in each column fraction was assayed by TF-1 cells (b).

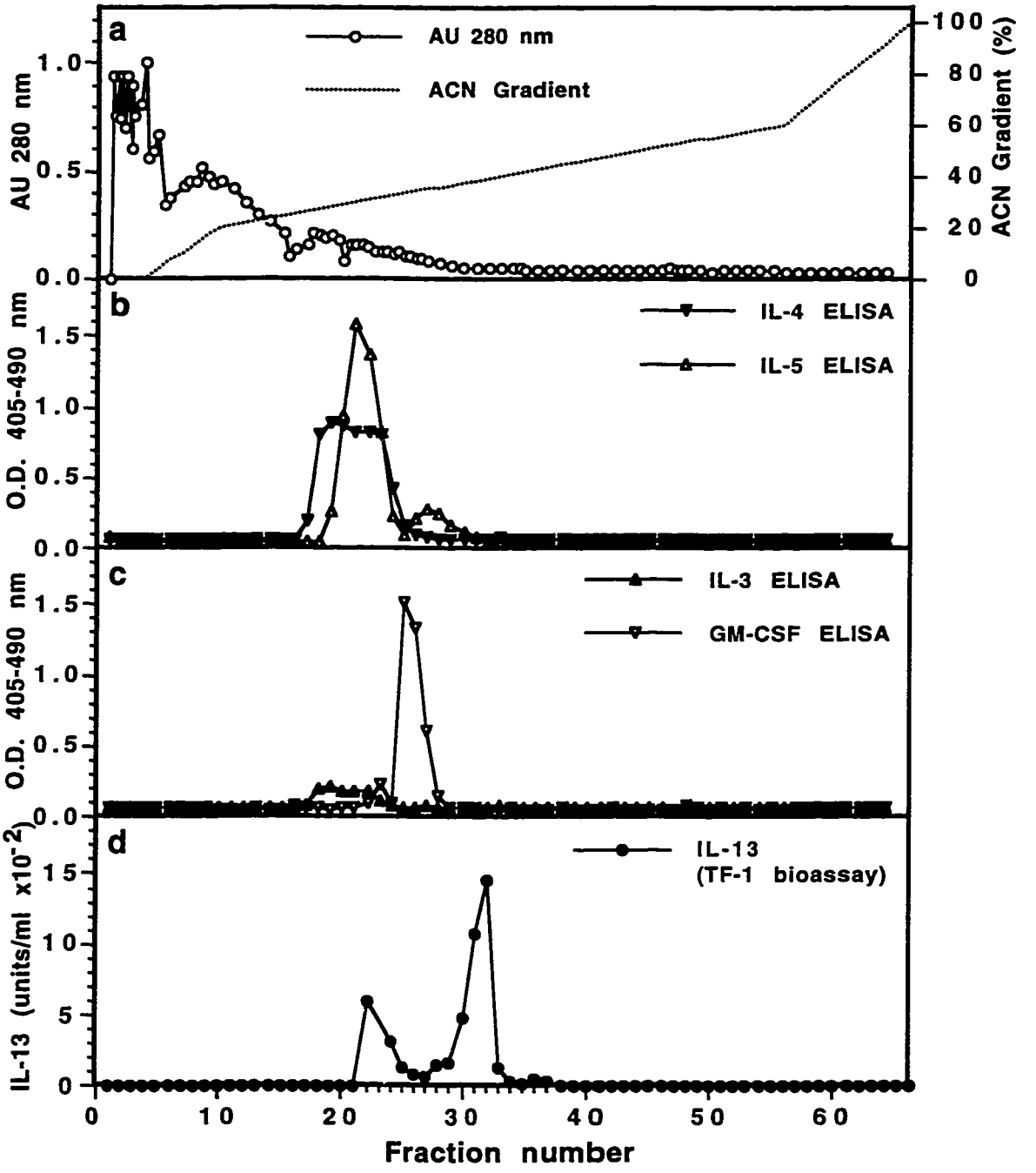


Figure 4.11. TF-1 cells responded to mouse IL-13 but not IL-3, IL-4, IL-5, or GM-CSF. Serum-free Con-A-stimulated D10 supernatant, equilibrated in 0.1% TFA-deionized water, was chromatographed over a ProRPC 5/10 reverse phase column and eluted with an acetonitrile gradient. Absorbance of each column fraction was recorded (a). Column fractions were assayed for IL-4 (b), IL-5 (b), IL-3 (c), GM-CSF (c) by ELISA or IL-13 by TF-1 cells (d).

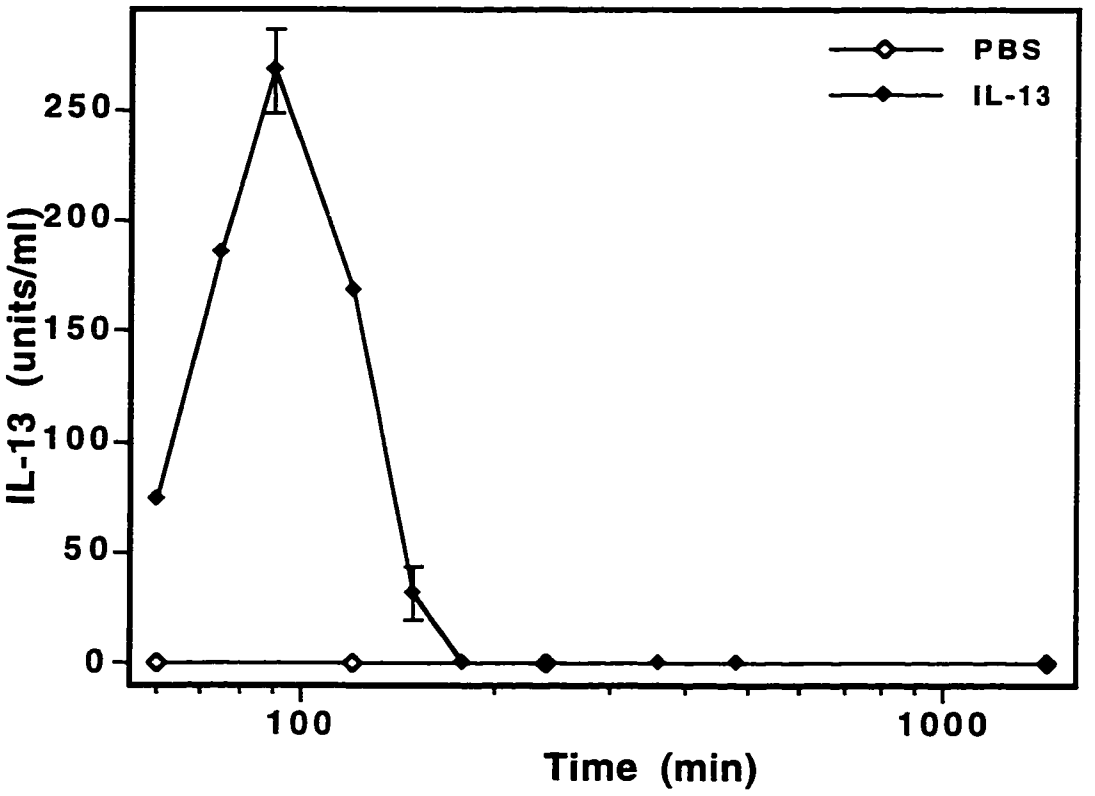


Figure 4.12. IL-13 was rapidly cleared from the circulation in mice. PBS or IL-13 was injected ip into groups of three mice. Blood was collected from a tail vein either every hour from the PBS-treated group (open circles) or every 30 min from alternate groups of IL-13-treated mice, for up to 24 h. IL-13 levels in the serum were measured by TF-1 cells. The mean and SD of triplicate serum samples are shown.

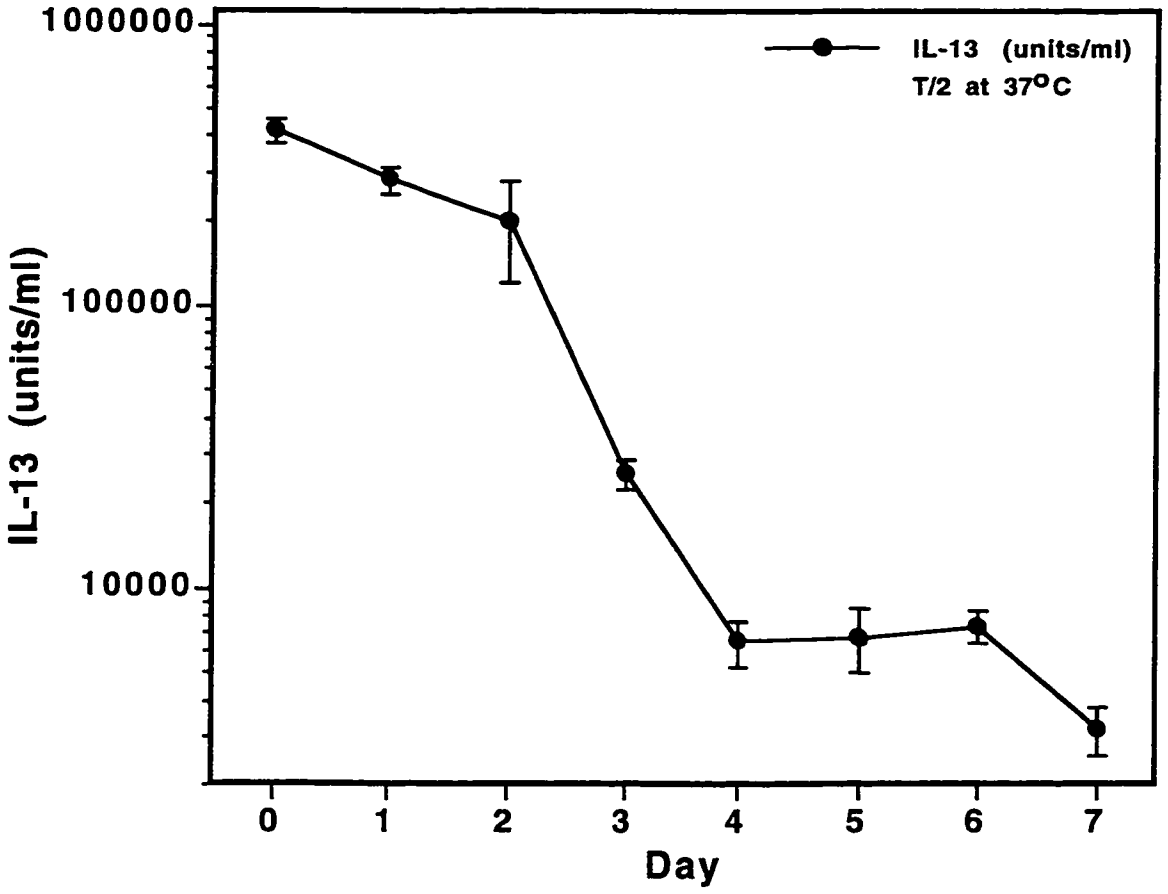


Figure 4.13. Stability of purified IL-13 at 37°C. Aliquots of purified IL-13 were incubated at 37°C for 0 to 7 days, and bioactivity of these aliquots was assayed by TF-1 cells. The mean and SD of triplicate cultures are shown.

CHAPTER V

CONTINUOUS ADMINISTRATION OF IL-13 TO MICE INDUCES EXTRAMEDULLARY HEMOPOIESIS AND MONOCYTOSIS

(The majority of the data presented in this chapter have been published in an article entitled "Continuous Administration of IL-13 to Mice Induces Extramedullary Hemopoiesis and Monocytosis" by Yew Hon Lai, Jean-Marie Heslan, Sibrand Poppema, John F. Elliott, and Tim R. Mosmann in *J. Immunol.* 156:3166-3173, 1996.)

A. Introduction

The distinctive features of hemopoiesis are lifelong, stable cell renewal, and complex multistage cellular and biological processes. These findings which are supported by the study of hemopoietic pluripotent stem cells, which in their steady-state appear to be dormant i.e. non-cycling (Till and McCulloch, 1961; Dexter et al., 1977). The entry into cell cycle of the dormant stem cells may be promoted by a number of growth factors. In the presence of these factors, stem cells leave G_0 and begin proliferation and differentiation into various committed progenitors which subsequently give rise to mature and functional hemopoietic cells (Wright and Pragnell, 1992; Wright and Lord, 1992; Moore, 1991).

In order to explore the transient nature of hemopoietic cells, functional assays have been developed to study the regulatory mechanisms of the hemopoietic systems. More than two decades ago, two laboratories independently developed a method to quantitate hemopoietic progenitors using double layer semisolid agar

culture systems in which the clonal proliferation of hemopoietic progenitors in the top layer is supported by factors released from the cells embedded in the feeder bottom layer (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). Subsequently, many modifications of the culture techniques have been developed. For example, the agar is substituted with other semisolid materials such as fibrin clot, plasma clot, and methylcellulose. During the ensuing two decades, the mechanisms of hemopoiesis have been significantly elucidated by studies using these clonal culture assays, and it is now well-known that cytokines support the survival and/or proliferation of hemopoietic progenitors. Cytokines demonstrating hemopoietic activities are constantly being discovered. Over the years, available experimental data indicate that the long list of cytokines that exhibits positive or negative biological effects on hemopoiesis includes GM-CSF, G-CSF, M-CSF, Meg-CSF, IL-1, IL-3, IL-4, IL-5, IL-6, IL-9, IL-11, erythropoietin, stem cell factor (SCF), basic fibroblast growth factor, leukemia inhibitory factor (LIF), TGF-β1, platelet factor-4, tetrapeptide acetyl-N-ser-ASp-Lys-Pro, macrophage inflammatory protein-1 (MIP-1), and IFN-α (Han and Caen, 1994). It is also known that some cytokines are lineage-specific, and some cytokines are pleiotropic and have multiple biological functions on various tissues and cells. IL-3, a stem cell factor, and GM-CSF appear to support the proliferation of early hemopoietic progenitors. Once the progenitors are committed to individual lineage, the subsequent maturation process appears to be supported by late-acting, lineage-specific factors, such as erythropoietin (for erythropoiesis), G-CSF (for neutrophilopoiesis), M-CSF (for macrophage/monocyte production), and IL-5 (for eosinophilopoiesis). In addition, different combinations of cytokines result in synergistic activities. For example, IL-6 significantly enhances the growth of granulocyte and macrophage progenitors (Rennick et al., 1989) in the presence of IL-3, G-CSF, GM-CSF, or

M-CSF. Thus, hemopoietic proliferation and differentiation appear to be regulated by a cascade of factors directed at different developmental stages.

IL-4 acts as a lineage-nonspecific early-acting factor as it does not support the differentiation of hemopoietic cells by itself but stimulates the differentiation of hemopoietic lineages in conjunction with other hemopoietic cytokines (Sonoda, 1994). In the presence of erythropoietin, IL-4 enhances erythroid colony growth in both mouse (Peschel et al., 1987; Rennick et al., 1987a) and human bone marrow cells (Broxmeyer et al., 1988). Addition of IL-4 results in increased numbers of G-CSF-induced granulocyte colonies (Peschel et al., 1987; Rennick et al., 1987b; Broxmeyer et al., 1988). However, colony formation by monocytic precursor cells is inhibited by IL-4 (Jansen et al., 1989). These results indicate that similar to IL-3, IL-4 is a direct-acting multi-CSF and acts on primitive hemopoietic progenitors.

IL-4 and IL-13 exhibit overlapping but different patterns in the regulation of proliferation and differentiation of primitive mouse hemopoietic progenitor cells (Jacobsen et al., 1994). Either cytokine alone does not stimulate the proliferation of $Lin^-Sca\text{-}1^+$ mouse bone marrow primitive hemopoietic progenitors *in vitro*. However, IL-13 synergizes with CSF-1, GM-CSF, or SCF to increase the number of colonies from $Lin^-Sca\text{-}1^+$ but not the more mature $Lin^-Sca\text{-}1^-$ bone marrow progenitors, suggesting that IL-13 preferentially acts on primitive progenitors. Unlike the potency of IL-4 versus IL-13 on B cells, IL-13 is more potent than its counterpart in stimulating SCF-induced proliferation of $Lin^-Sca\text{-}1^+$ progenitors (Jacobsen et al., 1994).

IL-13 shows a pattern of synergistic activity different from that of IL-4. While both IL-13 and IL-4 synergistically enhanced colony formation by $Lin^{-}Sca\text{-}1^{+}$ progenitors in response to G-CSF, only IL-13 synergizes with GM-CSF to increase colony formation frequencies of similar progenitor cells. In combination with SCF and G-CSF, IL-13 or IL-4 stimulates exclusively macrophage production by $Lin^{-}Sca\text{-}1^{+}$ progenitors, whereas predominantly granulocytes can be obtained in the absence of IL-13 (Jacobsen et al., 1994). Interestingly, both IL-13 and IL-4 synergistically suppress the proliferation of the late stage of committed macrophage progenitors as IL-13 and/or IL-4 inhibits the proliferation of macrophage colonies that form in the presence of IL-3, GM-CSF, M-CSF, and erythropoietin (Sakamoto et al., 1995), indicating that the IL-4 and IL-13 not only stimulate the differentiation of macrophages from hemopoietic progenitors but also suppress the proliferation of macrophages.

In summary, IL-13, the most recent cytokine member to join the Th2 family, shares some but not all of the *in vitro* functions of IL-4. As the biological functions of IL-13 *in vivo* are not well understood, and the majority of IL-13's reported functions are derived from *in vitro* systems, this study was carried out to investigate the *in vivo* functions of IL-13. In particular, the hemopoiesis and general immune aspects of normal BALB/c mice after continuous IL-13 administration were investigated.

B. Results

Continuous IL-13 administration for 7 days resulted in splenomegaly. In order to circumvent the short *in vivo* half-life of IL-13 in mice (see Chapter IV), possible toxic effects of bolus IL-13 injection, and stress due to repeated handling of the mice, Alzet osmotic pumps were chosen to continuously administer the purified cytokine. IL-13 or PBS control preparations were coded and continuously administered by Alzet osmotic pumps in the peritoneal cavities of normal BALB/c female mice. The codes were broken after all the results were obtained. The mice were monitored daily for any abnormalities, and after 7 days, the gross anatomy of the mice was analyzed. In four different experiments, the first striking observation was splenomegaly in IL-13-treated but not in control mice. In experiment 1, spleen weights were significantly increased by treatment with the initial dose of 5.7 μg IL-13 per mouse per day ($p< 0.03$, Fig. 5.1). Similar results were obtained in three other experiments, with initial IL-13 doses ranging from 0.6 to 9 μg/day (Fig. 5.1 and data not shown). Total numbers of nucleated spleen cells recovered from the IL-13-treated mice also increased (Fig. 5.1). Thus, the increase in spleen weight was probably due to the increase in cellularity of the spleen after IL-13 treatment. In contrast, liver weights were not significantly increased (Fig. 5.1).

Increased hemopoietic foci in the spleens and livers of IL-13-treated mice. To study the histological changes in the spleens and livers from PBS- or IL-13-treated mice, these organs were fixed, sectioned, and stained with hematoxylin-eosin. *In vivo* IL-13 treatment increased the numbers of cells with the morphology of megakaryocytes and immature erythroid progenitors in the red pulps of the spleen (Fig. 5.2a & b). Interestingly, small hemopoietic foci were

also observed in the liver of the IL-13- but not PBS-treated mice (Fig. 5.2c & d). Collectively, these data suggest that *in vivo* IL-13 treatment increased hemopoietic activity in the spleen and possibly liver.

Enhanced responsiveness to hemopoietic cytokines in spleen cells from IL-13-treated mice. As *in vivo* IL-13 treatment increased hemopoietic activity in the spleen, it was hypothesized that spleen cells should show increased responsiveness to cytokines that stimulate hemopoiesis. Spleen cells from PBS- or IL-13-treated mice were cultured in the presence of different concentrations of recombinant mIL-3, mIL-4, mGM-CSF, purified mIL-13, and hCSF-1. Proliferation/activation was assessed on day 6 by MTT cleavage (Fig. 5.3). After *in vivo* IL-13 treatment for 7 days, the spleen cells showed moderately higher responses to mIL-4 and IL-13 and substantially higher responses to mGM-CSF, mIL-3, and hCSF-1. Hence, IL-13 treatment induced an increase in the numbers and/or responsiveness of spleen cells that could be further stimulated *in vitro* by several cytokines with hemopoietic activity.

Increased numbers of colony-forming precursor cells in the spleen but not bone marrow after IL-13 treatment. In order to investigate whether the increased cytokine responsiveness of the spleen cells from IL-13-treated mice was accompanied by increased numbers of hemopoietic progenitor cells, hemopietic precursor frequencies were enumerated by colony formation in methylcellulose cultures. *In vivo* IL-13 treatment for seven days substantially increased the frequencies of CFU-E, BFU-E, and CFU-C in the spleens (either $p< 0.01$ or $p<0.05$, Fig. 5.4). Because IL-13 administration for seven days could be due to the upregulation of extramedullary hemopoiesis in general and/or mobilization of hemopoietic progenitors from bone marrow to the spleen, the

hemopoietic progenitor frequencies in the bone marrow were enumerated. In contrast to the spleen results, the frequencies of CFU-E and CFU-C progenitor cells in bone marrow did not increase significantly after IL-13 *in vivo* treatment (Fig. 5.5). In fact, because of the decreased total numbers of bone marrow cells obtained from the hind legs after IL-13 treatment ($p< 0.001$, Fig. 5.5), the absolute numbers of bone marrow hemopoietic progenitors in the whole mouse might have declined. However, testing of precursor frequencies in other tissues would be required before this conclusion could be drawn.

IL-13 induced slight anemia and pronounced monocytosis. Hematological analyses were performed on the heparinized blood from the mice in Experiment 1. Hemoglobin levels were on the average 28% lower in IL-13-treated mice compared to those of PBS controls (Fig. 5.6). There were no significant differences in the concentrations of total red blood cells, white blood cells, lymphocytes or neutrophils in the peripheral blood. No eosinophils were detected (<1 per 100 leukocytes) in any of the three IL-13-treated mice, whereas 1-2 eosinophils per 100 leukocytes were found in PBS-treated mice. The most striking alteration induced by *in vivo* IL-13 treatment was a significant increase (average of ten-fold, $p< 0.05$) in the concentration of monocytes in the peripheral blood (Fig. 5.6). These were identified as monocytes by standard morphological criteria (large nucleated cells with vacuolated cytoplasm).

Ficoll-purified peripheral blood cells from IL-13-treated mice in Experiment 2 contained similarly elevated levels of cells that morphologically resembled macrophages and were Mac-3^+ and Mac-1^+ (both $p< 0.05$, Fig. 5.7). Because neutrophils are also Mac-1^+, only data from mononuclear Mac-1^+ cells are shown (Fig. 5.7). The identity of these cells as mature cells rather than

progenitors was confirmed by hemopoietic colony forming assays. Peripheral blood from PBS or IL-13-treated mice contained undetectable levels of CFU-C or CFU-E (<5 per 10^6 nucleated cells, compared to the high spleen precursor levels (Fig. 5.4).

In vivo IL-13 treatment enhanced in vitro IL-6 production by LPS-stimulated spleen cells. We have observed that IL-13 induced the formation of macrophage-like cells from mouse bone marrow cells after 5 to 7 days of culturing. These cells often exhibit vacuolated cytoplasm, may contain multiple nuclei, and synthesize substantial amounts of IL-6 on LPS stimulation (J.-M. Heslan, Y. H. Lai, and T. R. Mosmann, unpublished). As $Mac1^+$ cells in the spleen were also increased after *in vivo* IL-13 treatment (Fig. 5.8) ($5.6 \pm 1.0\%$ vs. $2.5 \pm 0.8\%$, $p < 0.02$), spleen cells from PBS- or IL-13-treated mice were cultured with or without LPS for 48 h. IL-6 and IL-10 in the supernatants were measured by ELISA. IL-10 levels with or without LPS stimulation were variable between mice (all less than 1 ng/ml, Fig. 5.12), and *in vivo* IL-13 treatment did not significantly affect these levels (Fig. 5.9). In contrast, LPS induced high levels of IL-6 synthesis by spleen cells from IL-13-treated mice, but not PBS-treated mice (Expt. 1 and 2 both $p < 0.03$, Fig. 5.9).

In summary, 7 days of *in vivo* IL-13 treatment induced splenomegaly; increased spleen cell number; increased splenic and hepatic hemopoietic foci; increased spleen cell responsiveness to hemopoietic cytokines *in vitro*; increased the frequency of hemopoietic precursors in the spleen; peripheral monocytosis; and increased IL-6 production from LPS-stimulated spleen cells. Collectively, these data indicate that continuous *in vivo* IL-13 treatment increased extramedullary hemopoiesis and resulted in peripheral monocytosis.

Nippostrongylus brasiliensis (Nb) infection promotes a Th2 immune response. Knowing that IL-13 is one of the cytokines secreted by Th2 cells and that *in vivo* IL-13 treatment induces extramedullary hemopoiesis, it was hypothesized that extramedullary hemopoiesis might also be observed during the strong Th2 response induced by *Nb* infection in BALB/c mice. Eight days after infection, the spleen and blood were collected and analyzed. The average weight of the spleens from infected mice was increased (Fig. 5.10) (0.16 ± 0.04g, vs. 0.11 ± 0.02g). As expected, Con A-stimulated spleen cells from *Nb*-infected mice synthesized less IFN-γ and more IL-4 and IL-5 than negative controls (Fig. 5.11). Compared to the negative controls, the *Nb*-infected mice also demonstrated an increase in serum IgE levels (Fig. 5.11). These results confirmed that the *Nb* infection successfully induced the expected strong Th2 response (Urban et al., 1993). In a separate experiment, the synthesis of IL-13 by Con A-stimulated spleen cells was assessed by the proliferative or activation responses of the human premyeloid cell line TF-1, which respond to mouse IL-13 but not to mouse IL-3, IL-4, IL-5, or GM-CSF (Chapter IV). The moderate increase in the TF-1 bioassay signal (Fig. 5.11) was likely due to enhanced synthesis of IL-13 by cells from *Nb*-infected mice, although contributions by unknown cytokines could not be ruled out.

Nb infection enhanced spleen cell responsiveness to hemopoietic cytokines and extramedullary hemopoiesis in the spleen. Spleens isolated from *Nb*-infected mice also demonstrated an increase in hemopoietic foci as observed by histology and light microscopy (data not shown), and spleen cells showed enhanced responses to recombinant mIL-3, mIL-4, IL-13, mGM-CSF, and hCSF-1 (Fig. 5.12). In addition, spleen cells from controls or *Nb*-infected mice were cultured

in methylcellulose medium, and CFU-E and CFU-C were enumerated. *Nb* infection resulted in a significant increase in the frequencies of CFU-E and CFU-C colonies from the spleen (both $p< 0.02$, Fig. 5.13). Interestingly, spleen cells from *Nb*-infected mice produced significantly higher levels of IL-6 but not IL-10 in response to LPS stimulation (Fig. 5.14). Thus, extramedullary hemopoiesis in the spleen was induced both by IL-13 treatment and by a strong Th2 response to infection.

C. Discussion

After continuous administration by osmotic pumps for seven days, IL-13 induced extramedullary hemopoiesis in the spleen and liver, slight anemia, and profound monocytosis in the peripheral blood. Thus, IL-13 enhances production of monocytes, in contrast to its mostly suppressive effects on the functions of mature macrophages, including inhibition of cytotoxicity and synthesis of NO, IL-1, IL-6, and IL-12 (Zurawski and de Vries, 1994). This effect is not surprising in light of the observation that TF-1, a premyeloid cell line, also proliferates in response to IL-13 (McKenzie et al., 1993a; Lakkis and Cruet, 1993). Moreover, mIL-13 induces the formation of macrophage-like cells from mouse bone marrow *in vitro* (J.-M. Heslan, L. J. Guilbert, and T. R. Mosmann, unpublished).

The *in vivo* data with mouse IL-13 described in this chapter are consistent with the observation that hIL-13 synergizes with SCF and G-CSF to enhance the exclusive proliferation and differentiation of mouse primitive hemopoietic progenitor cells ($Lin^-Sca\text{-}1^+$) to form macrophages *in vitro*. In the absence of IL-13, SCF and G-CSF cultures of $Lin^-Sca\text{-}1^+$ result in the formation of 90% granulocytes (Jacobsen et al., 1994). However, no synergistic effect of IL-13 can be seen on SCF-induced proliferation of the more mature $Lin^-Sca\text{-}1^-$ progenitors, indicating that IL-13 is involved in early myelopoiesis (Jacobsen et al., 1994). In addition, IL-13 exerts a stimulatory effect on the growth of megakaryocyte progenitor cells obtained from non-adherent mononuclear cells or highly enriched $CD34^+$ cells of human cord blood (Xi et al., 1995). Collectively, these data are in line with our findings that *in vivo* IL-13 administration enhances monocytes, megakaryocytes, hemopoietic-cytokine-responsive spleen cells, CFU-E, CFU-C, and BFU-E colonies. However, these *in vivo* studies further

revealed that IL-13 enhances the frequencies of the primitive mouse hemopoietic progenitor cells, a function that *in vitro* studies fail to unravel. G-CSF has established such a precedent since it is not only a potent late-stage factor in the generation of granulocytes *in vitro* but also acts on several lineage-committed progenitors *in vivo* (Tamura et al., 1991).

Since neither IL-4 nor IL-13 alone stimulates the proliferation of $Lin^{-}Sca\text{-}1^{+}$, mouse primitive hemopoietic progenitors *in vitro* (Jacobsen et al., 1994), it is possible that IL-13 synergizes with other factors *in vivo* to induce extramedullary hemopoiesis and monocytosis in the peripheral blood. This discrepancy could also be due to the fact that hIL-13 but not mIL-13 was used in the *in vitro* studies (Jacobsen et al., 1994). Although hIL-13 is active on mouse cells (McKenzie et al., 1993a), hIL-13 is approximately 100-fold less active than mIL-13 on the mouse B9 plasmacytoma cell line (Zurawski and de Vries, 1994).

Although IL-13 was administered by continuous infusion to maintain constant exposure and avoid sudden changes in cytokine concentration, occasional deaths occurred in the IL-13-treated groups after four or more days of administration, particularly at initial doses above 5 μg/mouse/day. Overall, in five different experiments, seven out of 26 mice died when they were treated with of IL-13 ranging from 0.6 to 9 μg/day. The mechanism of this toxicity has not been investigated, but several IL-13-treated mice exhibited liver necrosis and/or flaccid hearts, and the related cytokine IL-4 induces a number of side effects, including hepatic and cardiac toxicity in Phase I and II clinical trials (Gilleece et al., 1992; Prendiville et al., 1993; Margolin et al., 1994).

In vivo IL-13 treatment increased the numbers of Mac-1^+ cells in the spleen and Mac-3^+ and Mac-1^+ monocytes in the peripheral blood. This is consistent with the findings *in vitro* in which hIL-13 synergizes with SCF and G-CSF to enhance the exclusive proliferation and differentiation of mouse macrophages from primitive hemopoietic progenitor cells (Jacobsen et al., 1994). Spleen cells from IL-13-treated mice also produced increased levels of IL-6 in response to LPS stimulation, suggesting the presence of increased numbers of functional monocytes/macrophages in the spleen. The origins of these cells are unclear at this stage as they may be derived directly from the ongoing splenic myelopoiesis or from the increased levels of circulating monocytes. IL-6 is able to support the proliferation and differentiation of granulocyte-macrophage progenitor cell, in the presence of IL-3, IL-4, G-CSF, GM-CSF, or M-CSF (Rennick et al., 1989; Kishimoto, 1989). As IL-4 is closely related to IL-13, it is conceivable that IL-13 acts indirectly through stimulating the differentiation of macrophages. These cells can in turn secrete cytokines, such as IL-6, that enhance hemopoiesis in the spleen and liver. Consequently, *in vivo* IL-13 treatment may directly and indirectly induce a microenvironment in the spleen to accommodate a massive multilineage increase in hemopoietic precursors and mature cells.

In the presence of SCF and G-CSF, IL-13 suppresses the differentiation of Lin$^-$ Sca-1^+ progenitor cells into granulocytes *in vitro* (Jacobsen et al., 1994). However, *in vivo* IL-13-treated mice did not exhibit a significant decrease in neutrophils in the circulation. Given that neutrophils have short half-lives of approximately 8 h in the circulation (Vincent, 1977; Lord et al., 1989; Lord et al., 1991), seven days of IL-13 *in vivo* administration is likely to be sufficient to deplete the granulocyte pool. Hence, these data indicate that either IL-13 may not suppress granulocyte differentiation *in vivo* or the increased number of

myeloid progenitors in the spleens of IL-13-treated mice may compensate for IL-13-induced suppression of differentiation.

Interestingly, eosinophil levels were undetectable in the peripheral blood after IL-13 treatment. IL-13 is not only a chemotactic factor for eosinophils (Luttmann et al., 1996; Horie et al., 1997) but also upregulates VCAM-1 expression on human endothelial cells. The upregulated-VCAM-1 can then interact with α4 integrin (CD49d) on eosinophils, facilitating the binding of eosinophils to endothelium (Bochner et al., 1995) and mediating selective eosinophil transendothelial migration (Horie et al., 1997). Thus, it is conceivable that *in vivo* IL-13 treatment upregulates VCAM-1 expression on the endothelial cells, subsequently sequestering eosinophils from the peripheral blood.

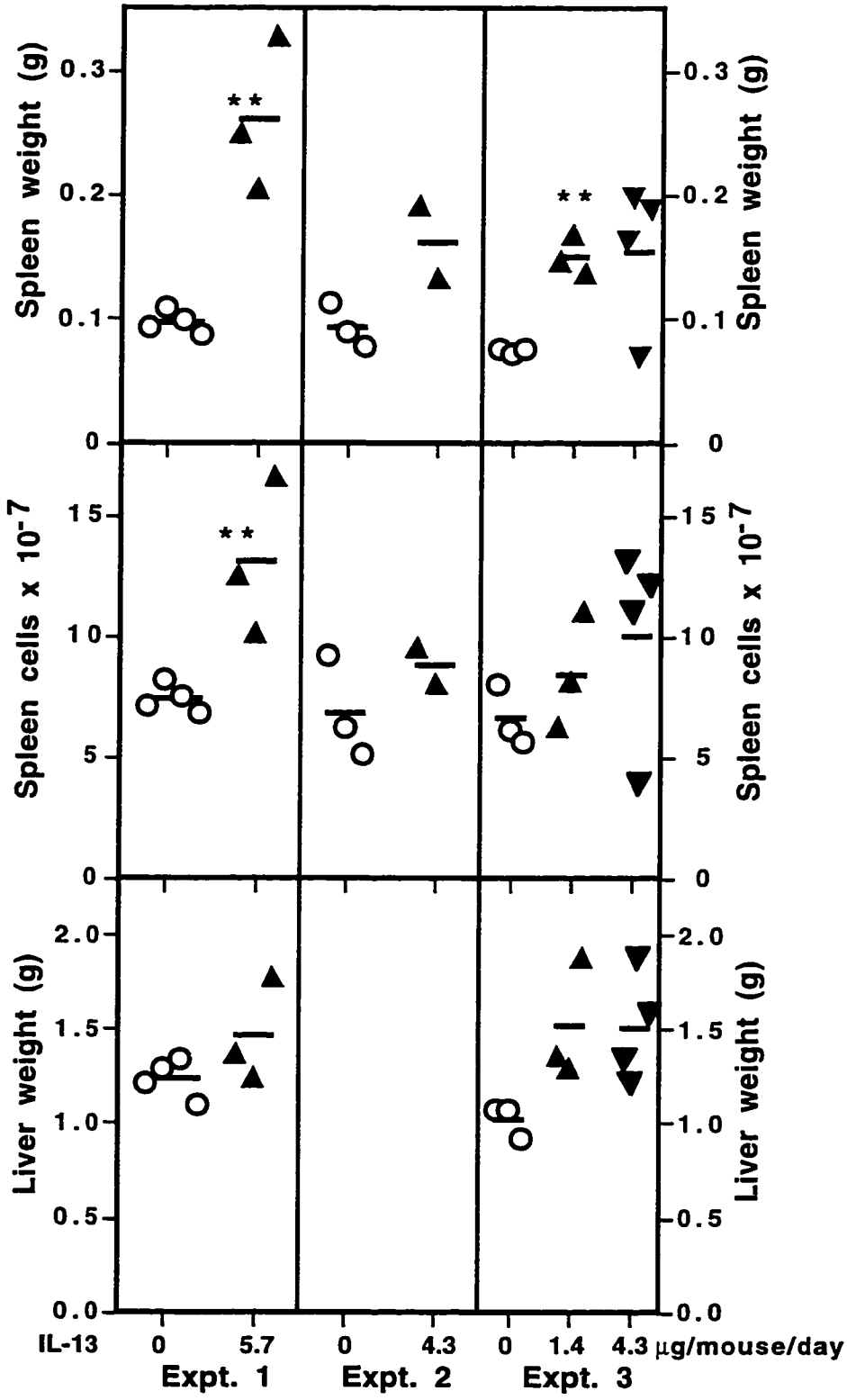
Despite the augmented extramedullary erythropoiesis in the spleen and liver, hemoglobin levels in the peripheral blood of IL-13-treated mice were not elevated but in fact slightly reduced. It is unclear whether this slight anemia was due to rapid destruction of red blood cells, increased plasma volume, or inhibition of *in vivo* maturation of the erythroid precursors.

In contrast to the spleen, bone marrow myeloid and erythroid hemopoietic progenitor frequencies did not increase in response to IL-13 administration. In fact, the total number of cells recovered from both hind legs was two-fold lower than the control value. This may indicate that IL-13 inhibits hemopoiesis in the bone marrow or induces the migration of hemopoietic progenitor cells to the spleen and liver. A similar reduction in mouse bone marrow progenitors can also accompany extramedullary hemopoiesis induced by *Salmonella* infection (Miyanomae et al., 1982). As the relative rates of proliferation and

differentiation of progenitors during infection or IL-13 treatment are unknown, the relative hemopoietic output of the bone marrow and spleen may not necessarily be reflected by the relative numbers of hemopoietic progenitors in these two organs. Moreover, the effects of IL-13 treatment on hemopoiesis in other organs were not investigated.

The strong Th2 response induced by *Nb* infection is accompanied by extramedullary hemopoiesis in the spleen and increased IL-6 production by spleen cells in response to LPS stimulation. Spleen hemopoiesis also occurs during other infections (Ali-Khan, 1978; Miyanomae et al., 1982; Asami et al., 1992) and can help to replenish the effector cells that are lost during strong immune responses (Kirikae et al., 1986). As effector cells are probably depleted during infections that provoke either Th1 or Th2 responses, other cytokine(s) may play a similar role in the induction of extramedullary hemopoiesis during both Th1 and Th2 immune responses. In a strong Th2 response, IL-13 synthesis was increased by spleen cells from *Nb*-infected mice, suggesting that IL-13 may play a major or minor role in extramedullary hemopoiesis during *Nb* infection and perhaps in the replenishment of effector cells to expel the worms. Blocking anti-mIL-13 mAb will help to determine the extent of IL-13 involvement, but our only anti-mIL-13 mAb (RAMP1) does not block IL-13 functions *in vitro*. Unlike IL-4-deficient mice which expel *Nb* normally, mice deficient in IL-4Rα (which is part of IL-4 and IL-13 receptor complexes) or STAT6 (a molecule involved in IL-4 and IL-13 signaling) fail to expel the mouse-adapted strain of *Nb*. In addition, IL-4-deficient mice treated with a specific IL-13-antagonist (a soluble IL-13Rα2-human IgGFc fusion protein), are unable to expel this parasite, strongly indicating that IL-13 may be more important than IL-4 in parasite expulsions (Urban et al., 1998).

Figure 5.1. Continuous IL-13 administration for 7 days resulted in splenomegaly. Mice in were administered continuously with PBS (○) or IL-13 (▲) by osmotic pumps for 7 days in the peritoneal cavity. Spleen weights, total number of splenic nucleated cells, and liver weights of mice were obtained. Each symbol represents data obtained from an individual mouse, and results from three different experiments are shown. Initial doses of IL-13 used were Expt. 1, 5.7 μg/mouse/day; Expt. 2, 4.3 μg/mouse/day; Expt. 3, 1.4 or 4.3 μg/mouse/day. The average value in each group is indicated by a solid horizontal bar. ** indicates that the spleen weight or numbers from IL-13-treated mice were significantly higher than mice treated with PBS, with $p<0.03$.


Spleen weight (g)
0.3
0.2
0.1
0
**
Spleen cells x 10^-7
15
10
5
0
Liver weight (g)
2.0
1.5
1.0
0.5
0.0
IL-13
0
5.7
0
4.3
0
1.4
4.3 µg/mouse/day
Expt. 1
Expt. 2
Expt. 3

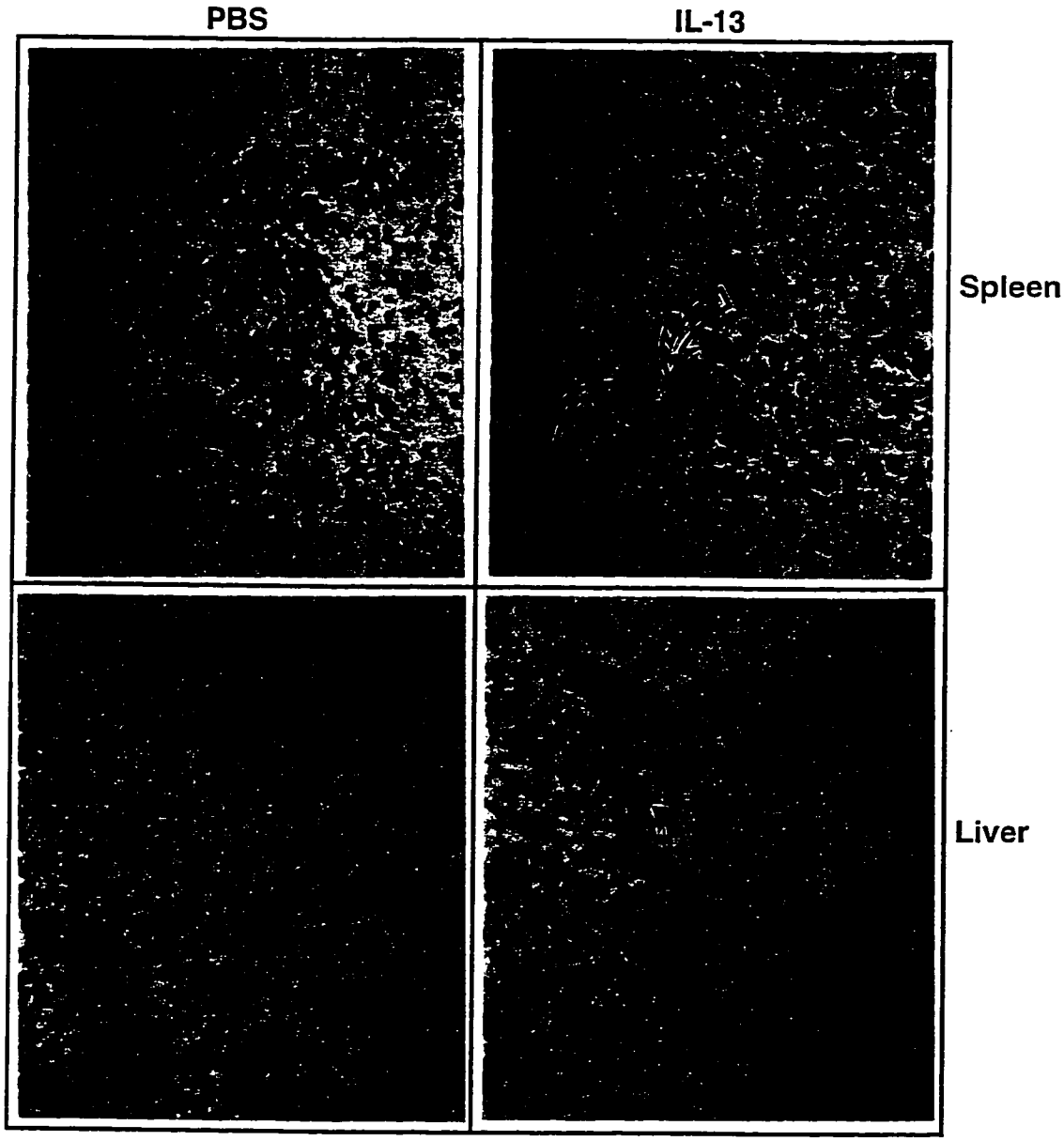


Figure 5.2. ***In vivo*** **IL-13 treatment for 7 days resulted in an increase in hemopoietic foci in the spleen and liver.** Spleen (top panels) and liver (bottom panels) sections were fixed and stained with hematoxylin-eosin. Tissue sections from PBS- (left panels) and IL-13- (right panels, 5.7 μg/mouse/day) treated mice from Expt. 1 are shown (250X magnification). Megakaryocytes and immature hemopoietic cell clusters are indicated by open and closed arrows respectively. Histological sectioning and staining were performed by Dr. S. Poppema.

Figure 5.3. *In vivo* IL-13 treatment resulted in an increase in hemopoietic-cytokine-responsive spleen cells. Spleen cells from *in vivo* PBS (○)- or IL-13 (▲)-treated mice in Expt. 1 were cultured in triplicate or quadruplicate with different concentrations mIL-4 (a), purified IL-13 (b), mGM-CSF (c), mIL-3 (d), and purified hCSF-1 (e). Proliferation or activation was measured on day 6 by MTT cleavage. The means and SDs of replicate cultures are shown. Each curve represents data obtained from an individual mouse.

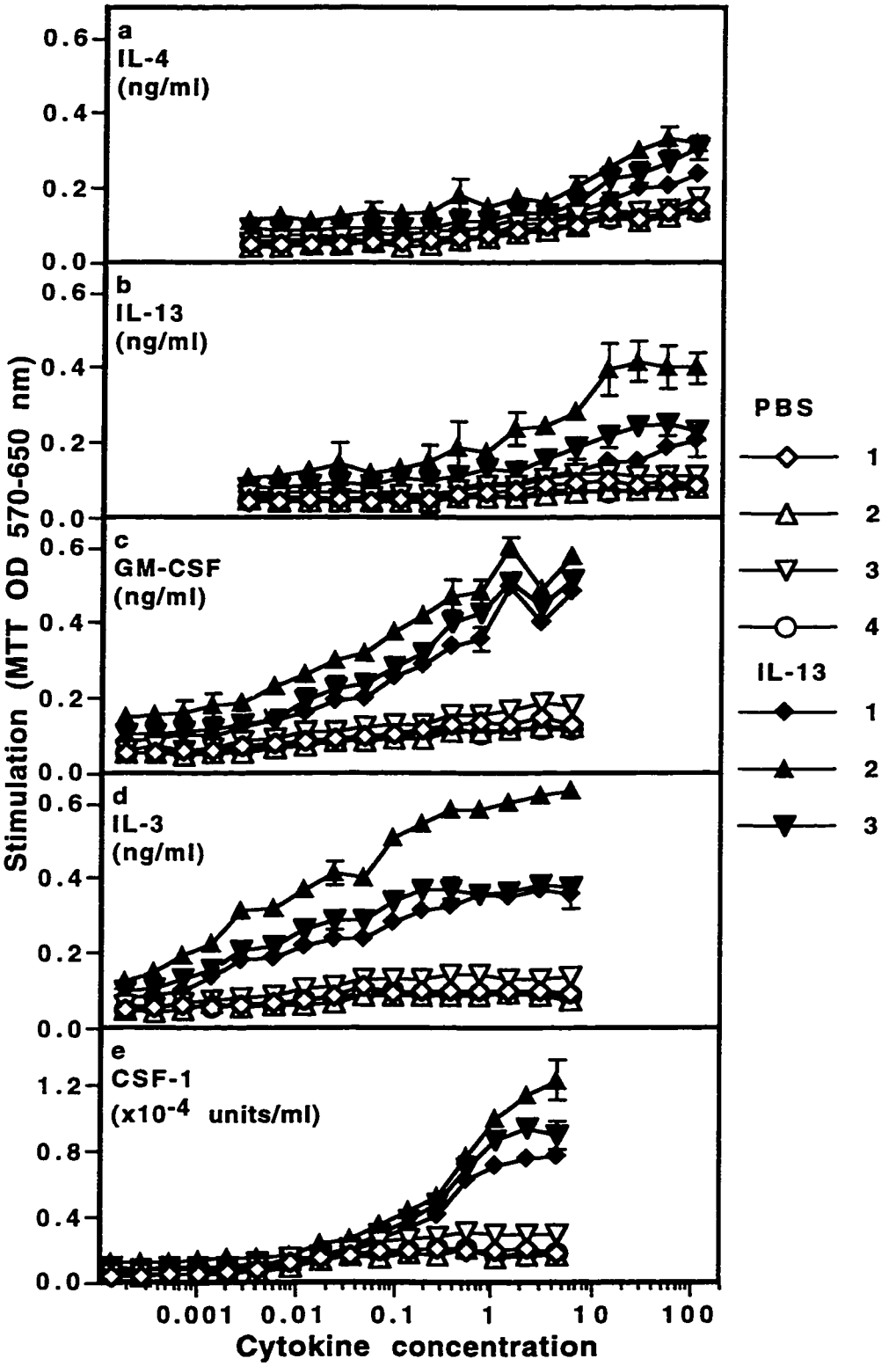
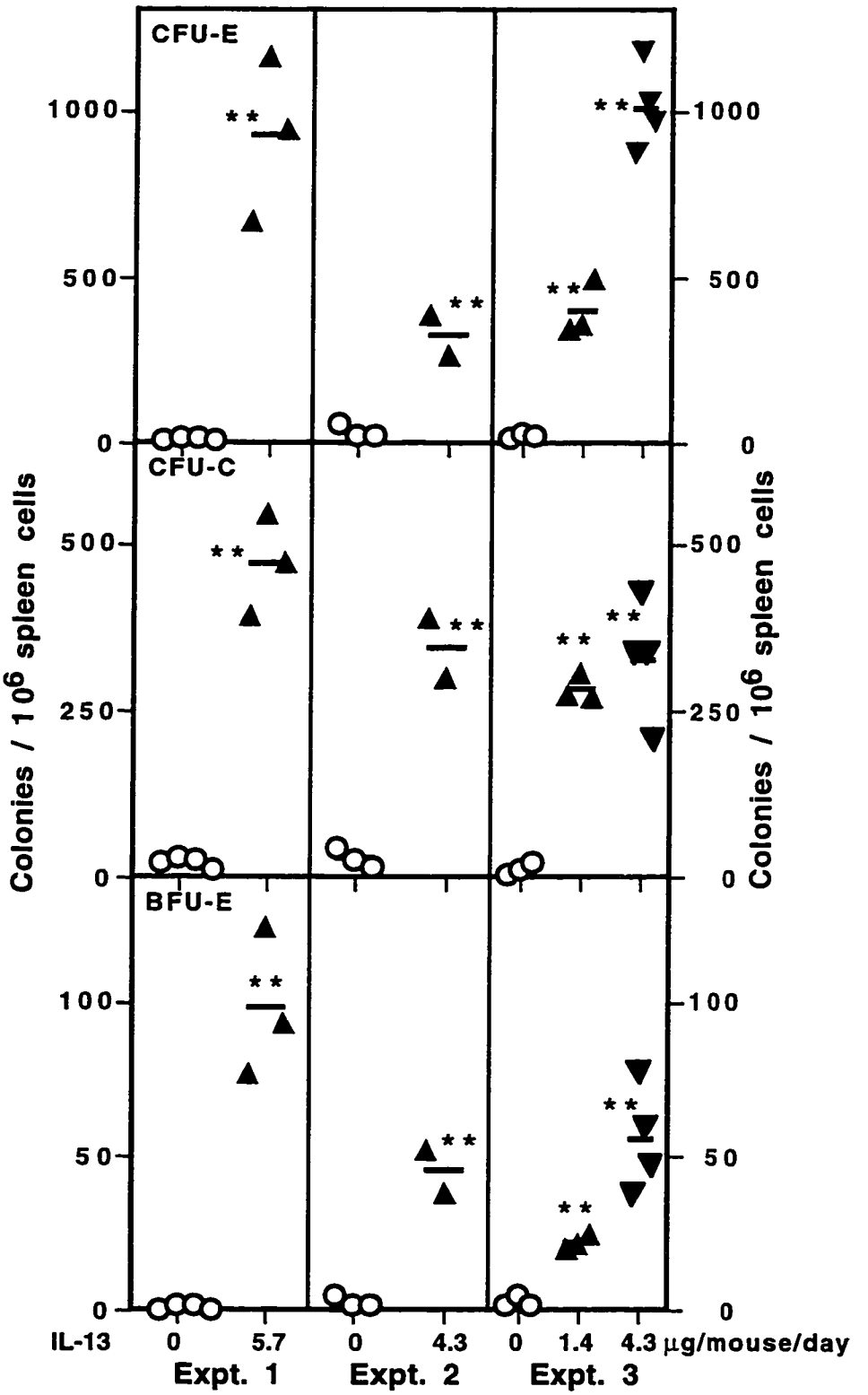

a
IL-4
(ng/ml)
b
IL-13
(ng/ml)
c
GM-CSF
(ng/ml)
d
IL-3
(ng/ml)
e
CSF-1
(x10-4 units/ml)
Stimulation (MTT OD 570-650 nm)
Cytokine concentration
0.001
0.01
0.1
1
10
100
PBS
1
2
3
4
IL-13
1
2
3

Figure 5.4. ***In vivo*** **IL-13 administration resulted in an elevation of hemopoietic precursor frequencies in the spleen.** Mice were treated for 7 days *in vivo* with PBS (○) or IL-13 (▲), and duplicate cultures of spleen cells were then established in methylcellulose medium. CFU-E, CFU-C, and BFU-E colonies were enumerated. Each symbol represents data obtained from an individual mouse, and results from three different experiments are shown. The average value in each group is indicated by a solid horizontal bar. Initial doses of IL-13 used were Expt. 1, 5.7 μg/mouse/day; Expt. 2, 4.3 μg/mouse/day; Expt. 3, 1.4 or 4.3 μg/mouse/day. ** or * indicates $p<0.01$ or $p<0.05$, respectively.


CFU-E
CFU-C
BFU-E
1000
500
0
250
100
50
Colonies / 10^6 spleen cells
IL-13
0
5.7
4.3
1.4
4.3 µg/mouse/day
Expt. 1
Expt. 2
Expt. 3

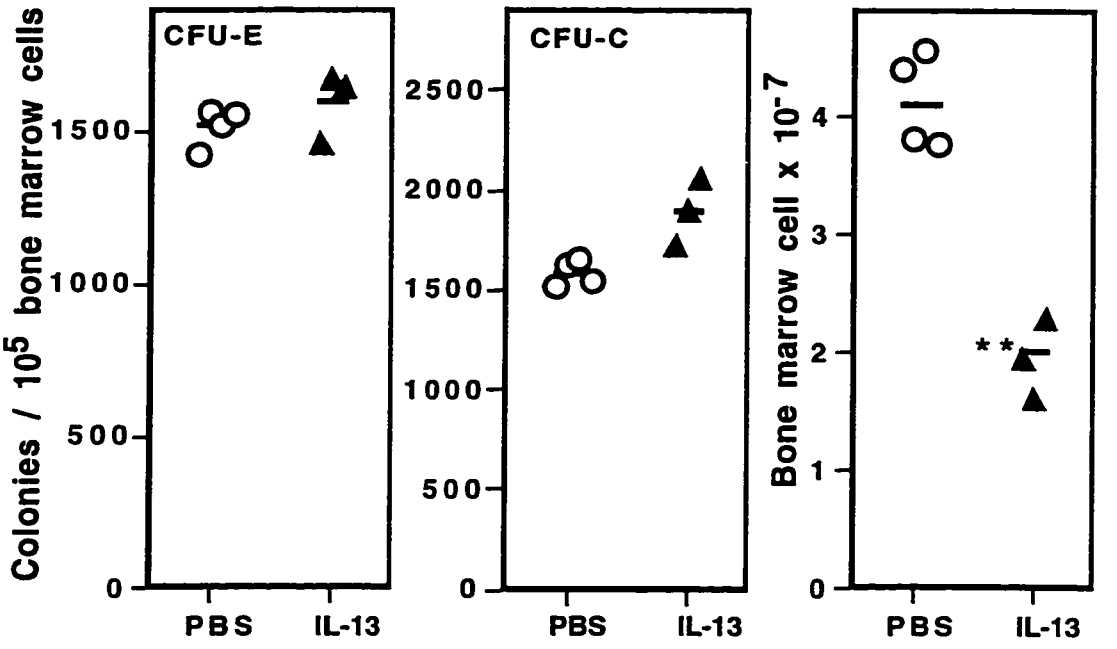


Figure 5.5. ***In vivo*** **IL-13 administration did not result in an elevation of hemopoietic precursor frequencies in the bone marrow, but decreased the total numbers of bone marrow cells.** Bone marrow cells from four PBS (○)- or three IL-13 (▲)-treated mice in Expt. 1 as described in Fig. 5.1 were counted and cultured in methylcellulose medium. CFU-E and CFU-C colonies were enumerated as indicated in Fig. 5.4. Each symbol represents data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. ** indicates $p<0.01$.

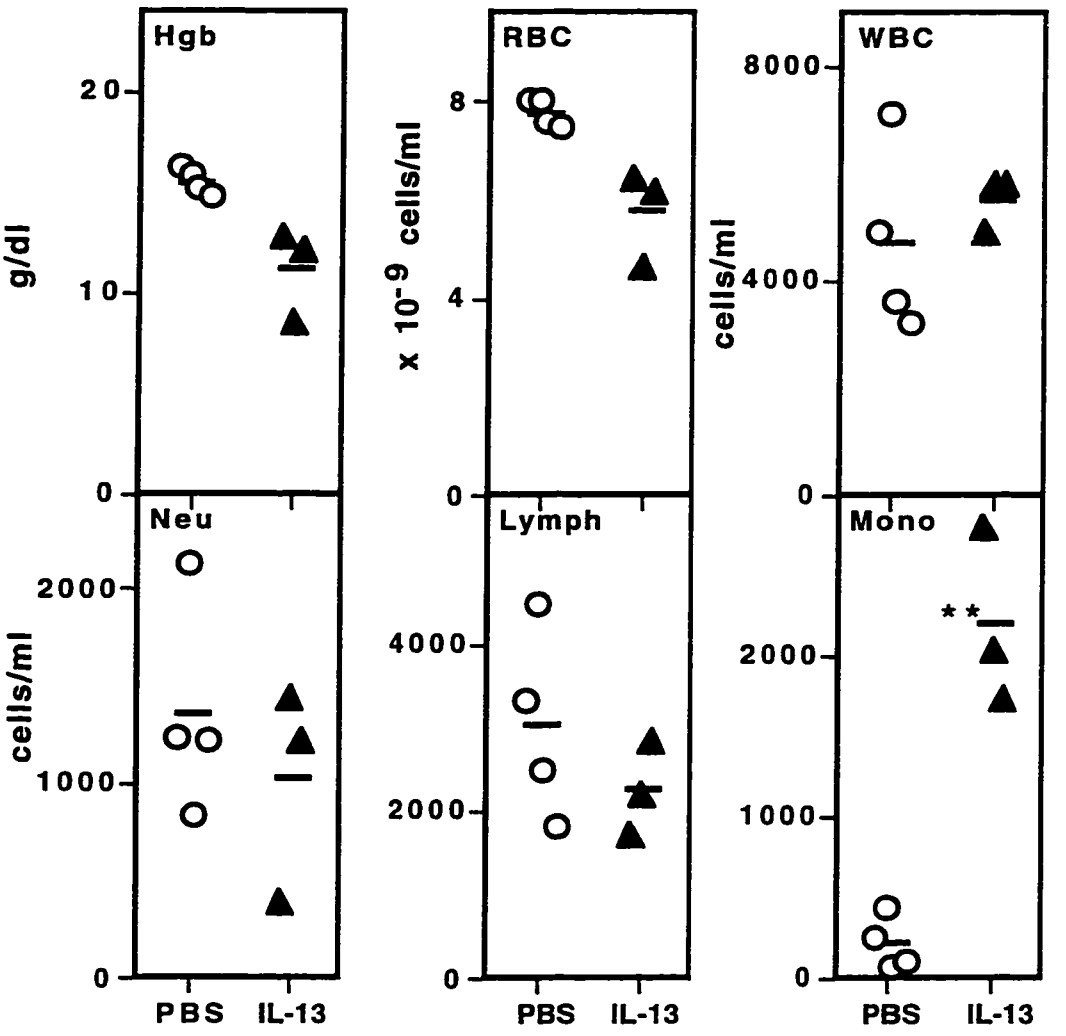


Figure 5.6. ***In vivo*** **IL-13 treatment for 7 days resulted in slight anemia and pronounced monocytosis.** Peripheral blood was collected from PBS (○)- or IL-13- (▲, 5.7 μg/mouse/day) treated mice in Expt. 1 as described in Fig. 5.1. The hemoglobin levels (Hgb) and number of neutrophils (neu), lymphocytes (lymph), leukocytes (WBC), erythrocytes (RBC), and monocytes (mono) were quantitated. Each symbol represents data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. ** represents $p<0.05$.

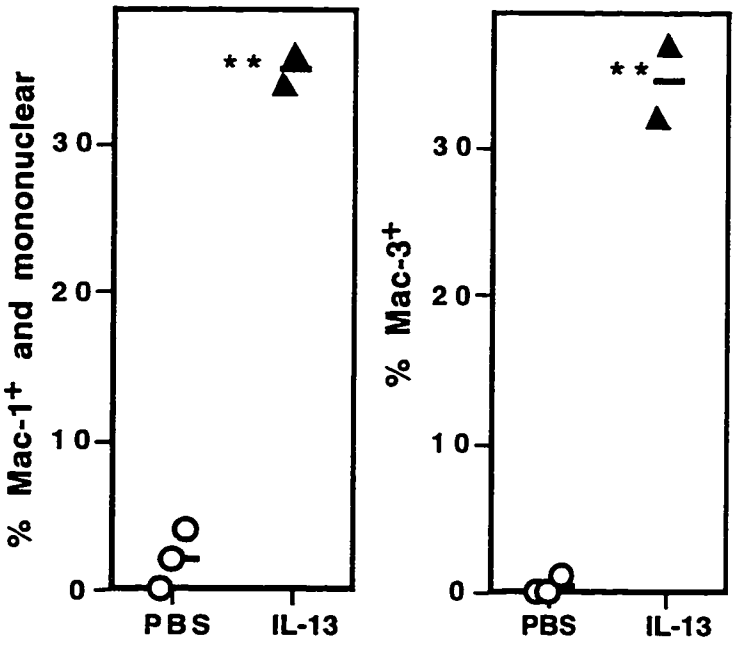


Figure 5.7. *In vivo* IL-13 administration for 7 days resulted in increased Mac-1$^+$ and Mac-3$^+$ mononuclear cells in the peripheral blood. Peripheral blood samples were collected from three PBS (○)- or two IL-13 (▲)-treated mice in Expt. 2 as described in Fig. 5.1. The percentages of Mac-1$^+$ and Mac-3$^+$ mononuclear cells were evaluated by immunohistochemistry. Each symbol represents data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. Fewer than 1 per 100 nucleated cells were stained with isotype control first mAb. ** represents $p<0.05$.

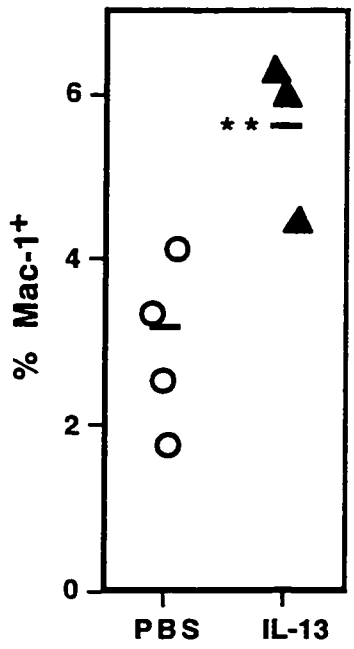


Figure 5.8. ***In vivo*** **IL-13 administration for 7 days resulted in increased Mac-1$^+$ cells in the spleen.** Spleen cells from four PBS(○)- or three IL-13 (▲)-(5.7 μg/mouse/day) treated mice in Expt. 1 as described in Fig. 5.1 were stained for surface Mac-1 expression. 50 000 spleen cells were analyzed by FACS. Each symbol represents % Mac-1$^+$ spleen cells obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. Fewer than 1% of the cells was stained with isotype control mAb. ** represents the percentage of Mac-1$^+$ cells from IL-13-treated mice that was significantly higher than mice treated with PBS, with $p<0.02$.

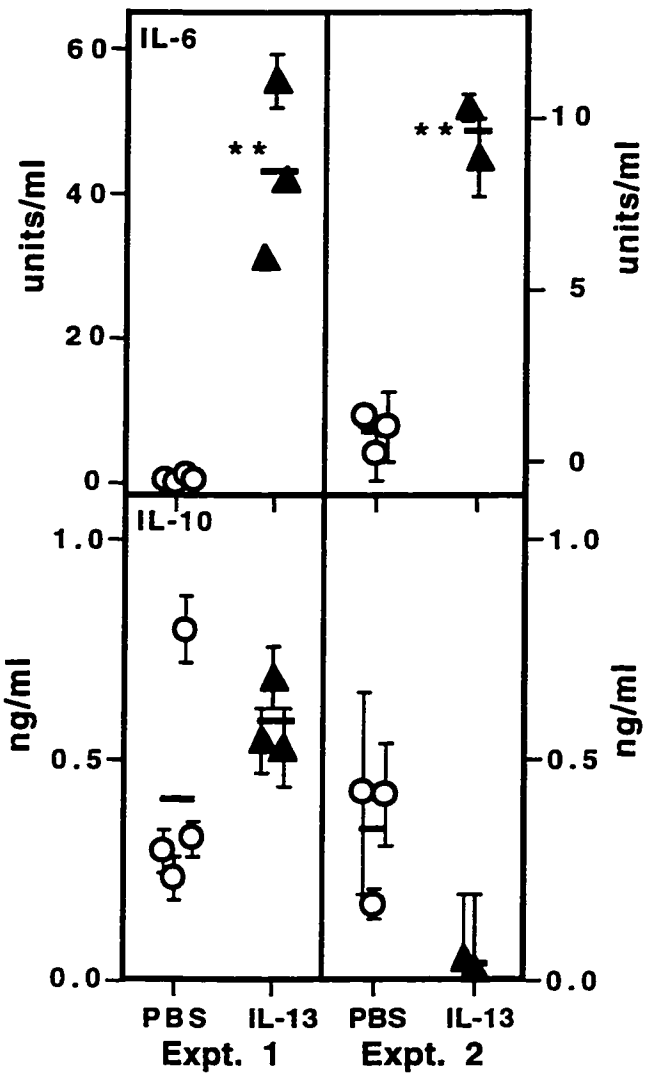


Figure 5.9. *In vitro* IL-6 production by LPS-stimulated spleen cells was enhanced by *in vivo* IL-13 treatment. Spleen cells from PBS (○)- or IL-13 (▲)-treated mice in Expt. 1 and Expt. 2 as described in Fig. 5.1 were stimulated with LPS for 48 h, and IL-6 and IL-10 levels in the supernatants were quantitated by sandwich ELISA. Each symbol represents data obtained from an individual mouse, and the SD of triplicate cultures from individual mouse as described in Fig. 5.1 are shown. The average values in each group are indicated by solid horizontal bars. ** indicates that IL-6 levels of LPS-stimulated spleen cells from IL-13-treated mice were significantly higher than the PBS-treated mice with $p<0.03$. In the absence of LPS stimulation, IL-6 or IL-10 levels in all samples were less than 2.5 units/ml or ranged from 0.19 to 0.79 ng/ml respectively.

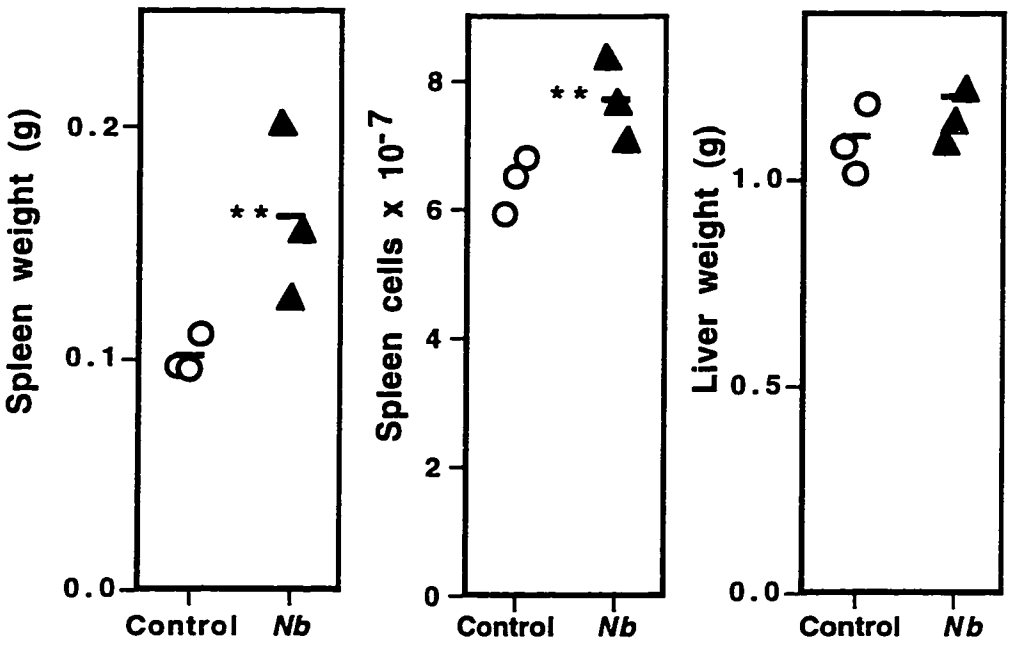


Figure 5.10. ***N. brasiliensis*** **infection for 8 days resulted in splenomegaly.** BALB/c mice were infected with 500 L3 infective *Nb* larvae for 8 days. Spleen weights, total numbers of splenic nucleated cells, and liver weights of mice from Control (○) or *Nb*-infected (▲) mice were obtained. The mean and SD of replicate cultures are shown. Each symbol represents data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. ** indicates $p<0.05$.

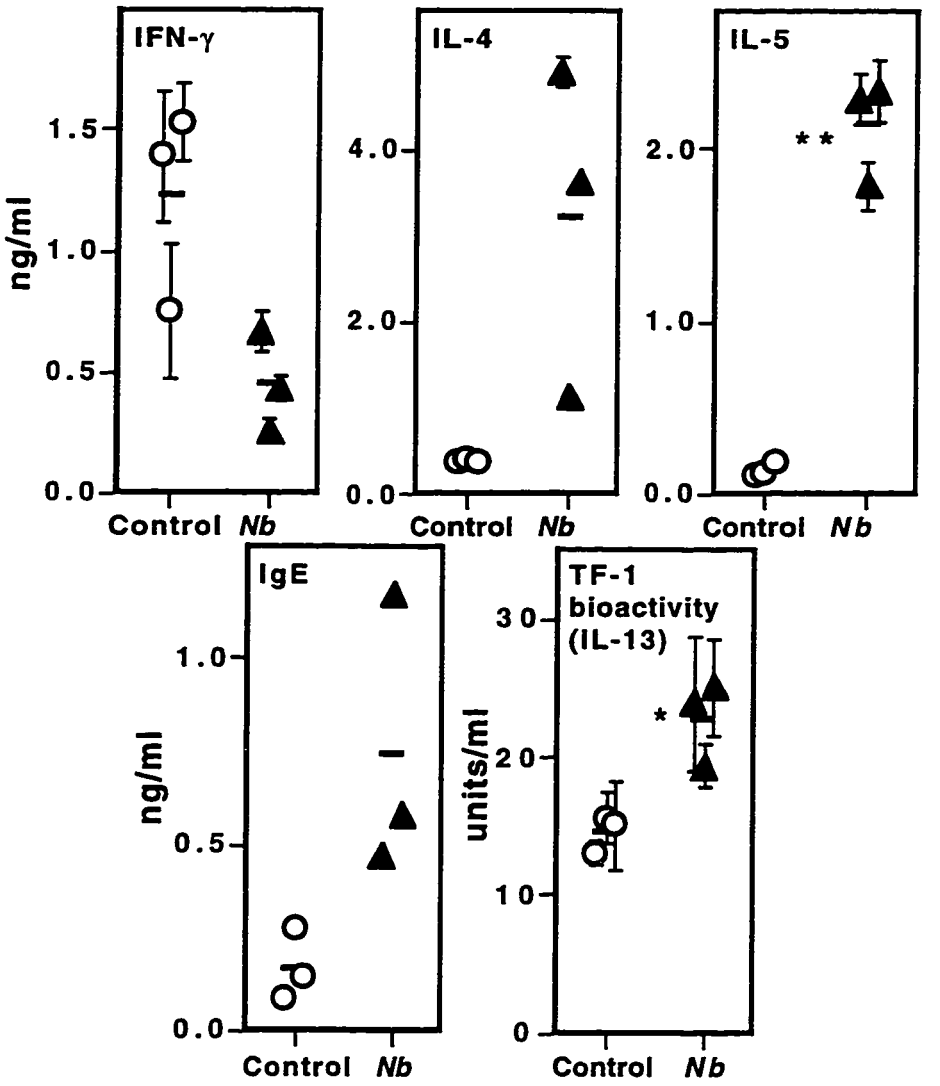
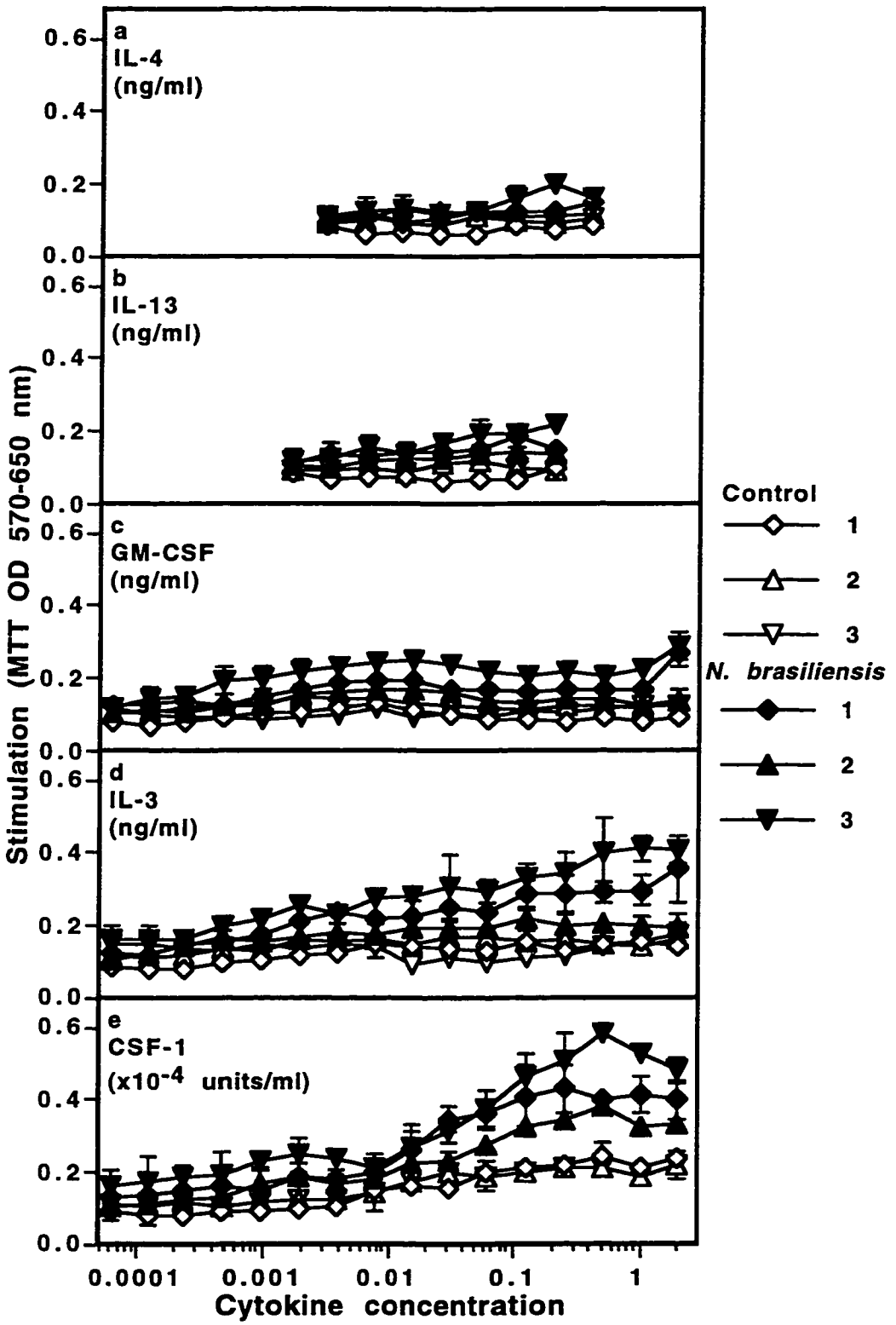


Figure 5.11. ***N. brasiliensis*** **infection enhanced serum IgE levels and** ***in vitro*** **IL-4, IL-5, and IL-13 production, but down-regulated IFN-γ production by Con A-stimulated spleen cells.** Spleen cells from Control (○) or 8-day *Nb*-infected (▲) mice were cultured for 48 h with Con A. IFN-γ, IL-4, and IL-5 levels in the supernatants or serum IgE levels were quantitated by sandwich ELISA. IL-13 levels in Con A-stimulated spleen cell cultures were assayed by TF-1 bioassay. Each symbol represents data obtained from an individual mouse, and the SDs of triplicate cultures from an individual mouse are shown. The average value in each group is indicated by a solid horizontal bar. Spleen cells from both group of mice cultured in the absence of Con A secreted less than 0.21 ng/ml of any of the above cytokines. *Nb*-infected vs. control mice : IFN-γ, $p<0.07$; IL-4, $p<0.12$; IL-5 $p<0.01$ (**); IgE, $p<0.11$; IL-13, $p<0.03$ (*).

Figure 5.12. ***N. brasiliensis*** **infection resulted in an increase in hemopoietic-cytokine-responsive spleen cells.** Spleen cells from control (open symbols) and 8-day *Nb*-infected mice were cultured in quadruplicate with different concentrations of mIL-4 (a), purified IL-13 (b), mGM-CSF (c), mIL-3 (d), and purified hCSF-1 (e). Proliferation or activation was measured on day 6 by MTT cleavage. The means and SDs of replicate cultures are shown. Each curve represents data obtained from an individual mouse.


a
IL-4
(ng/ml)
b
IL-13
(ng/ml)
c
GM-CSF
(ng/ml)
d
IL-3
(ng/ml)
e
CSF-1
(x10-4 units/ml)
0.6
0.4
0.2
0.0
Stimulation (MTT OD 570-650 nm)
0.0001
0.001
0.01
0.1
1
Cytokine concentration
Control
1
2
3
N. brasiliensis
1
2
3

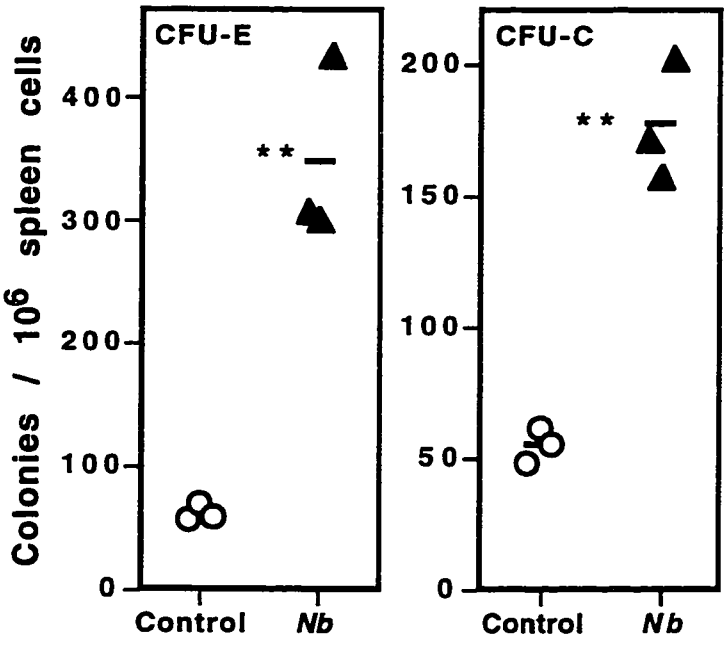


Figure 5.13. ***N. brasiliensis*** **infection induced extramedullary hemopoiesis in the spleen.** Spleen cells from Control (○) or 8-day *Nb*-infected (▲) mice were established in methylcellulose medium in duplicate. CFU-E and CFU-C colonies were enumerated. Each symbol represents data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. CFU counts of the duplicate cultures from each mouse were within 15% of the mean. For both CFU-E and CFU-C, Control versus *Nb*-infected mice, $p<0.02$ (**).

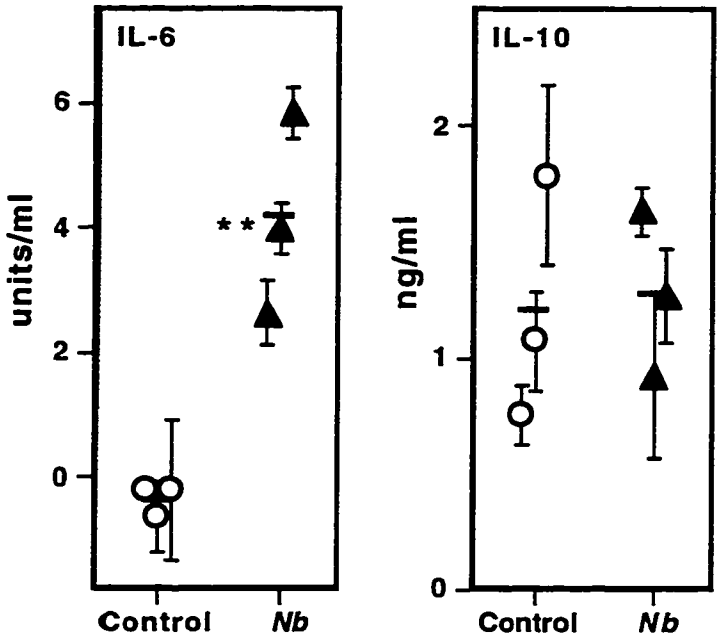


Figure 5.14. *N. brasiliensis* infection enhanced *in vitro* IL-6 production by LPS-stimulated spleen cells. Spleen cells from Control (○) or 8-day *Nb*-infected (▲) mice were stimulated with LPS for 48 h and IL-6 and IL-10 levels in the supernatant were quantitated by sandwich ELISA. Each symbol represents data obtained from an individual mouse, and the SDs of triplicate cultures from individual mouse are shown. The average value in each group is indicated by a solid horizontal bar. ** indicates that IL-6 levels in the supernatant of LPS-simulated spleen cells were significantly higher than uninfected controls with $p<0.01$.

CHAPTER VI

MOUSE IL-13 ENHANCES ANTIBODY PRODUCTION *IN VIVO* AND ACTS DIRECTLY ON B CELLS *IN VITRO* TO INCREASE SURVIVAL AND HENCE ANTIBODY PRODUCTION

(The majority of the data presented in this chapter have been accepted for publication in an article entitled "Mouse IL-13 enhances antibody production *in vivo* and acts directly on B cells *in vitro* to increase survival and hence antibody production" by Yew Hon Lai and Tim R. Mosmann in *J. Immunol.*)

A. Introduction

The generation of antigen-specific antibodies is the most efficient way for mammals to protect themselves from initial and recurring infections (Fuleihan et al., 1995; Klaus et al., 1994). The production of antigen-specific antibodies is a multi-step process which can be categorized as either T cell-independent or T cell-dependent activation. The antigen is recognized by membrane Ig, also known as the B cell antigen receptor. In the absence of T cells, B cells can be activated by polysaccharide antigens or polymeric structures with repeated epitopes, such as polymerized bacterial flagellin, lipopolysaccharide, dextran, ficoll, and pneumococcal capsular polysaccharide that cannot be processed and displayed as peptides together with MHC cII molecules on B cells. The polymeric nature of the antigen may facilitate the cross-linking requirement of B cell activation (Pike et al., 1987; Mond et al., 1980). At high doses, thymus-independent antigens act as mitogens to induce polyclonal responses. This humoral immune response is characterized by the production of IgM and the lack of memory cell generation.

On the other hand, T cell-dependent responses require the participation of T cells for B cell activation (Vitetta et al., 1991; Parker, 1993). Following the binding of an antigen on membrane Ig to provide signal 1 through the cross-linked antigen receptors, the antigen is internalized and processed by the B cell before being presented as antigenic peptides bound to MHC cII molecules to a T cell which is initially primed by antigen-bearing dendritic cells (Inaba et al., 1983; Inaba et al., 1984). Once a T cell recognizes the processed antigen on the membrane of a B cell, they interact and form a T-B conjugate. This conjugation induces directional release of cytokines by the activated T cell and the expression of accessory molecules on the activated T cell membrane. Cytokines such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and TGF-β can influence the growth and differentiation of B cells during the antibody response. However, it is clear that cytokines are not sufficient to provide all the necessary help as interaction of membrane-bound molecules is also required. In the presence of a contact-dependent signal delivered via interaction of B cells and T cells, B cells proliferate, differentiate and undergo class-switching, all of which are thought to occur primarily in the germinal centers of lymph nodes or spleen. Interestingly, T cell help can be delivered without antigen recognition *in vitro* via bystander responses. In the presence of cytokines, paraformaldehyde (PFA)-fixed plasma membranes derived from activated but not resting T cells can replace signal 1 in delivering contact-dependent help to B cells, resulting in proliferation and antibody secretion (Brian, 1988; Hodgkin et al., 1990; Noelle et al., 1989; Kawakami and Parker, 1993).

The number of ligand pairs in T-B interactions involved in the delivery of help is large and growing. CD40 is a B cell antigen but is also expressed on dendritic cells, macrophages, and epithelial cells (Clark and Ledbetter, 1986; Banchereau et

al., 1991; Banchereau et al., 1994; Stamenkovic et al., 1989; Smith et al., 1990; Smith et al., 1994; Johnson et al., 1986). The importance of CD40 in B cell activation is substantiated by its success in propagating B cells *in vitro* using an anti-CD40 mAb immobilized on Fc bearing stromal cells and, thus, bypassing the requirement for Ig receptor cross-linking (Banchereau et al., 1991). With the discovery of the ligand for CD40, CD40L, on the surface of activated-T cells (Armitage et al., 1988; Armitage et al., 1992b; Lane et al., 1992; Noelle et al., 1992; Armitage et al., 1992a; Noelle et al., 1992; Hollenbaugh et al., 1992; Spriggs et al., 1992) and mast cells (Gauchat et al., 1993), and the ability of soluble CD40L to substitute for the membrane-expressed ligand in contributing to T-dependent B cell activation (Hollenbaugh et al., 1992; Lane et al., 1993), the understanding of T-dependent B cell activation has been revolutionalised. CD40-CD40L signaling is necessary for Ig class switching because patients with defective CD40L exhibit hyper-IgM and little IgG, IgA, or IgE production and have no germinal centers in their lymph nodes (Tohma et al., 1991). As T-B interactions are not required for T-independent activations, these patients are capable of mounting normal T-independent and T-dependent IgM immune responses, but not class switching (Callard et al., 1993). Observations from mice with CD40 (Kawabe et al., 1994) or CD40L (Xu et al., 1994; Renshaw et al., 1994) deficiency further confirm the importance of this ligand/receptor pair during cognate immune responses.

Although CD40L exerts a dominant effect in B cell activation, other molecules contribute to T-B interactions. CD40L alone or even when overexpressed stimulates B cells inefficiently, whereas optimum T-dependent stimulation requires synergistic interactions with other molecules including MHC cII (Poudrier and Owens, 1994; Lane et al., 1990), adhesion molecules (Lane et al.,

1991; Owens, 1991; Poudrier and Owens, 1994), other CD40L family members (Macchia et al., 1993), and cytokines (Hodgkin et al., 1990; Isakson et al., 1982).

Activated T cells deliver both contact and cytokine mediated signals to stimulate B cell proliferation and differentiation. A variety of cytokines act at various stages of B cell activation, proliferation, and differentiation. Proliferation of activated B cells is induced by IL-2, IL-4, IL-5, human IL-10, and human IL-13. Differentiation into plasma cells, which produce different immunoglobulin isotypes, is induced by IL-2, IL-4, IL-5, IL-10, IFN-γ, or TGF-β, while IL-6 appears to stimulate late-stage B cell maturation (Hirano et al., 1986; Yamasaki et al., 1988) and antibody production (Splawski et al., 1990). TGF-β induces LPS-stimulated B cells to switch to IgA expression (Mosmann and Coffman, 1989; Sonoda et al., 1989; Coffman et al., 1989; van Vlasselaer et al., 1992). IL-4 is a potent mouse IgG1 (IgG4 in human) and IgE class switching factor but suppresses the expression of IgM, IgG3, IgG2a, and IgG2b in LPS-stimulated B cells (Isakson et al., 1982; Howard et al., 1982; Coffman et al., 1986; Pene et al., 1988a; Pene et al., 1988b). Depending on the appropriate experimental conditions, IL-5 boosts the production of most isotypes in mouse B cells (Gately et al., 1994; Takatsu et al., 1988). Interestingly, IL-5 demonstrates biological activities on human B cells only under certain mitogenic stimulations (Bertolini et al., 1993; Huston et al., 1996). On the other hand, IFN-γ enhances the expression of IgG2a, while inhibiting IgM, IgG3, IgG1, IgG2b, and IgE responses (Snapper and Paul, 1987). Thus, these two panels of Th1 and Th2 cytokines are antagonistic with respect to isotype expression.

In vivo studies support the role demonstrated *in vitro* for IL-4 in allergic disease. In humans, high levels of IL-4 are associated with allergic diseases such as atopic

dermatitis and hay fever, which are characterized by high IgE titers and high numbers of mast cells (Brown and Hanifin, 1989; Ricci, 1994). All transgenic mouse lines expressing the IL-4 transgene in T cells develop an allergic-like disorder of the eyelid (Tepper et al., 1990). The inflammatory infiltrate includes macrophages, eosinophils, and a marked increase in mast cells. Also, anti-IL-4 mAbs *in vivo* strongly inhibit IgE synthesis without affecting other Ig isotypes (Finkelman et al., 1986). Therefore, it is not surprising that IL-4-deficient mice do not produce IgE in response to nematode infection. However, these IL-4-deficient mice generated high titers of IgG1 responses against *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus* infection but produce considerably reduced IgG1 responses against *Trichuris muris* or *Trichinella sporalis* nematodes, indicating that IL-4 may not be required for IgG1 class-switching in certain nematode infections (Kuhn et al., 1991; Finkelman et al., 1997).

Up until the identification of IL-13, IL-4 was the only cytokine capable of inducing IgE class-switching in human and mouse B cells. Similar to IL-4, IL-13 induces CD23 expression, germ-line ε mRNA synthesis, and IgG4 and IgE switching in human B cells. The effects of IL-13 on human B cells are independent of IL-4, but the former is generally more potent in stimulating Ig production (McKenzie et al., 1993a; Punnonen et al., 1993).

In contrast to the effects of IL-13 on human B cells, IL-13 has been reported not to have any effects on mouse B cells (Zurawski and de Vries, 1994). Although this is consistent with the observation that IL-4-deficient mice do not produce IgE in response to *Nippostrongylus brasiliensis* infection (Kuhn et al., 1991; Lawrence et al., 1995; Morawetz et al., 1996), these mice produce IgE as a result of malaria (von der Weid et al., 1994) or *Leishmania major* infection (Noben-Trauth et al.,

1996) or during the course of retrovirus-induced immunodeficiency syndrome or anti-IgD treatment (Morawetz et al., 1996). These data suggest that an IL-4-independent mechanism for IgE synthesis also exists in mice and raises the possibility that mouse IL-13 may also induce IgE synthesis in some circumstances.

Because IL-13 is associated with the Th2 response in mice, including strong antibody production and IL-13 stimulates antibody production by human B cells, the potential effects of mouse IL-13 on mouse antibody production were re-examined in this study.

B. Results

IL-13 increased antibody production levels in vivo. In order to study the effects of IL-13 during a strong immune response, IL-13 or PBS was administered via osmotic pump in the peritoneal cavity of BALB/c female mice over a period of 7 days. Initial doses of IL-13 ranging from 0.5 to 6.5 μg/mouse/day were administered. After the pump implantation, the mice were immunized with chicken red blood cells (CRBC) in the peritoneal cavity. Consistent with the observations described in Chapter V (Fig, 5.1) (Lai et al., 1996), *in vivo* IL-13 administration induced splenomegaly (Expt. 2. 2.0-fold; $p<0.03$ and Expt. 3. 1.5-fold; $p<0.04$) due to increased splenocyte numbers, even during the strong immune response to CRBC. PBS-treated mice immunized with CRBC showed about a two-fold increase of total plasma IgG1, IgG2a, IgG2b, IgG3 and IgM compared to the Ig levels of untreated and non-immunized litter-mates (data not shown).

When CRBC-immunized mice were treated continuously with different doses of IL-13 in three separate experiments, significant further increases in total Ig levels were observed for IgG1, IgG2a and IgG2b (Fig. 6.1). IgM and IgG3 levels showed a significant increase in only one out of three experiments, and IgA did not show any significant changes. In one experiment (Expt. 3) IgE levels (IL-13, 137 ± 82.3 vs. PBS, 176.6 ± 93.5) were not significantly different between IL-13 and control groups. Consequently, *in vivo* administration of IL-13 enhanced total serum or plasma levels of at least three IgG subclasses during a strong immune response to CRBC.

IL-13 enhanced hemagglutination titers. The indirect and direct hemagglutination titers in the plasma of these mice were assayed in order to quantitate antigen-specific antibody production *in vivo*. The IL-13-treated groups consistently showed increased direct and indirect CRBC agglutination titers compared to those of the PBS-treated groups (Fig. 6.2). Isotype-specific indirect CRBC agglutination titers of IgG1, IgG2a, and IgG2b were also elevated in the IL-13-treated group (Fig. 6.3). Hence, *in vivo* treatment with IL-13 enhanced the antigen-specific antibody response.

In vivo IL-13 administration caused a slight increase in B220$^+$ splenocyte numbers. As treatment with IL-13 *in vivo* enhanced the anti-CRBC antibody response, it was investigated whether there was an increase in B220$^+$ splenocytes. Spleen cells from IL-13-treated mice demonstrated a slight but not statistically significant increase in B220$^+$ splenocytes compared to those of the PBS-treated mice ($19.3 \pm 4.2 \times 10^7$ vs. $13.6 \pm 0.8 \times 10^7$ cells/spleen). Two other experiments showed similar slight increases.

IL-13 in vivo treatment induced ex vivo antibody production. Next, *ex vivo* antibody production was determined by culturing different numbers of spleen cells from these mice. Supernatants were harvested after 3 days and quantitated for *ex vivo* Ig production. Consistent with the total plasma Ig levels, spleen cells from both IL-13-treated and control groups of CRBC-immunized mice secreted similar levels of IgM and IgG3 (Fig. 6.4). However, spleen cells from IL-13-treated mice secreted higher levels of total IgG1, ($p<0.05$), IgG2a ($p<0.05$) and IgG2b ($p<0.05$) under the same culture conditions (Fig. 6.4). These data indicate that spleen cells from IL-13-treated mice secreted higher levels of Ig, presumably due to *in vivo* activation.

In vivo IL-13 administration did not alter cytokine secretion in spleen cell cultures. The fact that *in vivo* IL-13 treatment induced higher levels of plasma IgG1, IgG2a, and IgG2b could have been due to direct or indirect effect of IL-13. Spleen cells were stimulated with Con A (Fig. 6.5) or CRBC (Fig. 6.6), and cytokine concentrations in the supernatants were quantitated. Spleen cells stimulated with Con A (Fig. 6.5) or CRBC (Fig. 6.6) from both control and experimental groups exhibited no consistent and significant differences in IFN-γ, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, or IL-10 secretion. In addition, the ratios of the sum of Th1/Th2 cytokines were not significantly different (IL-13, 137 ± 82.3 vs. PBS, 176.6 ± 93.5), suggesting that these cytokine patterns may differ in both groups of mice at earlier time points, IL-13 may not have any direct influence on T cell differentiation, or IL-13 may directly regulate *in vivo* antibody production.

IL-13 enhanced Ig levels in the presence of activated and fixed T cells. As IL-13 treatment *in vivo* did not alter the levels of cytokines secreted by Con A-(Fig. 6.5) or CRBC- (Fig. 6.6) stimulated spleen cells *in vitro*, it was hypothesized that mouse B cells might respond directly to IL-13, analogous to the direct effects of hIL-13 on human B cells (Punncnen et al., 1993; McKenzie et al., 1993a; Minty et al., 1993; Defrance et al., 1994; Cocks et al., 1993). In order to investigate the effects of IL-13 on mouse B cells, different numbers of PFA-fixed anti-CD3-activated M264-15 (Th1) or D10.G4.1 (Th2) T cells were cultured with $B220^+$ splenic B cells in the absence or presence of varying doses of IL-13 or IL-4 for 7 days. Both IL-13 and IL-4 consistently enhanced the levels of IgM and IgG1 in the supernatants (Fig. 6.7). However, compared to IL-4, IL-13 was consistently at least two-fold less effective in enhancing IgM production and more than ten-fold

less effective on IgG1 synthesis. IL-13 effects on the production of IgG2a, IgG2b, and IgG3 varied between experiments, possibly due to the release of endogenous cytokines such as IFN-γ, IL-4, or IL-13 from the PFA-fixed T cells. Similar findings were observed when Th1 or Th2 clones were replaced by irradiated primary allo-reactive Th1 cells (data not shown).

IL-13 enhanced Ig levels in the presence of CD40L-transfected cells. T cells fixed after activation provide a source of CD40L, which is expressed on activation and is the major cell-surface activating signal delivered by T cells to B cells (Armitage et al., 1992a; Noelle et al., 1992; Spriggs et al., 1992; Smith et al., 1994; Kawabe et al., 1994; Xu et al., 1994; Renshaw et al., 1994; Noelle et al., 1989; Kawakami and Parker, 1993; Banchereau et al., 1991). In order to examine the potential effects of IL-13 during CD40L stimulation, $sIgD^+$ splenic B cells were cultured with different numbers of irradiated and unstimulated cells of a CD40L-transfected T cell hybridoma, 40LDS, or the wildtype parental cell line, 2R50.20 (WT) (kindly provided by Dr. D. Parker), in the absence or presence of various doses of IL-13 or IL-4. As expected, IL-4, in the presence of irradiated CD40L-transfected cells, induced strong IgG1 and IgE class switching (Fig. 6.8). Various doses of IL-13 also cooperated with CD40L to increase the levels of IgM, IgG1, IgG2b, and IgG3 (Fig. 6.8). PFA-fixed CD40L-transfected cells gave similar results, suggesting that IL-13 exerted direct effects on B cells (data not shown).

IL-13 and anti-CD40 mAb stimulation upregulated Ig levels. The above results, particularly with PFA-fixed cells, strongly suggested that IL-13 stimulates naive mouse B cells. However, as activated T cells produce large amounts of cytokines, leakage of even a small fraction of cytokines from fixed cells might have

influenced the result. To exclude this possibility, B cells were stimulated in the absence of T cells by culturing sIgD$^+$ splenic mouse B cells with various doses of anti-CD40 antibody in the absence or presence of various doses of IL-13 or IL-4 for 6 to 7 days. In the presence of suboptimal doses of anti-CD40 antibody, IL-13 significantly enhanced IgM, IgG1, IgG2b, and IgG3 production by 3- to 8-fold (Fig. 6.9). By comparison, IL-4 induced similar levels of IgM, but higher levels of the IgG isotypes, particularly IgG1. In this mouse B cell system, IL-4 efficiently induced all isotypes of Ig levels tested, and in particular, at least 100-times more IgG1 production than IL-13. In the presence of anti-CD40 antibody and up to 100 ng/ml of IL-13, IgE levels in the cultures remained below the detection limit (6.25 ng/ml), in contrast to the induction of nearly 280 ng/ml IgE by anti-CD40 and 20 ng/ml of IL-4. Thus, low concentrations of anti-CD40 and IL-13 synergised in stimulating a moderate level of antibody production. Similar results were obtained in three other experiments, except that IL-4 often induced higher levels of IgM than IL-13.

IL-13 directly stimulated Ig production by mouse B cells. Although IL-13 enhanced Ig production in bulk culture, and the sIgD$^+$ sorted mouse B cells were >99.5% pure, each well contained 50,000 cells. Thus, up to 250 contaminating cells could have been present in each well, and indirect effects of IL-13 were theoretically possible. To exclude this possibility, sIgD$^+$ sorted mouse B cells were titrated to 90 to 100 cells per well and cultured in various combinations of anti-CD40 mAb, IL-13, and IL-4. Under these conditions, at least some wells must have contained only B cells, allowing a stringent test of the ability of IL-13 to enhance antibody production in the absence of any other cell type. After two days, the cells in each well were counted and transferred to affinity-purified goat anti-mouse IgM-coated nitrocellulose plates for an ELISPOT assay. In the

presence of anti-CD40 mAb, different doses of IL-13 not only enhanced the number of B cells that survived after 2 days of culture but also increased the numbers of IgM-secreting B cells (Fig. 6.10). Similar data were obtained in eight separate experiments (three are shown in Fig 6.10). Unlike IL-13-treated cells, IL-4 and anti-CD40-responsive sIgD$^+$ B cells formed tight clusters, and the number of antibody spots could not be accurately enumerated, supporting the idea that IL-4 and IL-13 have different effects on mouse B cells.

When sIgD$^+$ (naive) B cells were compared to B220$^+$ (naive plus memory) B cells, IL-13 and anti-CD40 induced similar levels of antibody-secreting cells from both populations (Fig. 6.11), indicating that the response of the naive population was not due to a stronger response of small numbers of contaminating IgD$^-$ memory B cells. In addition, the biological effects of IL-13 on mouse B cells were independent of IL-4 as the anti-IL-4 mAb failed to inhibit the response. Hence, these data indicate that IL-13 directly stimulated naive mouse B cells by an IL-4-independent pathway.

Unlike IL-4, IL-13 did not upregulate surface IgM (Fig. 6.12), CD23 (FcεRII) (Fig. 6.13), or MHC cII molecules (Fig. 6.14) on anti-CD40 stimulated sIgD$^+$ B cells, further indicating that IL-13 did not appear to activate mouse B cells. Neither IL-4 nor IL-13 upregulated MHC cI expression (Fig. 6.14). These IL-4 effects on surface molecule expression were consistent with the known functions of IL-4 (Noelle et al., 1984; Roehm et al., 1984b; Conrad et al., 1987; Pene et al., 1988a; Shields et al., 1989; Paul and Seder, 1994).

IL-13 effects on B cell proliferation. The increased numbers of B cells in the microwell experiments indicated that IL-13 enhanced either the survival or

proliferation of mouse B cells. To distinguish between these possibilities, $sIgD^+$ splenic mouse B cells were stimulated with anti-CD40 mAb in the absence or presence of IL-13 or IL-4, and thymidine incorporation was measured on day 3. IL-4 strongly enhanced proliferation of mouse B cells, particularly in the presence of anti-CD40 antibody (Fig. 6.15). However, IL-13 did not consistently enhance the proliferation of anti-CD40 stimulated $sIgD^+$ B cells although slight effects were seen in some experiments (e.g. Fig 6.15).

IL-13 enhanced mouse B cell survival. In order to distinguish whether IL-13 affected mouse B cell survival or proliferation, mouse $sIgD^+$ B cells were stained with CFSE and stimulated with 3 ng/ml of anti-CD40 antibody in the presence or absence of IL-13 or IL-4 for three days. Viable B cells in bulk cultures were counted and subjected to FACS analysis (Fig. 6.16). Consistent with the microculture observations, in the presence of a suboptimal dose of anti-CD40 antibody, cultures with IL-13 and IL-4 resulted in increased numbers of B cells. The fluorescent intracellular stain CFSE allows tracking of the proliferation or division history of individual cells (Lyons and Parish, 1994). After two days in anti-CD40 alone, the majority of the B cells demonstrated the characteristics of small non-proliferating lymphocytes as assessed by forward scatter (Fig. 6.16a) and high CFSE content (Fig. 6.16e), whereas a smaller subpopulation were larger and appeared to have divided once, as indicated by halving of the CFSE intensity. As a result of incubation in the combination of anti-CD40 and IL-13 (5 or 20 ng/ml), B cells maintained similar forward scatter and CFSE profiles compared to anti-CD40-stimulated B cells (Fig. 6.16b, c, f & g). This observation indicated that IL-13 did not induce any additional proliferation, and thus, the increase in viable cell number induced by IL-13 (Fig. 6.16j & k) was due to increased survival. In contrast, IL-4 induced activation and further proliferation in

anti-CD40-stimulated B cells, as shown by a marked increase in the forward scatter of the replicating cells (Fig. 6.16d), and the appearance of CFSE fluorescence peaks indicating two and three divisions (Fig. 6.16h). Collectively, the above proliferation and antibody data indicate that IL-13 enhanced antibody synthesis by increasing survival rather than inducing proliferation of anti-CD40-activated B cells.

C. Discussion

After continuous administration by osmotic pumps for 7 days, mouse IL-13 enhanced antigen-specific antibody levels in the plasma indicating that IL-13 can play a role in enhancing antibody production *in vivo*. At least part of this effect may be due to direct stimulation of B cells, as IL-13 induced B cells *in vitro* to secrete higher Ig levels in response to a number of stimuli: PFA-fixed anti-CD3 activated M264-15 (Th1) or D10.G4.1 (Th2) cells; irradiated allo reactive primary Th1 cells; and most significantly, anti-CD40 antibody acting on $sIgD^+$ or $B220^+$ B cells. The magnitude of the IL-13 effect was generally lower than that induced by IL-4, even at saturating levels of IL-13. Higher levels of IL-13 were also required before saturation was observed. These observations are consistent with the weak effects of IL-13 on human B cells compared to those of IL-4 (Defrance et al., 1994). Unlike IL-4 (Noelle et al., 1984; Roehm et al., 1984b; Conrad et al., 1987; Pene et al., 1988a; Shields et al., 1989), IL-13 did not upregulate surface MHC cII, IgM, or CD23 expression on activated mouse B cells. However, mouse IL-13 induced parallel increases in the numbers of total B cells and antibody-secreting cells in microcultures containing only B cells, confirming the direct and IL-4-independent biological effects of IL-13 on mouse B cells. Interestingly, treatment of mice with anti-IL-13 polyclonal antibody reduces humoral immune responses at mucosal surfaces, further supporting a role for IL-13 during humoral responses (Bost et al., 1996). As IL-13 did not increase cell division, it is thus concluded that mouse IL-13 increased antibody production by enhancing B cell survival.

In agreement with previous reports (Zurawski and de Vries, 1994; Welham et al., 1995), this study did not detect any IL-13 effects on LPS-stimulated mouse B cells, or B cells stimulated strongly by anti-CD40 antibody (>100 ng/ml) (data not

shown). Hence, the biological effects of IL-13 on mouse B cells may not be revealed during potent stimulation from either anti-CD40 antibody or LPS. Data from this study reveal that IL-13 has biological effects on mouse B cells at suboptimal doses of anti-CD40 antibody, which may represent more physiological stimulation levels. Thus, effects of IL-13 may not be observed in all B cell stimulatory systems, which may account for a previous report that IL-13 failed to exhibit any biological functions on mouse B cells (Zurawski and de Vries, 1994).

IL-4 enhances proliferation of mouse and human B cells and moderately stimulates synthesis of most Ig subclasses. In addition, IL-4 strongly stimulates the cell surface expression and secretion of IgG1 (mouse)/IgG4 (human) and IgE (mouse and human) (Vitetta et al., 1985; Coffman et al., 1986) by the induction of specific class switching (Snapper and Paul, 1987; Lutzker et al., 1988). Human IL-13 stimulates $sIgD^+$ B cells to produce IgG4 and IgE (Punnonen et al., 1993; Defrance et al., 1994; Cocks et al., 1993). However, the data in this study with mouse IL-13 suggested that although this cytokine might share some of the general B cell stimulatory effects of IL-4, there was no strong isotype selectivity. IgG1 levels were enhanced only to a similar extent as other isotypes, and no significant levels of IgE production could be detected when B cells were stimulated by IL-13 and fixed, activated T cells or anti-CD40 antibody, although IL-4 induced high levels of IgE in both conditions. If IgE production was stimulated by IL-13, the levels were below the detection limit (6.25 ng/ml), or at least 50-fold below the levels induced by IL-4. The difference between IL-4- and IL-13-induced levels was more than ten-fold for IgG1; thus, the possibility of IL-13 in enhancing IgE levels to a low extent, for example by increasing B cell survival, cannot be excluded.

Interestingly, IgE was produced in IL-4-deficient mice treated with anti-CD40 mAb (Ferlin et al., 1996) or infected with malaria (von der Weid et al., 1994) or *Leishmania major* (Noben-Trauth et al., 1996) or during the course of retrovirus-induced immunodeficiency syndrome or anti-IgD treatment (Morawetz et al., 1996), but not during *Nippostrongylus brasiliensis* infection (Kuhn et al., 1991; Lawrence et al., 1995; Morawetz et al., 1996), suggesting that there is an IL-4-independent mechanism that can also stimulate IgE production in some circumstances. Although the results of this study do not provide evidence for a selective role of IL-13 in IgE production, it remains possible that IL-13 may enhance IgE levels under certain conditions that have not been tested. IL-5 does not induce class switching but efficiently enhances antibody production in B cells that have undergone class-switching (Sonoda et al., 1989; Yokota et al., 1987; Yuan et al., 1990; Webb et al., 1991).

The capacity of IL-4 and IL-13 to enhance DNA synthesis and proliferation of human B cells stimulated with anti-IgM (Howard et al., 1982) or anti-CD40 (Valle et al., 1989; Banchereau et al., 1991; Banchereau and Rousset, 1991; McKenzie et al., 1993a; Cocks et al., 1993; Defrance et al., 1994) is widely established. Mouse IL-4 also enhances mouse B cell proliferation and survival (Howard et al., 1982; Hodgkin et al., 1991). The results in Fig. 6.16 also show that IL-4 induced additional divisions of mouse B cells stimulated with anti-CD40 antibodies. Under similar conditions, IL-13 did not significantly induce proliferation of mouse B cells beyond that induced by anti-CD40 alone. In contrast, mouse IL-13 enhanced proliferation of the B9 mouse plasmacytoma cell line (Zurawski and de Vries, 1994; He and Malek, 1995), suggesting that IL-13 might enhance normal B cell proliferation under other circumstances. The effects of IL-13 on mouse B cells were consistent with the known effects of human IL-13 in providing survival

signals to anti-CD40-stimulated human peripheral blood B cells (Lomo et al., 1997), B-chronic lymphocytic leukemia cells (Fluckiger et al., 1994) and non-Hodgkin's lymphoma B cells (Billard et al., 1997). Similar to IL-13, IL-4 prolongs the viability of not only normal human and mouse B cells (Go et al., 1990; Hodgkin et al., 1991; Simons and Zharhary, 1989; Brams et al., 1993; Parry et al., 1994) but also malignant human B cells (Dancescu et al., 1992; Panayiotidis et al., 1993).

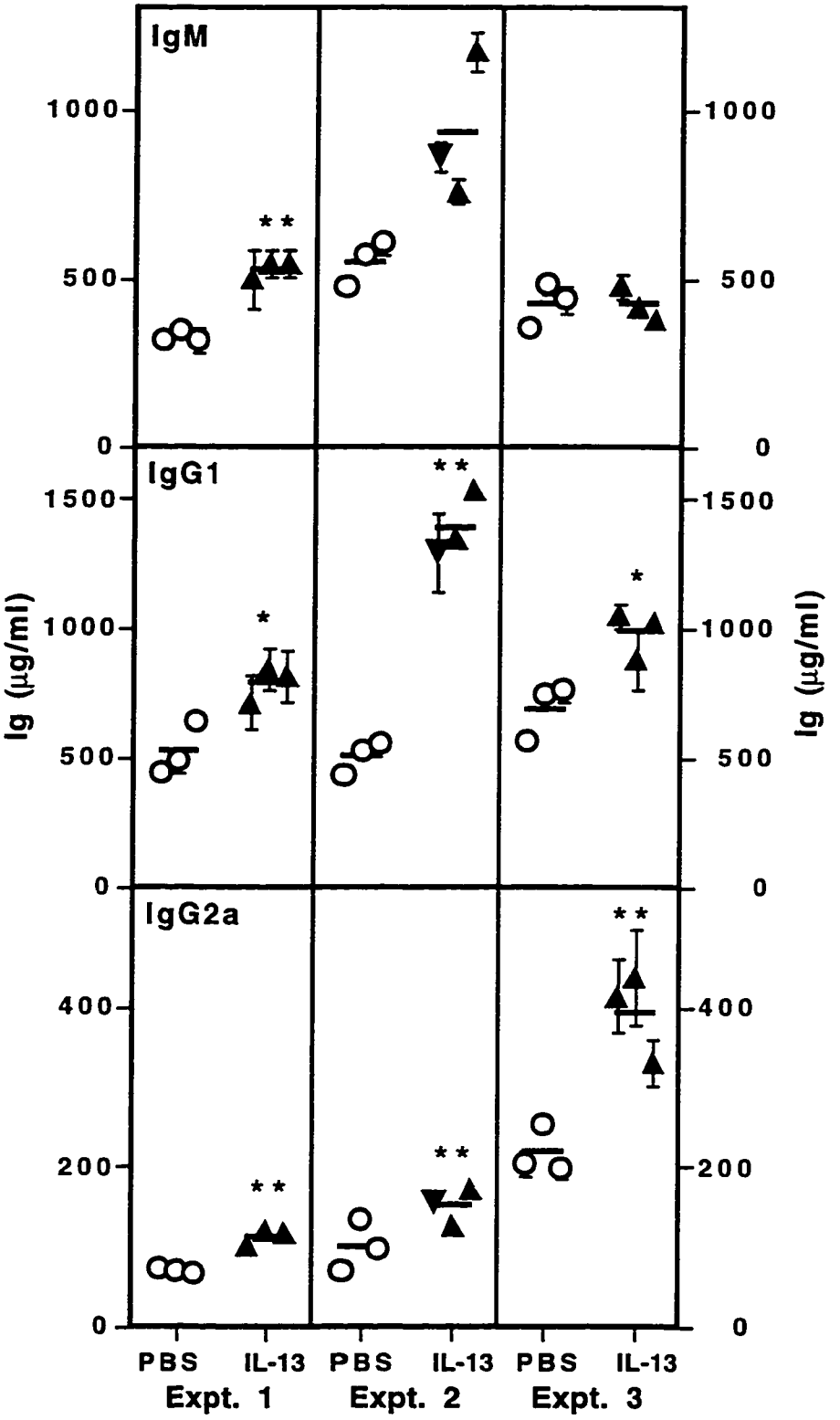
Interestingly, high concentrations of anti-CD40 induced purified B cells to produce antibodies in the absence of any exogenous cytokine. This effect could not be inhibited by anti-IL-4 mAb, indicating an IL-4-independent stimulation pathway. Furthermore, in the presence of IL-4, anti-CD40 stimulated B cells formed tight clusters, in sharp contrast to cultures with anti-CD40 and IL-13. As the Epstein-Barr virus-transformed human B cell lines express IL-13 mRNA and produce a small amount of IL-13 (de Waal Malefyt et al., 1995; Fior et al., 1994; Kindler et al., 1995), it is conceivable that mouse splenic B cells can produce IL-13 upon strong anti-CD40 activation and that IL-13 can function in an autocrine fashion. This would be consistent with the inability of exogenous IL-13 to further enhance antibody production at high anti-CD40 concentrations.

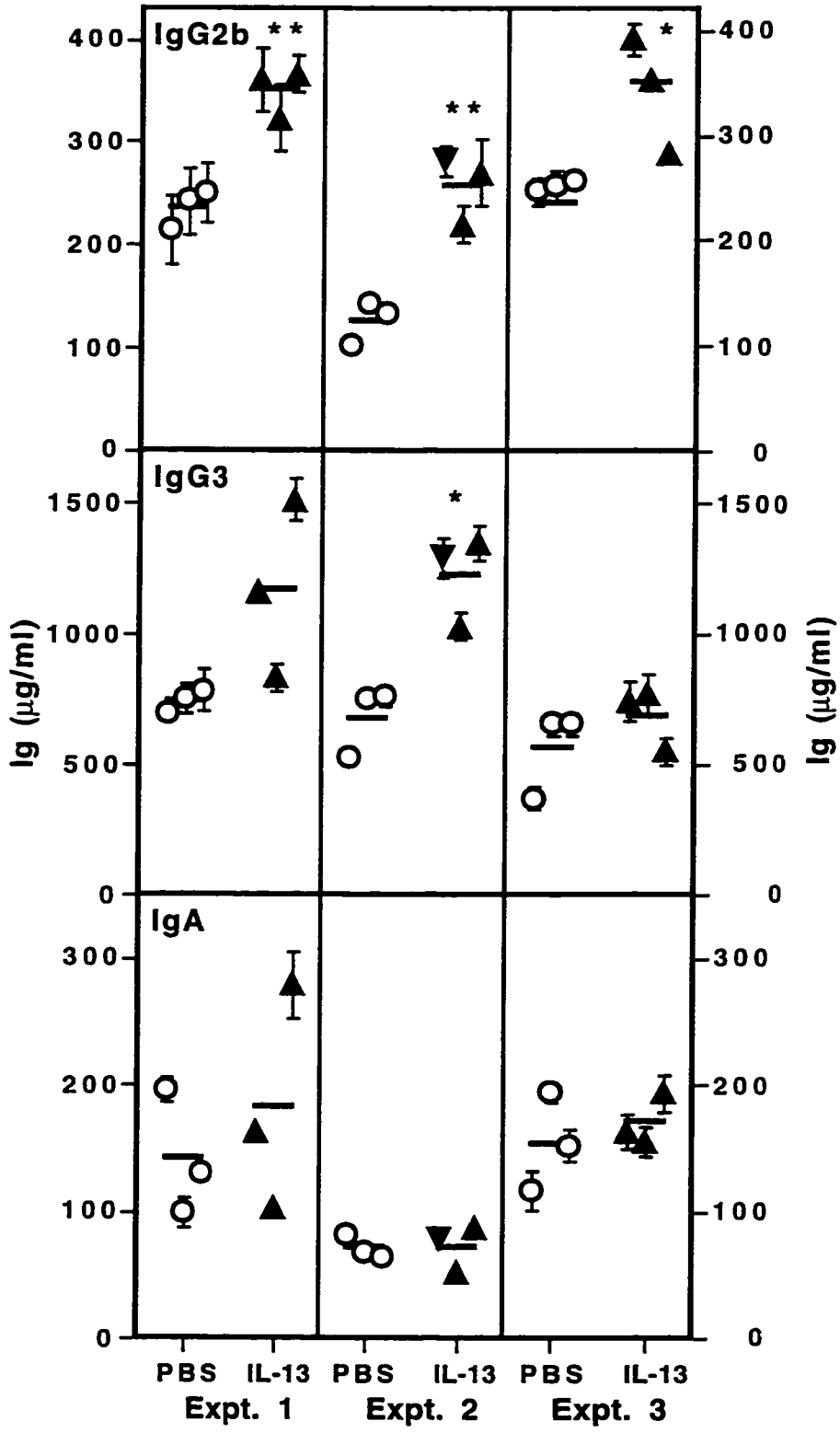
In addition to the direct effect of IL-13 on B cells that is reported in this chapter, other factors may also be involved in upregulating antibody production during *in vivo* IL-13 administration and CRBC immunization. IL-13 induces extramedullary hemopoiesis and monocytosis in mice (Lai et al., 1996) (Chapter V). It is conceivable that the increased numbers of macrophages may secrete soluble mediators that enhance the antibody response or that enhanced antigen presentation to T cells may provide more B cell help. This may also explain the

isotype differences between the enhancement mainly of IgM levels *in vitro* compared to the enhancement of IgG levels *in vivo*. Unlike IL-4, which plays an important role in T cell differentiation (Le Gros et al., 1990; Swain et al., 1990; Sad et al., 1995; Croft et al., 1994), IL-13 does not appear to bind to or exhibit any biological functions on either human or mouse T cells (Zurawski and de Vries, 1994; Zurawski et al., 1993a; de Waal Malefyt et al., 1995). Thus, IL-13 is unlikely to affect B cells by directly altering T cell differentiation *in vivo*.

In comparison to IL-4, the results in this chapter suggest that IL-13 may play a more minor role during antibody responses *in vivo*. However, there is potential for a more substantial role in circumstances in which other B cell stimulatory cytokines, such as IL-4, are absent or ineffective. The IL-4 receptor α chain (IL-4Rα) constitutes part of the IL-4 and IL-13 receptors (Mosley et al., 1989; Zurawski et al., 1993b; Zurawski et al., 1995; Obiri et al., 1995; Smerz-Bertling and Duschl, 1995; Hilton et al., 1996; Zhang et al., 1997; Obiri et al., 1997). IL-4Rα-deficient mice fail to produce antigen specific IgG1 responses (Urban et al., 1998), whereas IL-4-deficient mice generate lower antigen specific IgG1 levels (Kuhn et al., 1991; Kopf et al., 1993), suggesting the involvement of IL-13 in Ig production *in vivo*. In addition, IL-13 is not produced in complete concordance with IL-4, and so there may be circumstances when IL-13 but not IL-4 is expressed. Also, hIL-13 is expressed within 2 h of activation, and hIL-13 mRNA can still be detected after 72 h. In contrast to the early yet sustained hIL-13 mRNA expression, hIL-4 mRNA expression in T cells is transient and can only be detected 24 h after activation (Zurawski and de Vries, 1994). If mouse IL-13 has similar expression kinetics it may play an important role in stimulating an initial antibody response prior to the expression of IL-4 or sustaining antibody production at later times.

Figure 6.1. *In vivo* IL-13 administration increased the levels of three IgG isotypes. Mice that were immunized with CRBC were continuously administered with PBS (○) or IL-13 (▲ or ▼) (Expt. 1, 3.5 μg/mouse; Expt. 2, 4.5 (▼) or 45 (▲) μg/mouse, or Expt. 3, 25 μg/mouse) by osmotic pumps for 7 days in the peritoneal cavity. Serum or plasma from three PBS (○)- or three IL-13 (▲ or ▼)-treated mice from each experiment were quantitated for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA levels by sandwich ELISA. Each symbol represents data obtained from an individual mouse, and the error bars indicate the SD of quadruplicate or triplicate assay determinations from each mouse sample. The average Ig level in each group is indicated by a solid horizontal bar. * or ** indicates Ig levels from IL-13-treated mice significantly higher than mice treated with PBS, with $p<0.05$ or $p<0.01$, respectively.


IgM
IgG1
IgG2a
Ig (µg/ml)
1000
500
0
1500
400
200
**
*
PBS
IL-13
Expt. 1
Expt. 2
Expt. 3


IgG2b
IgG3
IgA
Ig (μg/ml)
400
300
200
100
0
1500
1000
500
PBS
IL-13
Expt. 1
Expt. 2
Expt. 3

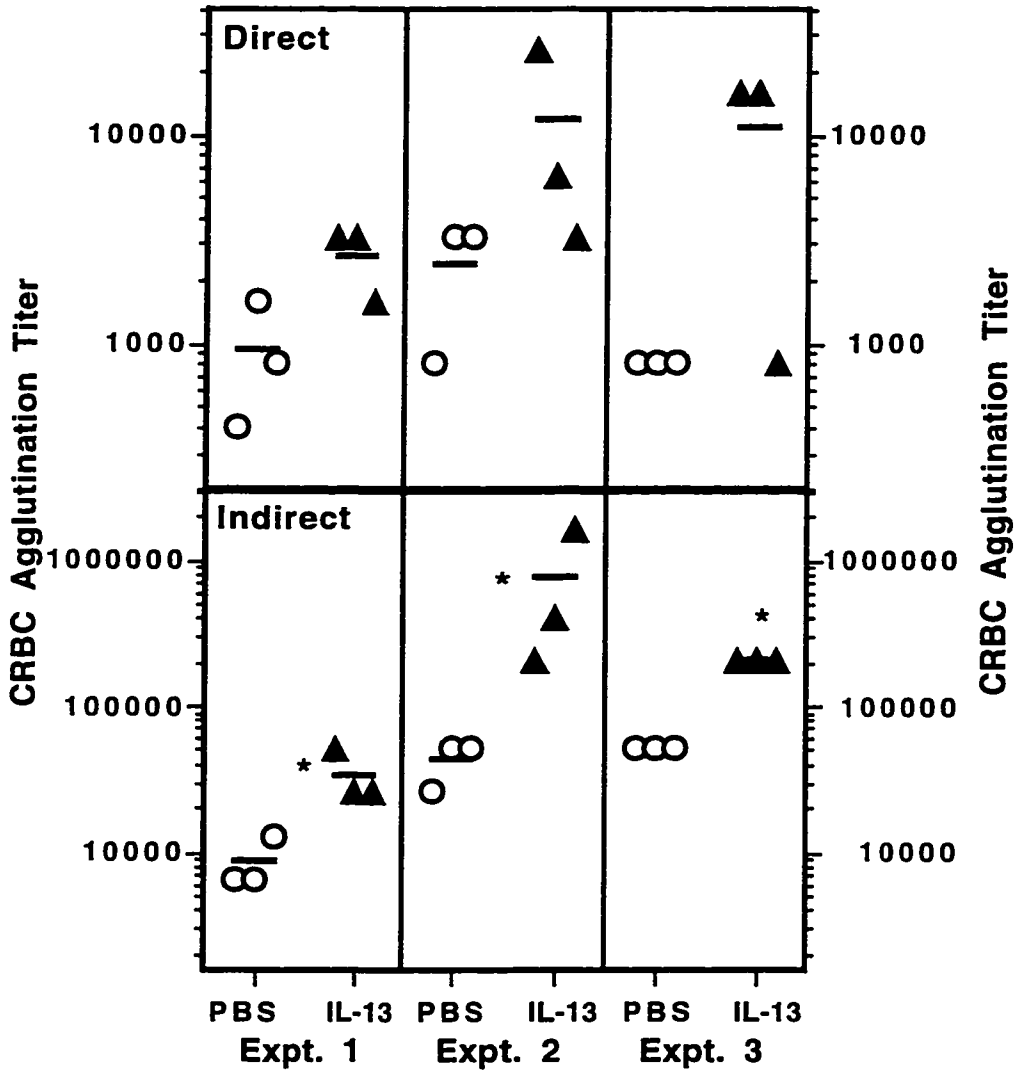


Figure 6.2. ***In vivo*** **IL-13 administration during a CRBC immune response enhanced direct and indirect CRBC hemagglutination titers.** Doubling dilutions of plasma from PBS (○)- or IL-13 (▲)-treated mice from Expt. 1 in Fig. 6.1 were tested for the ability to agglutinate CRBC directly or indirectly by using a pan-specific anti-Ig antiserum. Titers are expressed as the reciprocal of the last dilution that gave positive CRBC agglutination. Each symbol represents data obtained from an individual mouse. The average hemagglutination titer in each group is indicated by a solid horizontal bar. Plasma from unimmunized mice had direct or indirect CRBC agglutination titers of 1:200 or less. * indicates $p<0.05$.

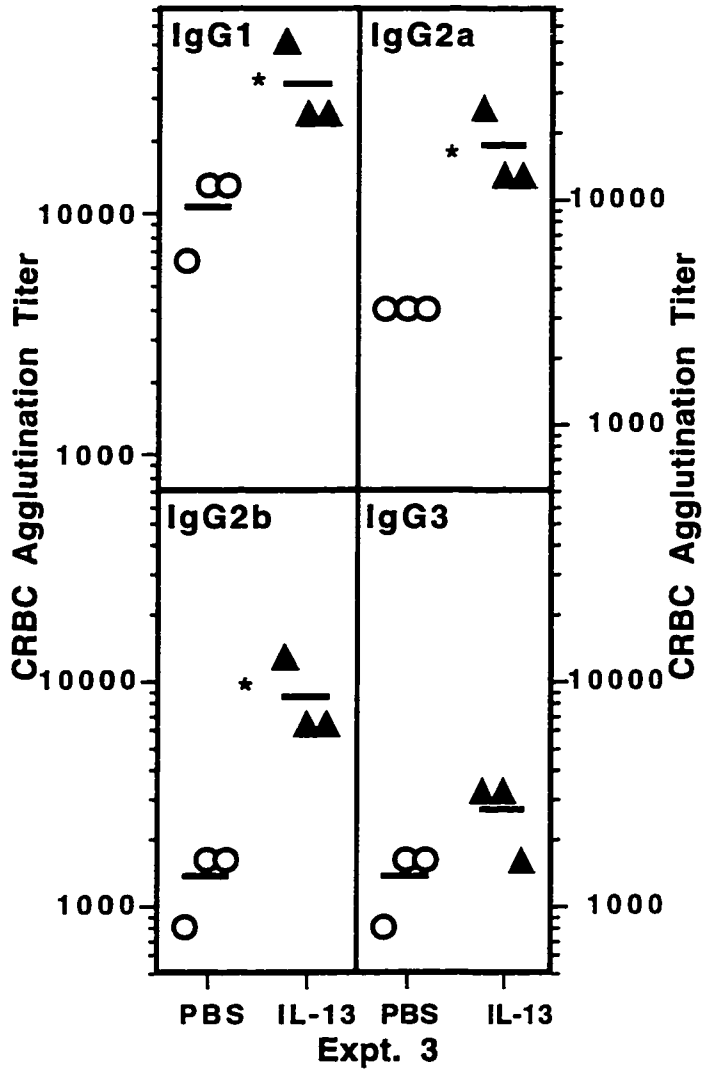


Figure 6.3. ***In vivo*** **IL-13 administration during a CRBC immune response enhanced isotype specific CRBC hemagglutination titers.** Doubling dilutions of plasma from PBS (○)- or IL-13 (▲)-treated mice from Expt. 1 in Fig. 6.1 were tested for the ability to agglutinate CRBC indirectly by using isotype-specific antisera for IgG1, IgG2a, IgG2b, or IgG3. Titers are expressed as the reciprocal of the last dilution that gave positive CRBC agglutination. Plasma from unimmunized mice had isotype-specific CRBC agglutination titers of 1:200 or less. * indicates $p<0.05$.

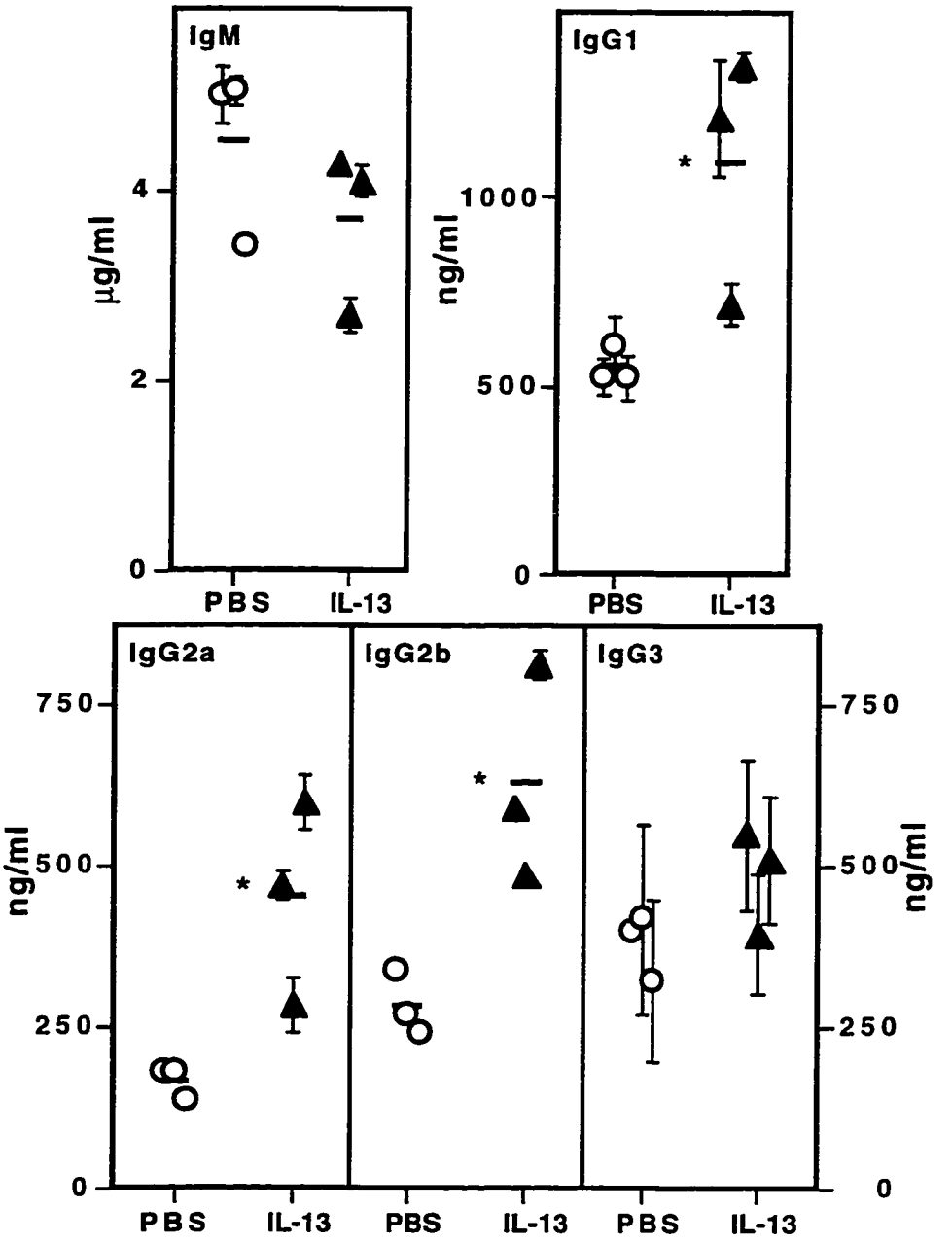


Figure 6.4. IL-13 *in vivo* treatment of immunized mice increased *ex vivo* antibody production. $5x10^6$ cells/ml of spleen cells from PBS (○)- or IL-13 (▲)-treated mice from Expt. 1 in Fig. 6.1. were cultured in triplicate for 6 days, and IgM, IgG1, IgG2a, IgG2b, and IgG3 Ig levels in the supernatant were quantitated by sandwich ELISA. The mean and SD of replicate cultures are shown. The average Ig level in each group is indicated by a solid horizontal bar. * indicates $p<0.05$. Similar results were obtained with $1x10^6$ or $2x10^6$ spleen cells/ml, and in the presence or absence of CRBC.

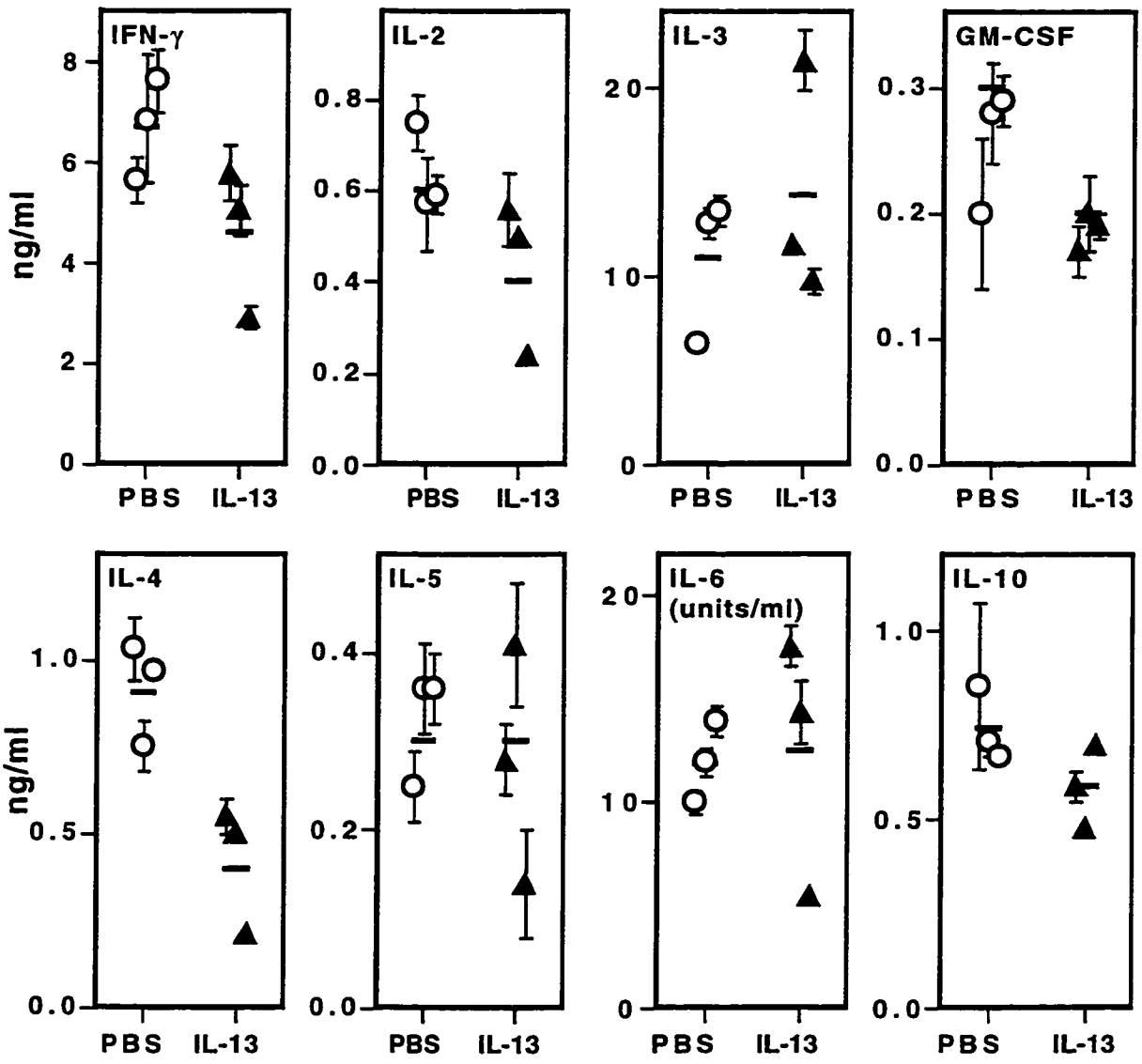


Figure 6.5. In the presence of Con A stimulation, spleen cells from IL-13-treated and CRBC-immunized mice did not show a significant difference in cytokine production profiles compared to those of PBS-treated and CRBC-immunized mice. $5x10^6$ cells/ml of spleen cells from PBS (○)- or IL-13 (▲)-treated mice from Expt. 1 in Fig. 6.1 were cultured in triplicate for 48 h with 5 μg/ml of Con A. IFN-γ, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10 levels in the supernatant were quantitated by sandwich ELISA. The mean and SD of replicate cultures are shown. The average cytokine level in each group is indicated by a solid horizontal bar. Similar results were obtained with $1x10^6$ or $2x10^6$ spleen cells/ml.

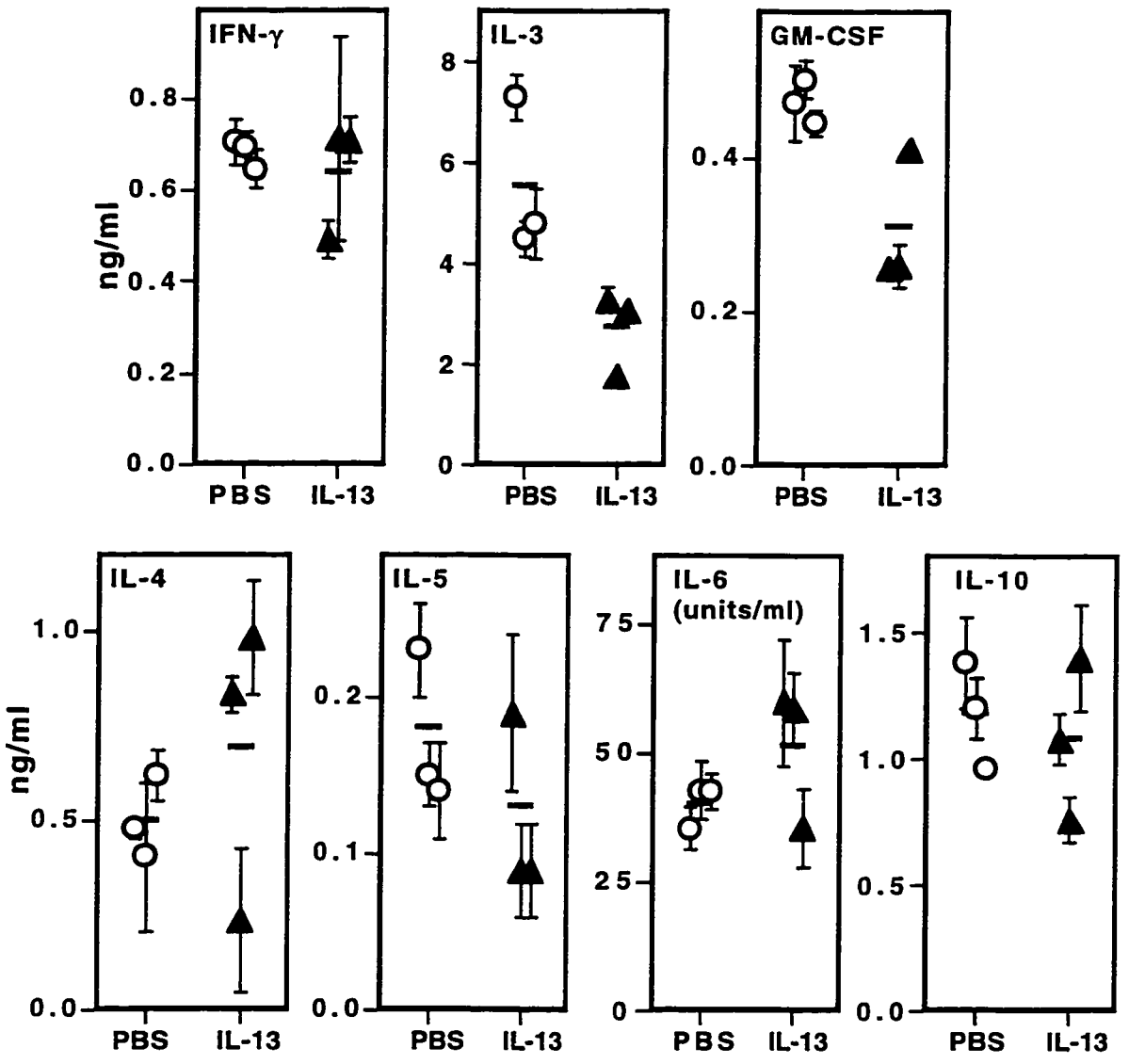


Figure 6.6. In the presence of CRBC stimulation, spleen cells from IL-13-treated and CRBC-immunized mice did not show a significant difference in cytokine production profiles compared to those of PBS-treated and CRBC-immunized mice. $5x10^6$ spleen cells/ml from PBS (○)- or IL-13 (▲)-treated mice from Expt. 1 in Fig. 6.1. were cultured in triplicate for 6 days in the presence of 0.4% CRBC. IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, and GM-CSF levels in the supernatants were quantitated by sandwich ELISA. The average cytokine levels in each group are indicated by solid horizontal bars. The mean and SD of replicate cultures are shown. Similar results were obtained with $1x10^6$ or $2x10^6$ spleen cells/ml in the presence or absence of CRBC.

Figure 6.7. IL-13 enhanced immunoglobulin levels *in vitro* in the presence of fixed, activated Th1 or Th2 cells. $3x10^5$ sorted $B220^+$ mouse splenic B cells were cultured alone (○) or with $5x10^4$ (▲) or $1x10^5$ (▼) PFA-fixed anti-CD3 activated M264-15 or D10.G4.1 cells, in the absence or presence of IL-13 (3 or 30 ng/ml) or IL-4 (3 or 30 ng/ml) in triplicate for 7 days. IgM and IgG1 levels were quantitated, and the mean and SD of replicate cultures are shown. The insets in the IL-13 panels for IgG1 production show an expanded scale. M264-15 and D10.G4.1 data were from two separate experiments.

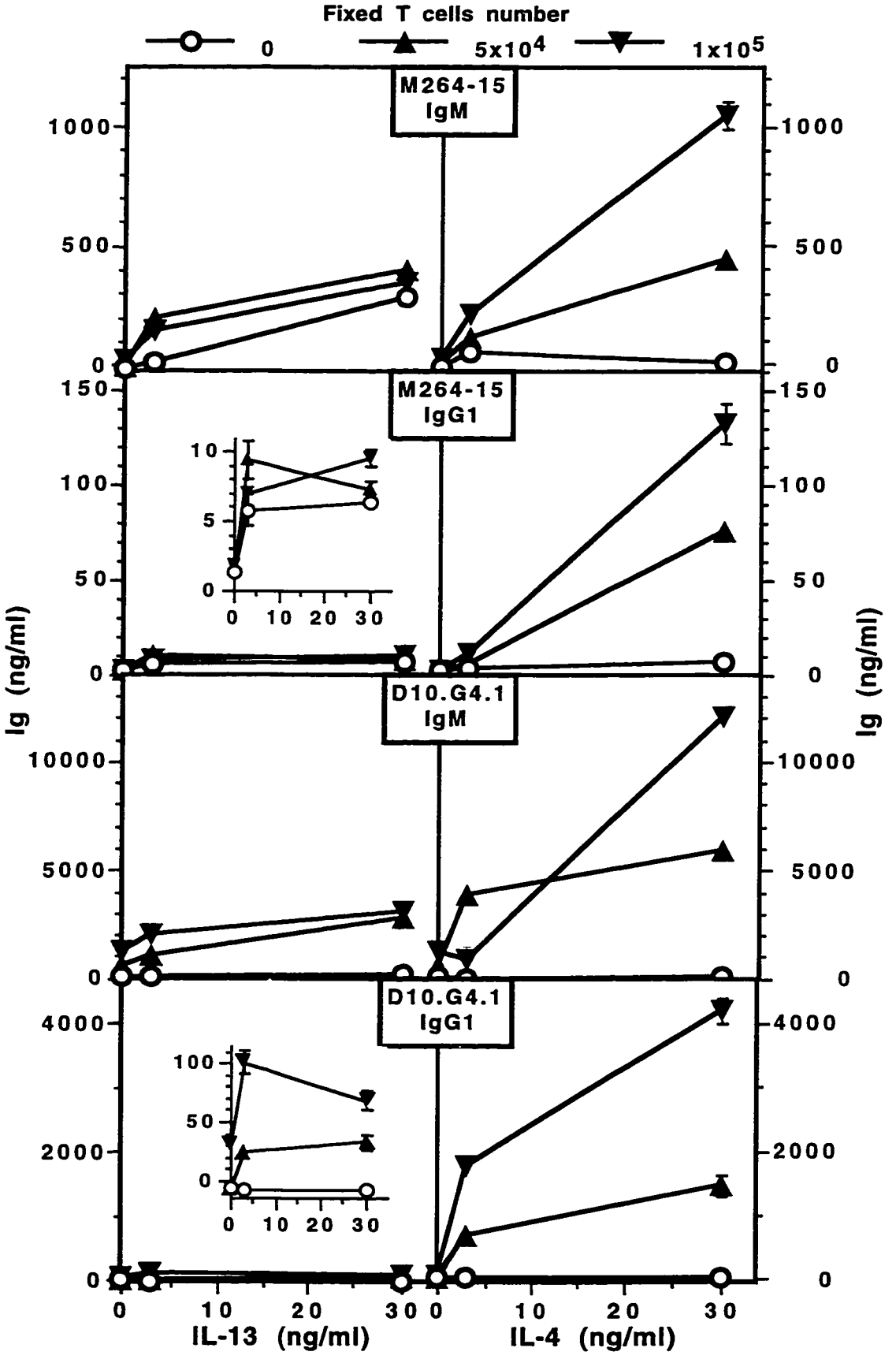
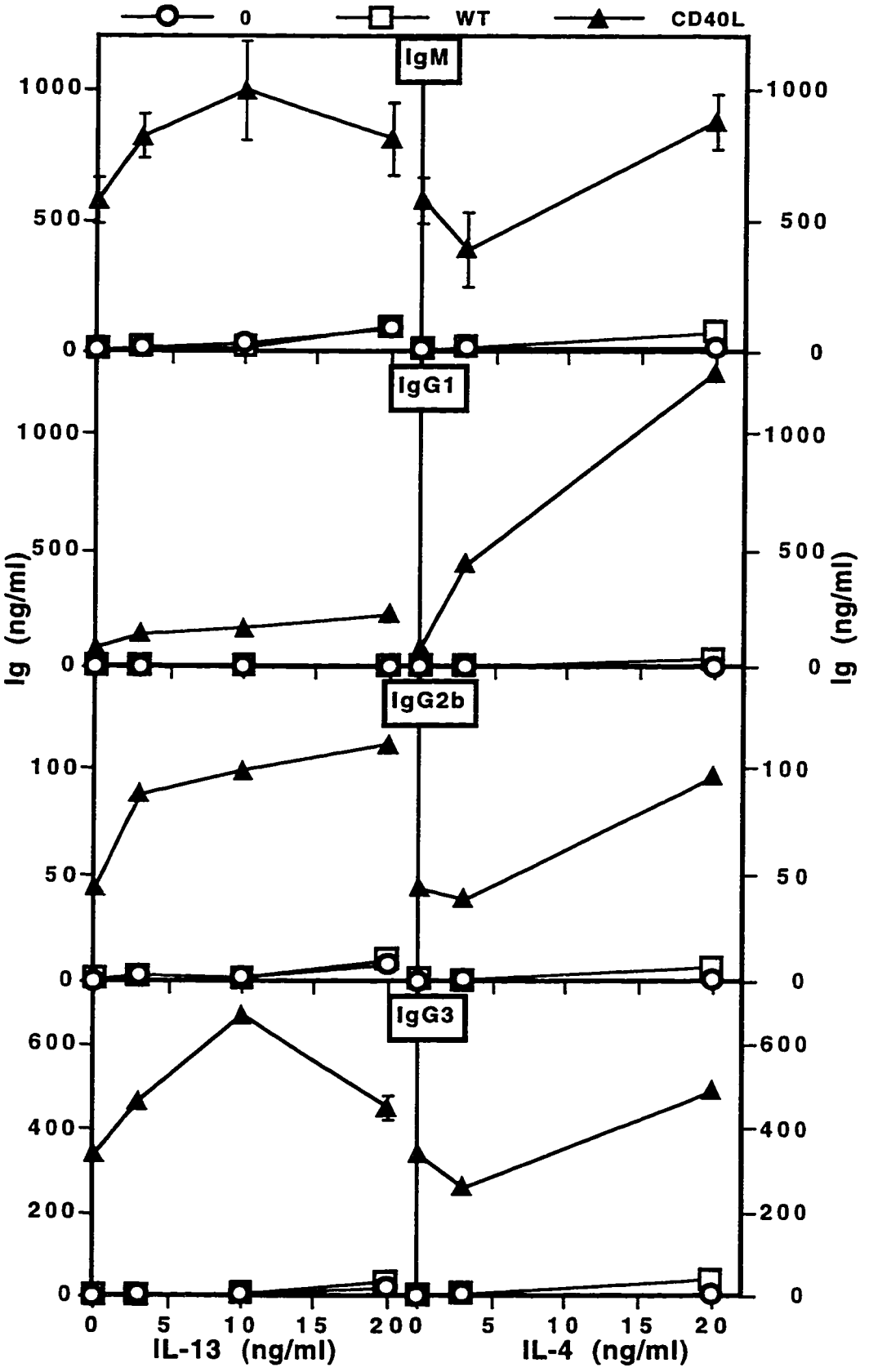

Fixed T cells number
0
5x10^4
1x10^5
M264-15 IgM
M264-15 IgG1
D10.G4.1 IgM
D10.G4.1 IgG1
Ig (ng/ml)
IL-13 (ng/ml)
IL-4 (ng/ml)

Figure 6.8. IL-13 enhanced immunoglobulin levels *in vitro* in the presence of the irradiated CD40L-transfected T hybridoma cell line, 40LDS. $5x10^5$ sorted $B220^+$ mouse splenic B cells were cultured alone (○) or with $5x10^3$ or $5x10^3$ irradiated parental (WT, ❑) or CD40L-transfected (CD40L, ▲) cells in the absence or presence of IL-13 or IL-4 in triplicate for 7 days. IgM, IgG1, IgG2b, and IgG3 levels were quantitated, and the mean and SD of replicate cultures are shown.


0
WT
CD40L
IgM
IgG1
IgG2b
IgG3
1000
500
0
100
50
600
400
200
Ig (ng/ml)
0 5 10 15 20
IL-13 (ng/ml)
IL-4 (ng/ml)

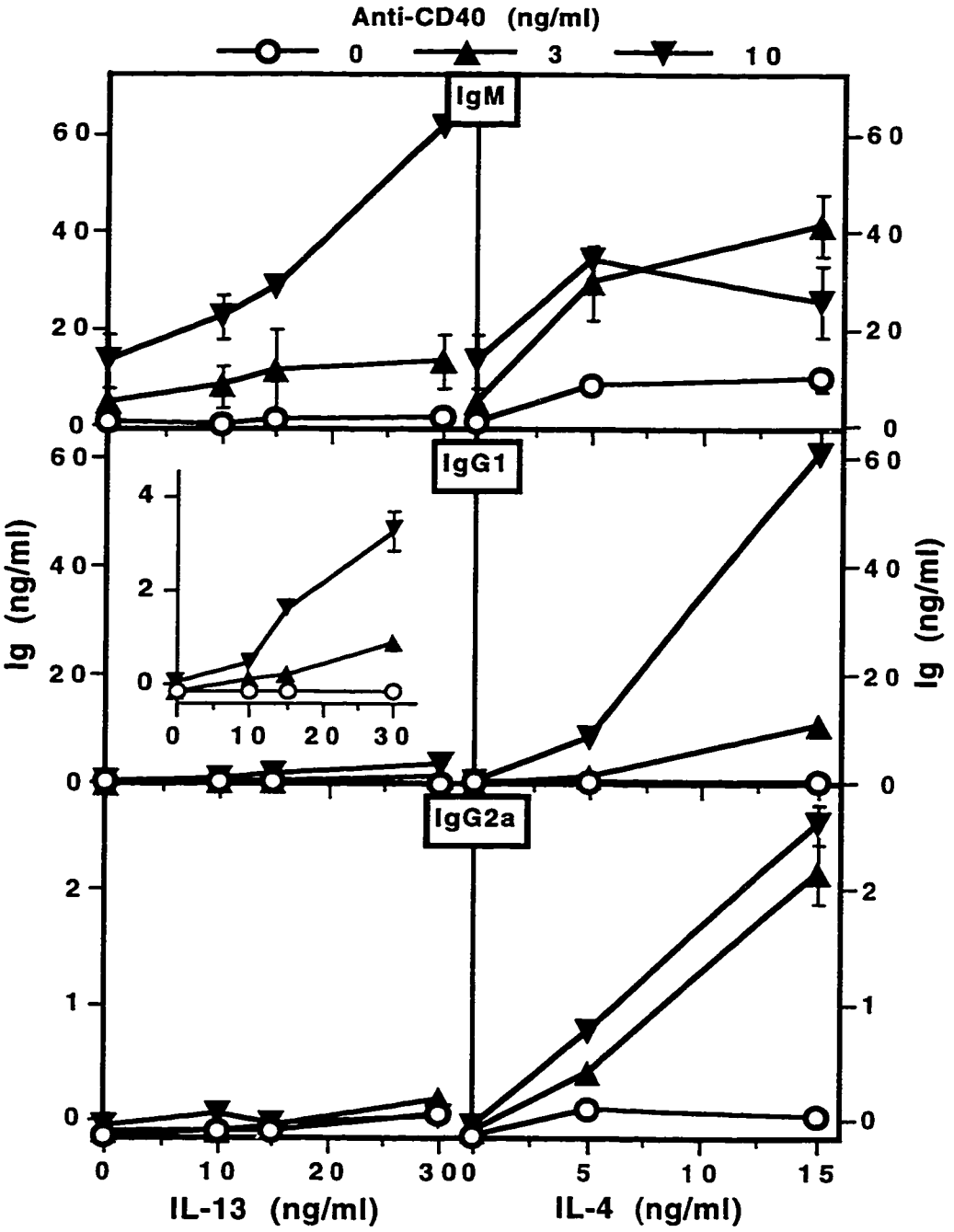
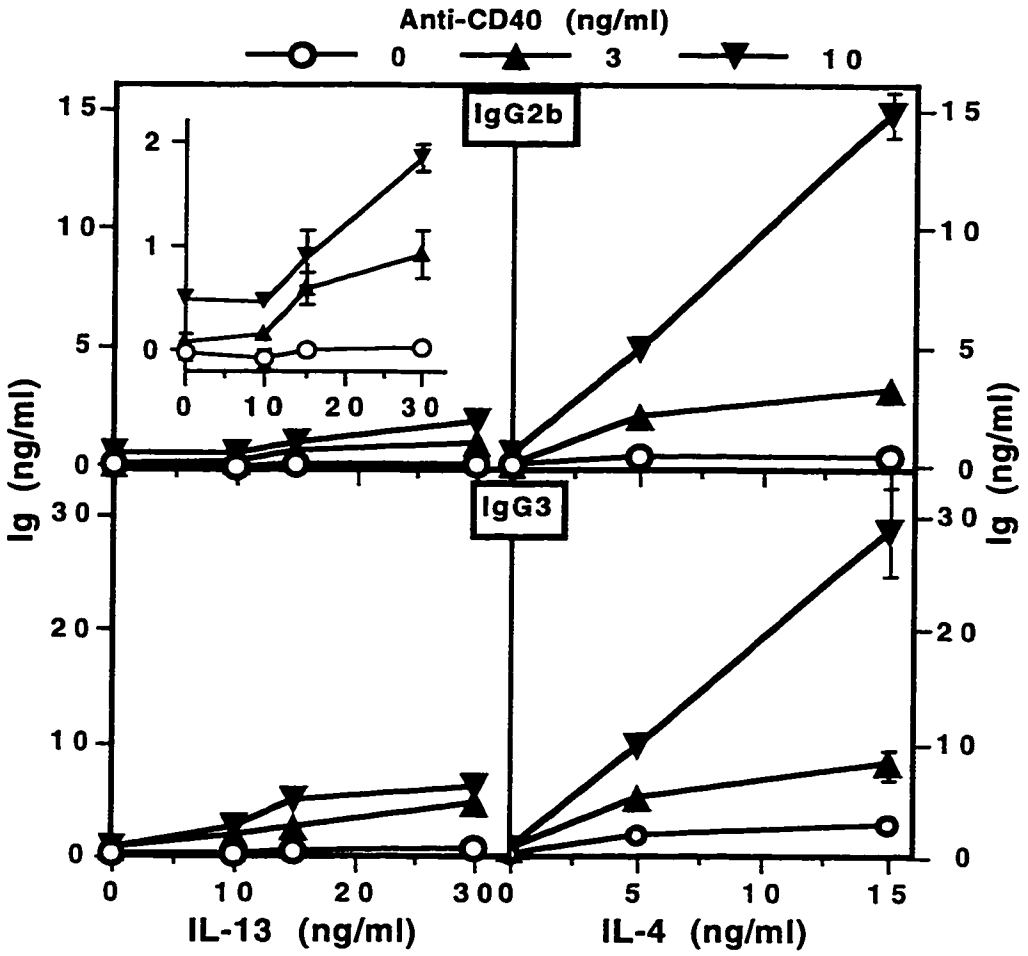


Figure 6.9. IL-13 enhanced Ig production from anti-CD40-stimulated sIgD+ B cells. $5x10^4$ sorted sIgD+ mouse splenic B cells were cultured without (○) or with 3 (▲) or 10 (▼) ng/ml anti-CD40 antibody, in the absence or presence of IL-13 or IL-4 for 7 days. IgM, IgG1, IgG2a, IgG2b, and IgG3 levels were quantitated. These data are representative of three other experiments. The mean and SD of quadruplicate or triplicate cultures are shown. The insets in the IL-13/IgG1 and IL-13/IgG2b panels show expanded scales.


Anti-CD40 (ng/ml)
0
3
10
IgG2b
IgG3
Ig (ng/ml)
Ig (ng/ml)
IL-13 (ng/ml)
IL-4 (ng/ml)

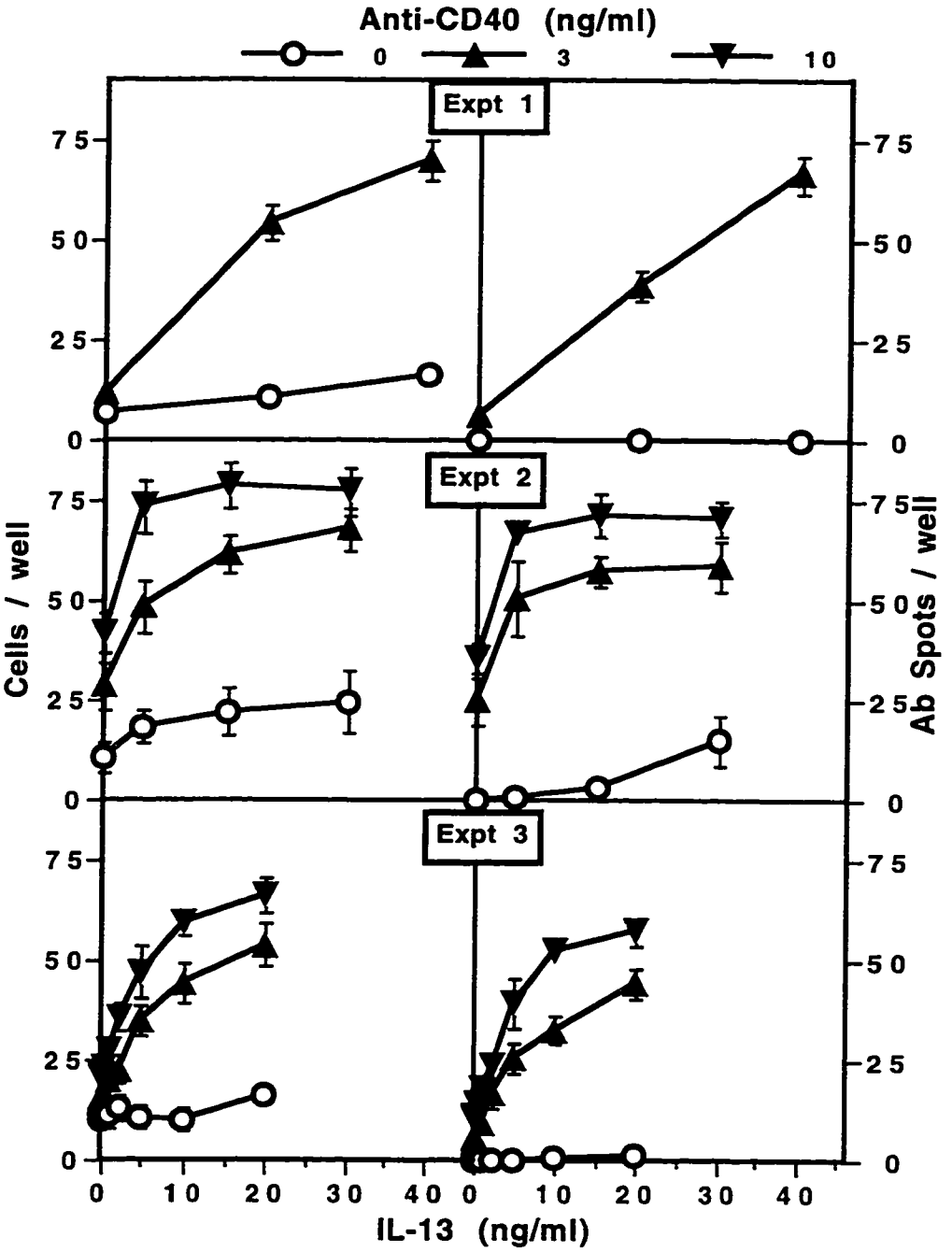


Figure 6.10. IL-13 directly stimulated mouse IgD$^+$ B cells. Sorted sIgD$^+$ mouse B cells (>99.5% pure, 100/well, Expt. 1 & 2; or 90/well, Expt. 3) were cultured in Terasaki wells for 2 days in the absence (○) or presence of 3 (▲) or 10 (▼) ng/ml anti-CD40 mAb with different doses of IL-13. The cells in each well were counted and transferred to affinity purified goat anti-mouse IgM antibody-coated nitrocellulose plates for ELISPOT assay. The mean and SD of three (Expt. 1) or six (Expts. 2 & 3) replicate cultures are shown.

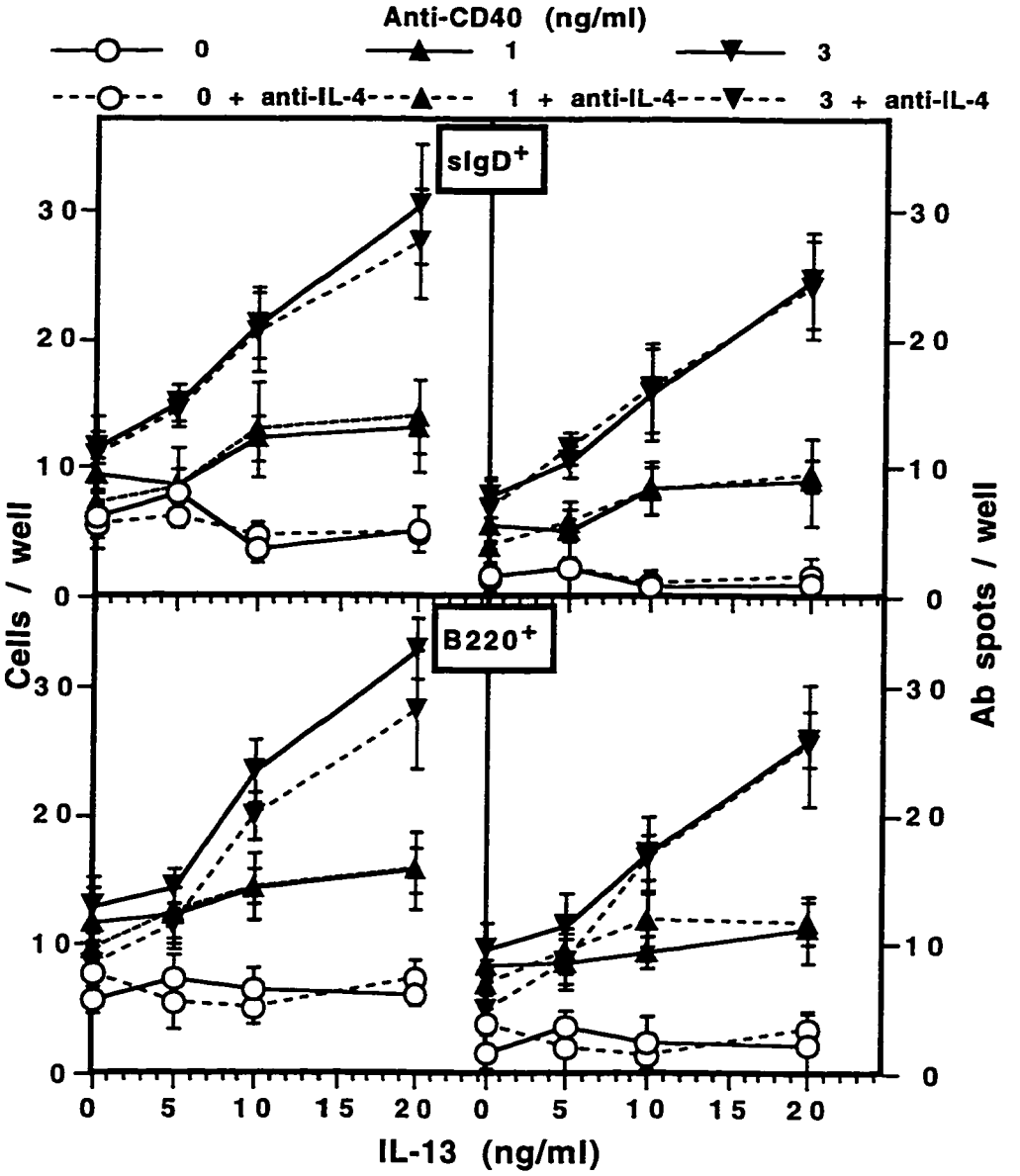


Figure 6.11. IL-13 directly stimulated mouse IgD$^+$ or B220$^+$ B cells, independent of IL-4. Sorted mouse IgD$^+$ (>98.5% pure) or B220$^+$ (>99.0% pure) B cells from the same pool of spleen cells were cultured in Terasaki wells for 2 days at 90 /well in the absence (○) or presence of 1 (▲) or 3 (▼) ng/ml anti-CD40 mAb with different doses of IL-13. 10 μg/ml of anti-IL-4 blocking mAb (dotted lines) was used in the corresponding cultures. ELISPOT assays were performed as described in Fig. 6.10. The mean and SD of quadruplicate cultures are shown.

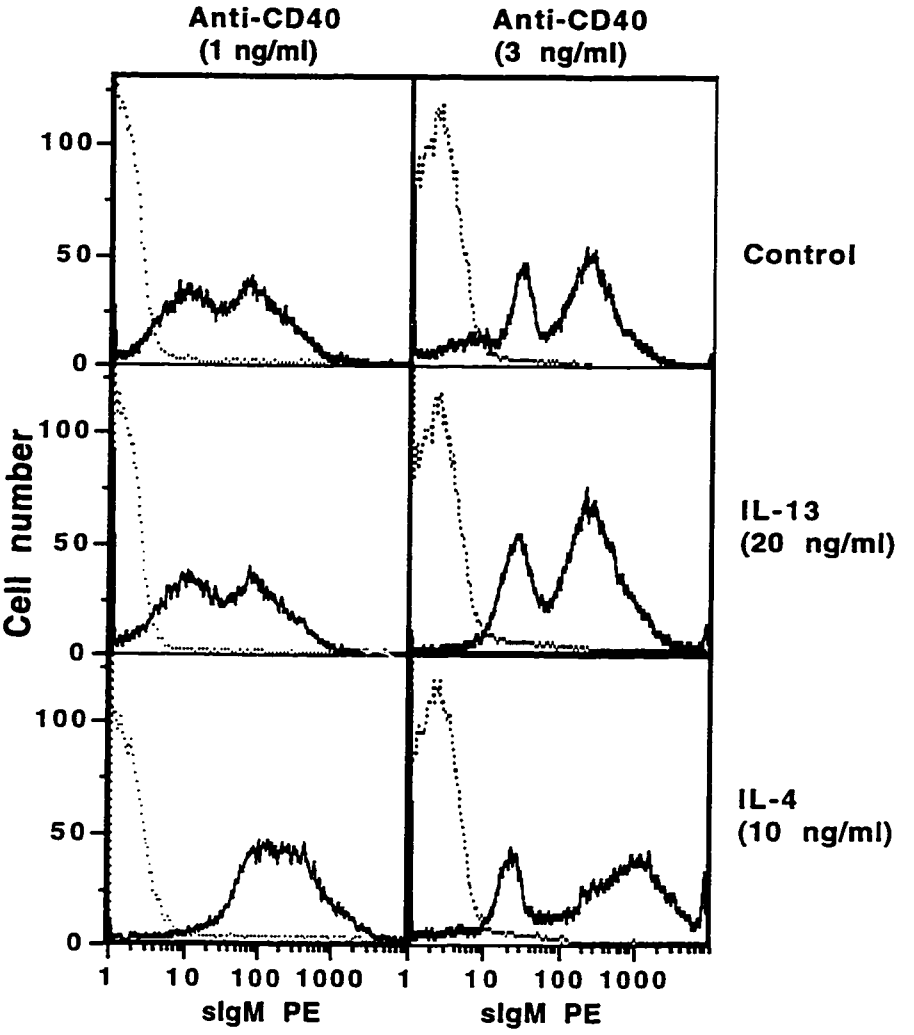


Figure 6.12. IL-13 did not enhance surface IgM expression on anti-CD40 mAb activated B cells. Sorted IgD^+ B cells were cultured with 1 or 3 ng/ml (from two separate experiments) of anti-CD40 mAb in the absence or presence of IL-13 (20 ng/ml) or IL-4 (10 ng/ml) for 40 to 48 h. Cells were harvested, counted, blocked with anti-Fc receptor mAb, and stained with PE-conjugated anti-IgM mAb. At least 15 000 cells were analyzed by FACScan. Dotted or solid lines represent cells that were stained with isotype control or anti-IgM mAb, respectively.

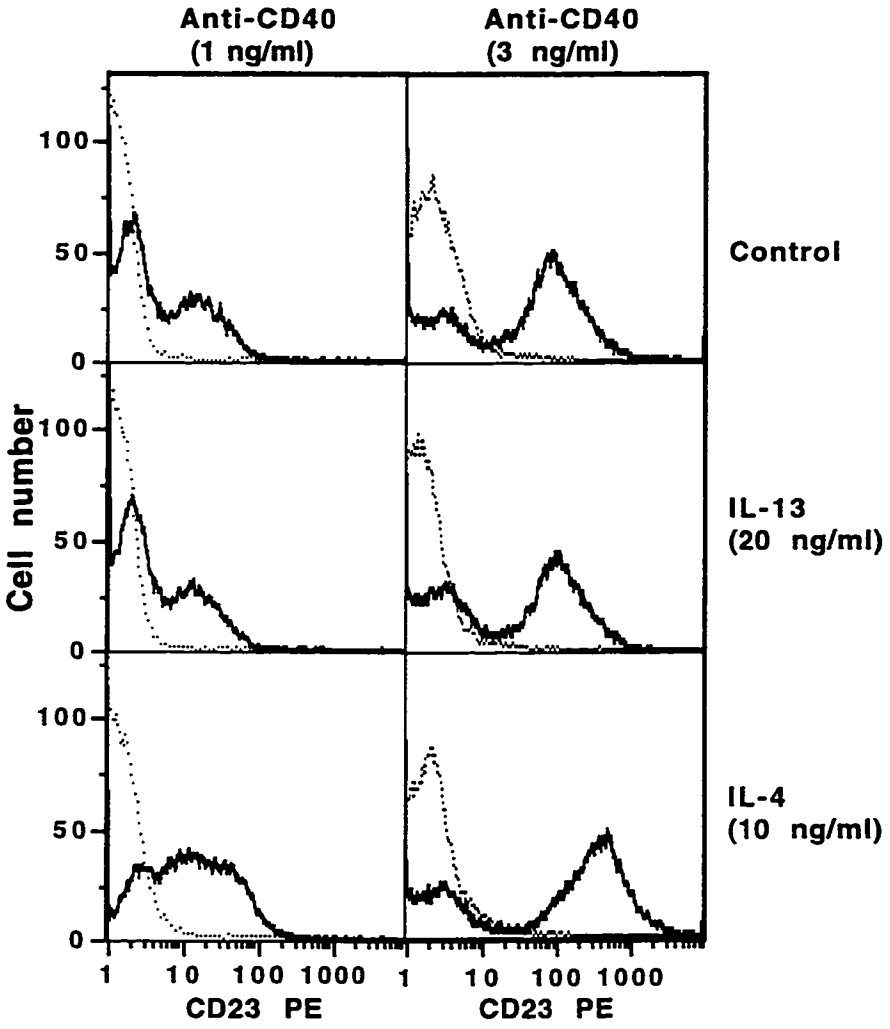


Figure 6.13. IL-13 did not enhance surface CD23 expression on anti-CD40 mAb activated B cells. Sorted IgD^+ B cells were cultured with 1 or 3 ng/ml (from two separate experiments) of anti-CD40 mAb in the absence or presence of IL-13 (20 ng/ml) or IL-4 (10 ng/ml) for 40 to 48 h. Cells were harvested, counted, blocked with anti-Fc receptor mAb, and stained with PE-conjugated anti-CD23 mAb. At least 15 000 cells were analyzed by FACScan. Dotted or solid lines represent cells that were stained with isotype control or anti-CD23 mAb, respectively.

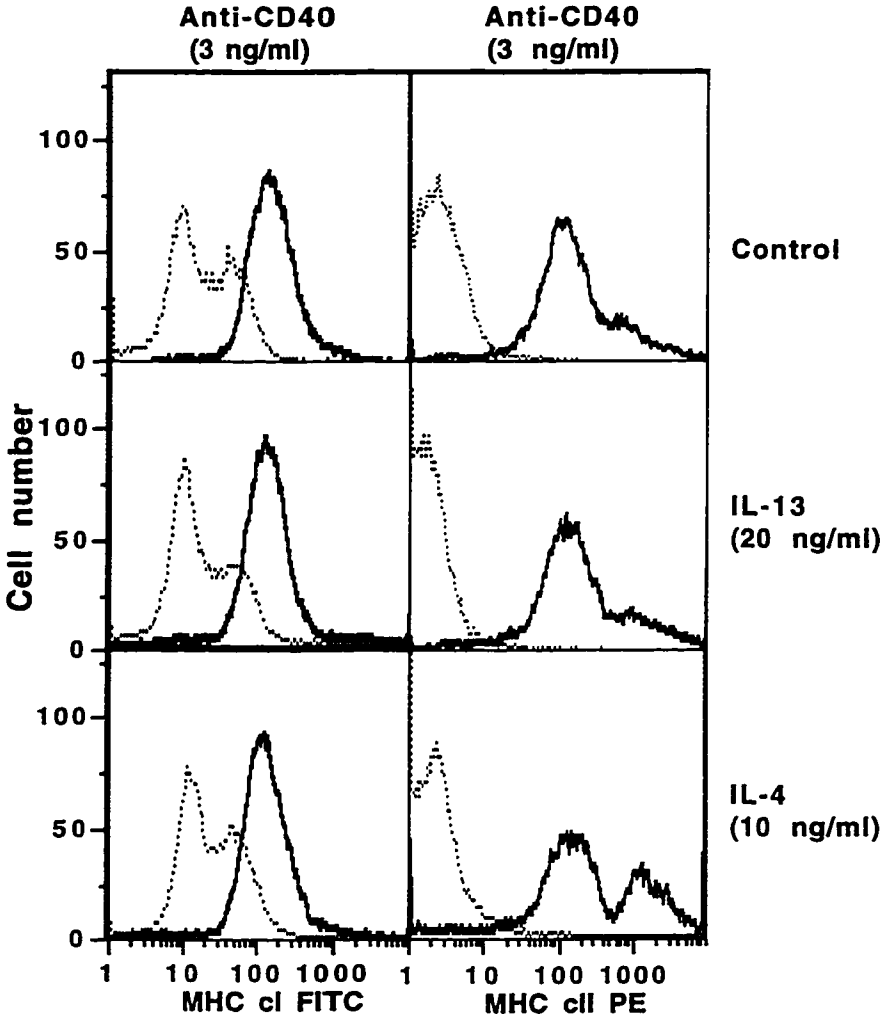


Figure 6.14. IL-13 did not enhance surface MHC class I or class II expression on anti-CD40 mAb activated B cells. Sorted IgD^+ B cells were cultured with 1 or 3 ng/ml (from two separate experiments) of anti-CD40 mAb in the absence or presence of IL-13 (20 ng/ml) or IL-4 (10 ng/ml) for 40 to 48 h. Cells were harvested, counted, blocked with anti-Fc receptor mAb, and stained with FITC-conjugated anti-MHC cI or PE-conjugated anti-MHC cII mAb. At least 15 000 cells were analyzed by FACScan. Dotted line, cells stained with isotype control; and solid line, cells stained with anti-MHC cI or cII mAb.

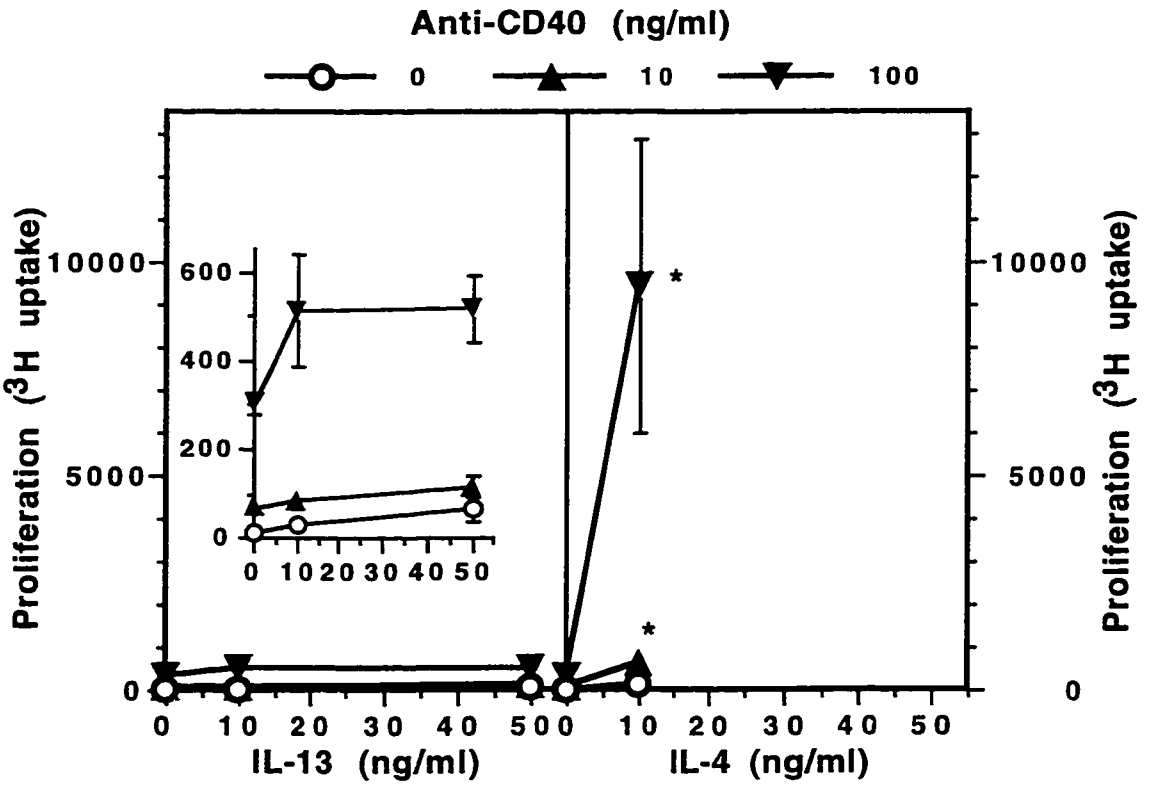


Figure 6.15. Effect of IL-13 on thymidine incorporation by sIgD$^+$ B cells in the presence of suboptimal concentrations of anti-CD40. Sorted sIgD$^+$ mouse splenic B cells were cultured at 1x10^4/ml without (○) or with 10 (▲) or 100 (▼) ng/ml of anti-CD40 mAb and in absence or presence of IL-13 (10 or 50 ng/ml) or IL-4 (10 ng/ml) in triplicate for 3 days. Thymidine was added during the final 18 h. The inset shows an expanded scale for the IL-13 results. An asterisk (*) indicates data significantly greater (p<0.05) than cultures in the absence of exogenous cytokines.

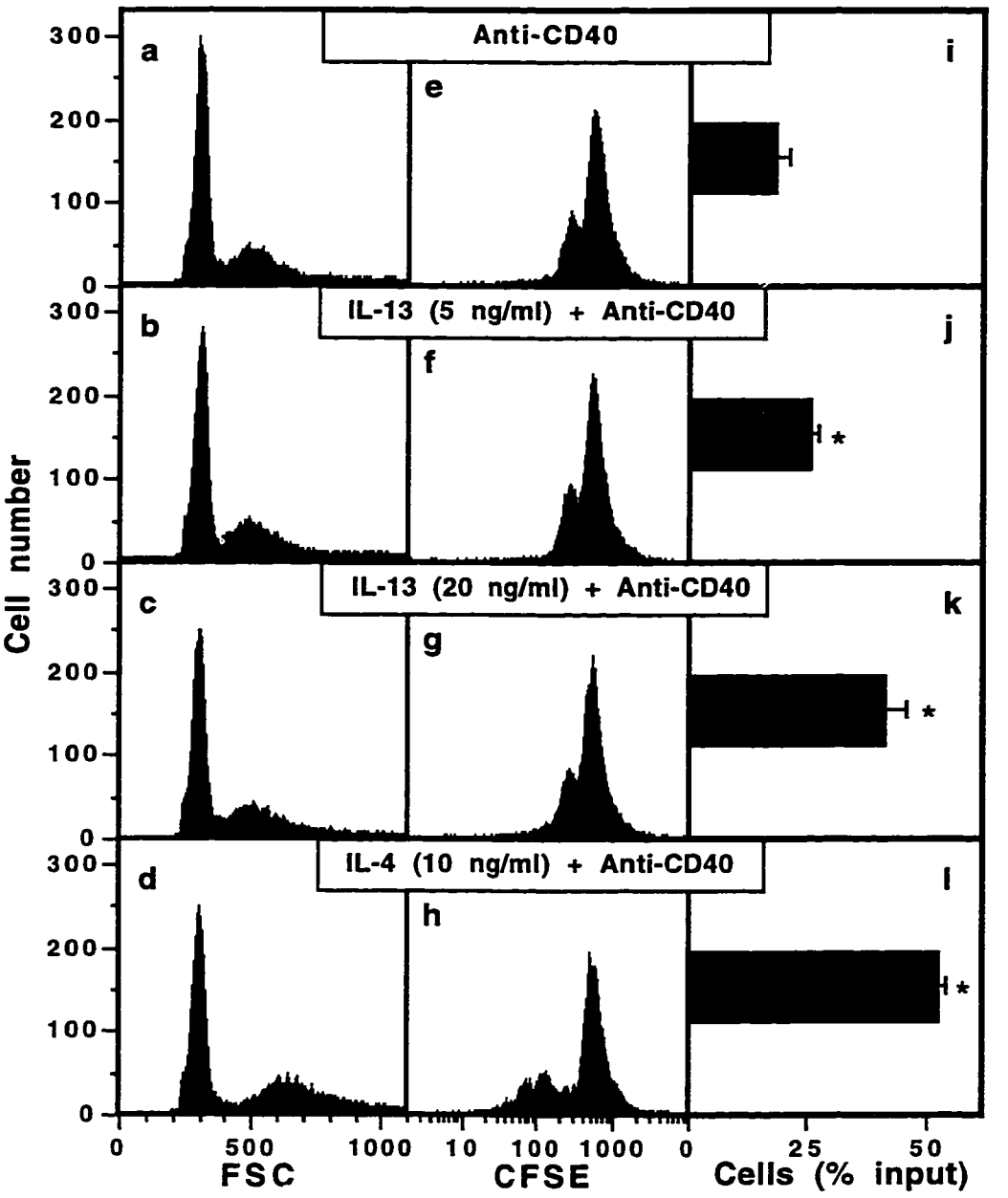


Figure 6.16. IL-13 enhanced survival but not proliferation of anti-CD40-stimulated sIgD$^+$ B cells. Sorted sIgD$^+$ mouse splenic B cells were stained with CFSE and cultured in triplicate at 1.25 x 10^5 cells/ml with 3 ng/ml anti-CD40 mAb in the absence (a, e, & i) or presence of 5 ng/ml IL-13 (b, f & j) or 20 ng/ml IL-13 (c, g & k), or 10 ng/ml IL-4 (d, h & l) for 3 days. Viable B cells were counted, and the number expressed as a percentage of the starting population (i - l). Forward light scatter (a - d) and CFSE levels (e - h) were analyzed by flow cytometry. Similar data were obtained in two other experiments. An asterisk (*) indicates data significantly greater ($p<0.01$) than the anti-CD40 cultures in the absence of exogenous cytokines (i).

CHAPTER VII

GENERATION AND ANALYSES OF IL-13 TRANSGENIC MICE

A. Introduction

For the second part of the IL-13 *in vivo* studies, efforts were channeled into producing genetically engineered mice carrying the IL-13 transgene. Successful generation of such transgenic mice would be beneficial for a broad initial survey of the biological activity of IL-13 during embryogenesis, development, and immune responses. Initially, IL-13 transgenic mice under the control of a broad and general expression promoter, human cytomegalovirus (hCMV) transcriptional regulatory sequences, have been generated. However, if this transgene is lethal to embryogenesis and no founders can be obtained, tissue-specific IL-13 transgenic constructs driven by CD3δ plus TCR Vβ regulatory regions offer another alternative.

B. Results

Generation and screening of the IL-13 transgene driven by the hCMV regulatory region (pCRβTP600 construct). In order to subclone IL-13 cDNA into the pCRβT vector containing the hCMV transcriptional regulatory sequences, the 427 bp fragment of IL-13 cDNA was initially excised with restriction enzymes, XbaI and EcoRI, from the pJFEΔ123 plasmid, which was generated as a stable source of recombinant IL-13 when transfected into mouse thymoma cell line, BW5147 (Chapter IV). As the overhangs of IL-13 cDNA were not compatible with the XhoI cloning site in the pCRβT vector, the IL-13 cDNA fragment was blunt-ended with Klenow enzyme, and XhoI-linkers were added to both ends. After digestion with XhoI and gel purification, the IL-13 cDNA modified fragment was subcloned into the XhoI-digested pCRβT vector. The ligated plasmids were transformed into DH5α bacteria hosts. Bacteria colonies were screened with probes specific for IL-13 cDNA fragments, and pCRβT clones with IL-13 cDNA inserts were identified. The identity of these clones was further verified through multiple restriction enzyme digestion. Plasmids containing the vector only were linearized with XhoI and migrated as a 4.5 kb band (Fig. 7.1). When digested with XhoI, vectors containing IL-13 cDNA gave rise to two bands migrating at 4.5 and 0.43 kb, corresponding to the linearized vector and IL-13 cDNA, respectively (Fig. 7.1). As the IL-13 cDNA fragment contains an XhoI overhang on both sides, IL-13 cDNA can be inserted into the vector in the proper or reversed direction. The clones containing the IL-13 cDNA fragment from the initial hybridization screening were subjected to PstI restriction digest. Upon PstI digestion, clones with vector alone migrated as two bands at 2.6 and 1.8 kb (Fig. 7.1). PstI-digested pCRβTP600 clones with the correct IL-13 cDNA orientation migrated as 2.6, 1.29, and 0.96 kb fragments (Fig. 7.1). On the other hand,

Pst1-digested pCRβTP600 clones with the reversed IL-13 cDNA orientation migrated as 2.6, 1.65, and 0.59 kb fragments (Fig. 7.1). Identification of these pCRβTP600 clones was further verified by Southern blotting (Fig. 7.1b). The complete pCRβTP600 plasmid contains a hCMV regulatory region, rabbit β-globin splice region, IL-13 cDNA, rabbit β-globin poly-A region, and an Ampicillin antibiotic resistant gene (Fig. 7.2).

In vitro functional determination of the IL-13 transgene construct, pCRβTP600. To assess the functionality of the pCRβTP600 plasmid, two different clones of pCRβTP600 (L56.1 and L56.2) were transfected into COS 7 cells, and secreted IL-13 protein was assayed by mouse bone marrow bioassay. COS 7 transfected with either of the pCRβTP600 clones produced similar levels of IL-13 compared to the IL-13 cDNA clone, pCDSRαP600, which was isolated from a D10 cDNA library by Dr. J. -M. Heslan (Fig. 7.3). However, COS 7 cells transfected with the pJFEΔ123 plasmid, which was constructed for stable and high levels of IL-13 production (Chapter IV), secreted at least two-fold more biologically active recombinant IL-13 (Fig. 7.3). High levels of IL-13 were detected in EL4-IL-2 mouse T lymphoma cells transfected with pJFEΔ123 plasmid (Fig. 7.3). In addition, good secretion levels of IL-13 can be detected in the supernatant of EL4-IL-2 cell lines (Fig. 7.3), mouse fibroblast L929 cells (data not shown), or BW5147 mouse thymoma cells transfected with the pCRβTP600 construct (data not shown). Collectively, these data verified that the pCRβTP600 construct can drive the *in vitro* expression of IL-13 protein in different cell types.

Generation and screening for hCMV-IL-13 transgenic mice. Next, a pCRβTP600 clone, L56.2, was sequenced to verify the IL-13 cDNA and the polylinker regions and expanded for transgenic production. pCRβTP600 was

digested with SphI, and the 2.3 kb fragment was purified (Fig. 7.4) and sent to Dr. J. Marth (University of British Columbia, Vancouver, British Columbia) for mouse embryo microinjection. Subsequent screening and breeding were performed at the University of Alberta. Out of 31 mice produced, only two founders with 2-3 copy numbers (338-2 and 349-1) were positive for the hCMV regulatory region fragment (Fig. 7.5a). The 349-1 founder transmitted the transgene to his offspring in the expected Mendelian fashion as approximately half of the litter hybridized to the radiolabelled hCMV fragment (Fig. 7.5b). However, 338-2 did not transmit the transgene to any of a total of 42 offspring in 4 litters (Fig. 7.5b and data not shown). No further analysis was possible with the 338-2 line.

The presence of IL-13 cDNA in the transgene after random genomic integration was verified by PCR with two sets of IL-13 cDNA transgene specific primers. The 5'P600 set of primers amplified the 266 bp amplicon containing the 5' polylinker and first 254 bp of IL-13 cDNA in the 349-1 line (Fig. 7.6 a & b), whereas the 3'P600 set of primers amplified the 260 bp amplicon spanning the 245 bp of 3' IL-13 cDNA and 3' polylinker (Fig. 7.6 a & b). Amplicons from both sets of primers contained 56 overlapping bp. The two sets of primers detected as low as 1 to 0.1 pg of pCRβTP600 plasmid (Fig. 7.6b). The amplicons from Fig. 7.6b were blotted on to nitrocellulose membrane and probed with IL-13 oligonucleotides (Fig. 7.6c). Littermate controls did not possess the transgene (Fig. 7.6b & c). This was not due to the presence of inhibitors in the littermate control genomic DNA samples as the appropriate amplicons were observed after the addition of 1 pg of exogenous pCRβTP600 (Fig. 7.6 b & c). Hence, this hCMV-IL-13 transgenic founder not only was positive for the hCMV regulatory

region and IL-13 transgenic cDNA but also transmitted the transgene to his offspring.

Transgenic IL-13 mRNA detection by RPA (349-1 line). An RNase Protection Assay (RPA) was established to detect endogenous versus transgenic IL-13 mRNA in different tissues. 330 bp fragments of anti-sense RNAs spanning 107 bp of the 3' end of the coding region and 223 bp of the following 3' untranslated region of natural IL-13 were cloned into the pBluescript II SK^+ vector. Anti-sense IL-13 RNA was transcribed with T3 RNA polymerase *in vitro,* and the full length anti-sense IL-13 RNA was gel purified. After hybridization and RNAse digestion, the anti-sense RNA protects a fragment of 330 bp or 107 bp for natural or transgene-derived IL-13 mRNA, respectively (Fig. 7.7). As expected, a 330 or 107 bp protected band was observed when purified RNA samples from D10 (Fig. 7.8) or IL-13-transfected BW5147 cells (Fig. 7.8) were used, respectively. Neither natural nor transgene-derived IL-13 mRNA was detected in the spleen or thymus RNA samples from littermate controls (Fig. 7.8) or hCMV-IL-13 transgenic mice (Fig. 7.8). Similar results were obtained with RNA samples purified from the uterus and liver. Reconstruction studies revealed that IL-13 mRNA could be detected by RPA if 5% of the cells produced transgene-derived IL-13 mRNA (data not shown). In addition, β-actin anti-sense RNA protected the expected 102 bp fragments from littermate controls and the 349-1 hCMV-IL-13 transgenic line (Fig. 7.8).

Detection of transgene-derived IL-13 mRNA by RT-PCR (349-1 line). Alternatively, RT-PCR was utilized to increase the sensitivity of IL-13 mRNA detection. When the 3'P600 primer set was used in the RT-PCR, transgene-derived IL-13 mRNA was not detected in the DNAse I-treated spleen

RNA samples from littermate controls (Fig. 7.9) or the 349-1 hCMV-IL-13 transgenic line (Fig. 7.9). However, the expected 260 bp PCR product could be detected when 0.1 pg of exogenously added BamHI-digested pCRβTP600 plasmid was added to the mouse spleen RNA samples (Fig. 7.9), suggesting that RT-PCR would have detected transgene-derived IL-13 mRNA if 1% of the cells were to synthesize the corresponding mRNA. The expected 260 bp amplicon was also detected in the RNA sample from BW5147 cells stably transfected with IL-13 cDNA but not in the parental cells. The 540 bp amplicons of β-actin control were detected in all the samples. Based on RPA and RT-PCR, no transgene-derived IL-13 mRNA could be detected in the 349-1 hCMV-IL-13 transgenic line, suggesting that mice from this transgenic line did not express the transgene.

Immune and hemopoietic systems of the 349-1 hCMV-IL-13 transgenic mice. Mice of the 349-1 hCMV-IL-13 transgenic line appeared to be normal with no detectable abnormality in their gross anatomy or serum Ig levels. In addition, IL-13 levels in the serum from 349-1 line and litter control mice were below the detection limit of the TF-1 bioassay (data not shown). In the presence of 10% mouse serum and exogenous IL-13, the detection limit of the TF-1 bioassay was determined to 20 units/ml (7.8 ng/ml). As *in vivo* IL-13 administration enhanced extramedullary hemopoiesis in the spleen (see Chapter V), hemopoietic precursor frequencies of the transgenic mice were quantitated. Spleen cells from littermate controls or the 349-1 hCMV-IL-13 transgenic line did not show significant differences in the CFU-E or CFU-C frequencies (Fig. 7.10).

Immune and hemopoietic systems of the 349-1 hCMV-IL-13 transgenic mice during an immune response. During a strong immune response, elevated IL-13 levels are associated with extramedullary hemopoiesis during *N. brasiliensis*

infection (see Chapter V), suggesting that cytokines and DNA transcription factors induced during immune response may regulate the expression of the hCMV-IL-13 transgene. In addition, it is conceivable that the hCMV-IL-13 transgene may interact with other promoters or enhancers during a strong immune response. Thus, it was hypothesized that the expression of the hCMV-IL-13 transgene may be detected during *N. brasiliensis* infection or CRBC immunization. Eight days after *N. brasiliensis* infection or 7 days after CRBC immunization, splenic hemopoietic precursor frequencies of these mice were quantitated. No significant differences in CFU-E, CFU-C, or BFU-E frequencies were obtained in the littermate controls or the 349-1 hCMV-IL-13 transgenic mice when challenged with *N. brasiliensis* or CRBC (Fig. 7.11). In fact, splenic CFU-E, CFU-C, and BFU-E frequencies appeared to be decreased in response to *N. brasiliensis* infection but increased when immunized with CRBC (Fig. 7.11). In contrast to the observation that *in vivo* IL-13 administration during a strong response to CRBC enhanced serum Ig levels (see Chapter VI), littermate controls and 349-1 hCMV-IL-13 transgenic mice did not exhibit significant differences in their serum Ig levels (data not shown). In summary, although the hCMV-IL-13 transgene was successfully transmitted to the offspring, the transgene did not appear to be expressed, even during strong immune response to *N. brasiliensis* or CRBC.

Identification of a high copy number hCMV-IL-13 transgenic mouse (150 line). Collaborations were also set up to generate additional hCMV-IL-13 transgenic mice with HSLAS (U. of Alberta) and Dr. Sol Zackson (U of Calgary), and a total of 120 and 54 mice were obtained through the pCRβTP600 construct microinjections, respectively. I have also learned to perform embryo microinjection and implantation, which resulted in two possible founders. Among all the mice produced, a high copy number hCMV-IL-13 transgenic mouse, 150,

which successfully transmitted the transgene to his offspring, was generated (Fig. 7.12) by HSLAS. This transgenic line was tested positive for the IL-13 cDNA transgene when both 5'P600 and 3'P600 primer sets were used in the PCR (data not shown). Hence, the hCMV-IL-13 transgene was successfully integrated in the genome of the 150 line and could be transmitted to his offspring.

Analysis of the 150 hCMV-IL-13 transgenic mice. Expression of IL-13 mRNA was not detected by RT-PCR in the 150 hCMV-IL-13 transgenic line. These mice appeared to be normal, with no detectable abnormality in their gross anatomy or serum Ig levels (data not shown). The spleen CFU-E, CFU-C, and BFU-E hemopoietic progenitor frequencies of these mice did not exhibit significant differences when compared to littermate controls. (Fig. 7.13). In addition, the splenic hemopoietic frequencies of littermate controls and mice of the 150 hCMV-IL-13 transgenic line were similar after 8 days of *Nb* infection (Fig. 7.14). Collectively, these date indicate that although both hCMV-IL-13 transgenic lines successfully transmitted the transgene to their offspring, they did not appear to express the transgene.

Construction and analysis of IL-13 transgene driven by CD3δ plus TCR Vβ regulatory regions (pML179P600). As IL-13 is produced by T cells, a transgene that can be expressed in T cells only has been generated. IL-13 cDNA, excised from the pJFEΔ123 plasmid with XhoI and NotI restriction enzymes, was blunt-ended, and BamHI linkers were added to both ends. The modified IL-13 cDNA was subcloned into a BamHI-digested T cell specific vector, pML179 which contains a CD3δ regulatory region, T cell Vβ promoter, and human growth hormone minigene (Fig. 7.15). These pML179P600 clones with the correct orientation of the inserted IL-13 cDNA were identified by restriction mapping

(data not shown). Next, two pML179P600 clones were transfected into COS 7 (data not shown) and EL4-IL-2 mouse T lymphoma cell lines (Fig. 7.16) Supernatants from both cell types transfected with two different pML179P600 clones, L110.1 and L110.6, did not contain a detectable amount of IL-13 as assayed by TF-1 bioassay. As expected, pCRβT or pML179 vectors alone did not drive detectable IL-13 production, whereas transfection with pCDSRαP600, pCRβTP600, and pJFEΔ123 plasmids resulted in IL-13 production. Thus, it appeared that pML179P600 did not drive the secretion of IL-13 in a mouse T lymphoma cell line, and no further investigation was carried out with this construction.

C. Discussion

In order to study the *in vivo* effects of IL-13, a transgene under the influence of the hCMV regulatory region was constructed. Subsequently, three hCMV-IL-13 transgenic lines were generated. Although the 338-2 line did not transmit the transgene to her offspring, the other two lines, 349-1 and 150, successfully transmitted the transgenes to their offspring, allowing further analyses. Despite germ-line transmission in the two lines, no transgene-derived IL-13 mRNA or gross abnormality was detected. In addition, the hCMV-IL-13 transgenic mice or littermate controls did not show significant differences with respect to their hemopoietic and immune systems. Moreover, these transgenic mice did not differ significantly from littermate controls in their hemopoietic and immune systems when challenged with a strong antigen (CRBC) or infection (*N. brasiliensis*). Thus, these mice did not appear to express the transgene.

By using a ubiquitous transgene under the control of hCMV regulatory region, the hCMV-IL-13 transgene should result in the general expression of IL-13 in all cell types. When transfected with the transgene, COS 7, mouse fibroblast, EL4-IL-2 mouse T lymphoma, and BW5147 mouse thymoma cell lines all secreted good levels of IL-13, indicating that the transgene was able to promote IL-13 in a few cell types *in vitro*. However, IL-13 expression was not detected in the low or high copy number transgenic animals. One possible explanation could be the toxicity of IL-13 as observed in mice administered high levels of IL-13 (see Chapter V). Out of more than 200 mice, only two transgenic lines with low or high copy numbers of the transgene were obtained. As the transgene products were not detected in either transgenic line, it is conceivable that the transgenes may have

been integrated into silent or negatively-regulated locations in the genome. Alternatively, IL-13 is toxic during embryogenesis and only non-expression hCMV-IL-13 transgenic mice can be obtained. Consistent with this idea, transgenic mice produced with exogenous genomic mouse IL-4 under the control of the potent human Ig enhancer and mouse Ig promoter indicate that the fully active form of the transgene is lethal in newborn mice (Tepper et al., 1990). Viable mice demonstrating marked effects on B cell function, an increase in serum IgG1 and IgE levels, and blepharitis were obtained only when the potent Ig promoter/enhancer activity was reduced by inserting attenuating *Escherichia coli lac* operator sequences (Tepper et al., 1990; Burstein et al., 1991). Interestingly, transgenic lines generated with IL-4 cDNA under the control of mouse Ig enhancer/promoter regions (Muller et al., 1991) or the *lck* proximal promoter segment which results in selective thymic expression (Lewis et al., 1991; Lewis et al., 1993) do not exhibit this lethal effect nor enhanced serum Ig concentrations.

As an alternative approach, the pML179P600 plasmid which contains a CD3δ regulatory region, a T cell Vβ promoter, and a human growth hormone minigene was constructed. This T cell specific transgene which selectively regulates IL-13 expression in T cells could potentially result in lower IL-13 levels *in vivo* and perhaps circumvent the toxicity effects due to high IL-13 expression during embryogenesis. For reasons that are unclear, this transgene did not appear to promote IL-13 secretion in cell lines. As the functionality of this transgene could not be confirmed *in vitro*, it was not utilized in subsequent transgenic mice production.

The efficiency of transgenes in most cases is unpredictable. As DNA microinjection results in random insertion, the site of integration and surrounding

genes or sequences may influence the expression of the transgene. In addition, native genes containing all or part of their introns and their terminators are generally much better expressed than the corresponding cDNAs. In contrast to *in vitro* expression, the presence of introns greatly benefits transgene expression (Brinster et al., 1988; Palmiter et al., 1991). Apparently, DNA sequences with specific and unknown regulatory mechanisms located in the introns, while not required in cultured cells, are necessary to maintain a transgene in an active configuration (Petitclerc et al., 1995). This is consistent with the observations that hCMV-IL-13 transgenic mice derived from IL-13 cDNA exhibit no detectable phenotype while the transgene is fully functional in cell lines and IL-4 transgenic mice generated with IL-4 cDNA exhibit less severe phenotypes (Muller et al., 1991; Lewis et al., 1991; Lewis et al., 1993).

The major hCMV immediate early (IE) enhancer/promoter is widely reputed to be one of the strongest and most broadly active regulatory elements for directing transcription of heterologous genes in cell culture DNA transfection experiments (Boshart et al., 1985; Muller et al., 1991). This is consistent with the observations that the pCRβTP600 construct was efficient in promoting IL-13 secretion in COS 7, mouse fibroblast, EL4-IL-2 mouse T lymphoma, and BW5147 mouse thymoma cell lines. On the other hand, the efficiency of the hCMV IE enhancer/promoter in directing heterologous gene expression in a variety of tissues in transgenic animals remains controversial. Although the hCMV IE enhancer/promoter results in ubiquitous expression of two different transgenes (Schmidt et al., 1990; Peppel et al., 1993), this regulatory region also results in several different transgene expressions in restricted sites (Mikkelsen et al., 1992; Furth et al., 1991), including the target tissues of congenital hCMV infection in human fetuses (Koedood et al.,

1995; Fritschy et al., 1996; Baskar et al., 1996). The inherent cell specificity of the hCMV IE enhancer/promoter in transgenic animals is not fully supported by the unrestricted expression of the heterologous gene in transient transfectants. Interestingly, once the hCMV IE enhancer/promoter is integrated in the cellular genome, this regulatory region can be gradually downregulated or inactivated in some cultured cell types or *in vivo* (Palmer et al., 1991; Scharfmann et al., 1991). This is further supported by the fact that *de novo* methylation can result in the inhibition of hCMV-regulated transgene expression *in vivo* (Gibbs et al., 1994). Collectively, these data indicate that the nature of the transgene, copy numbers, transgene integration sites, and expression levels dictate the phenotype and outcome of these genetically manipulated mice.

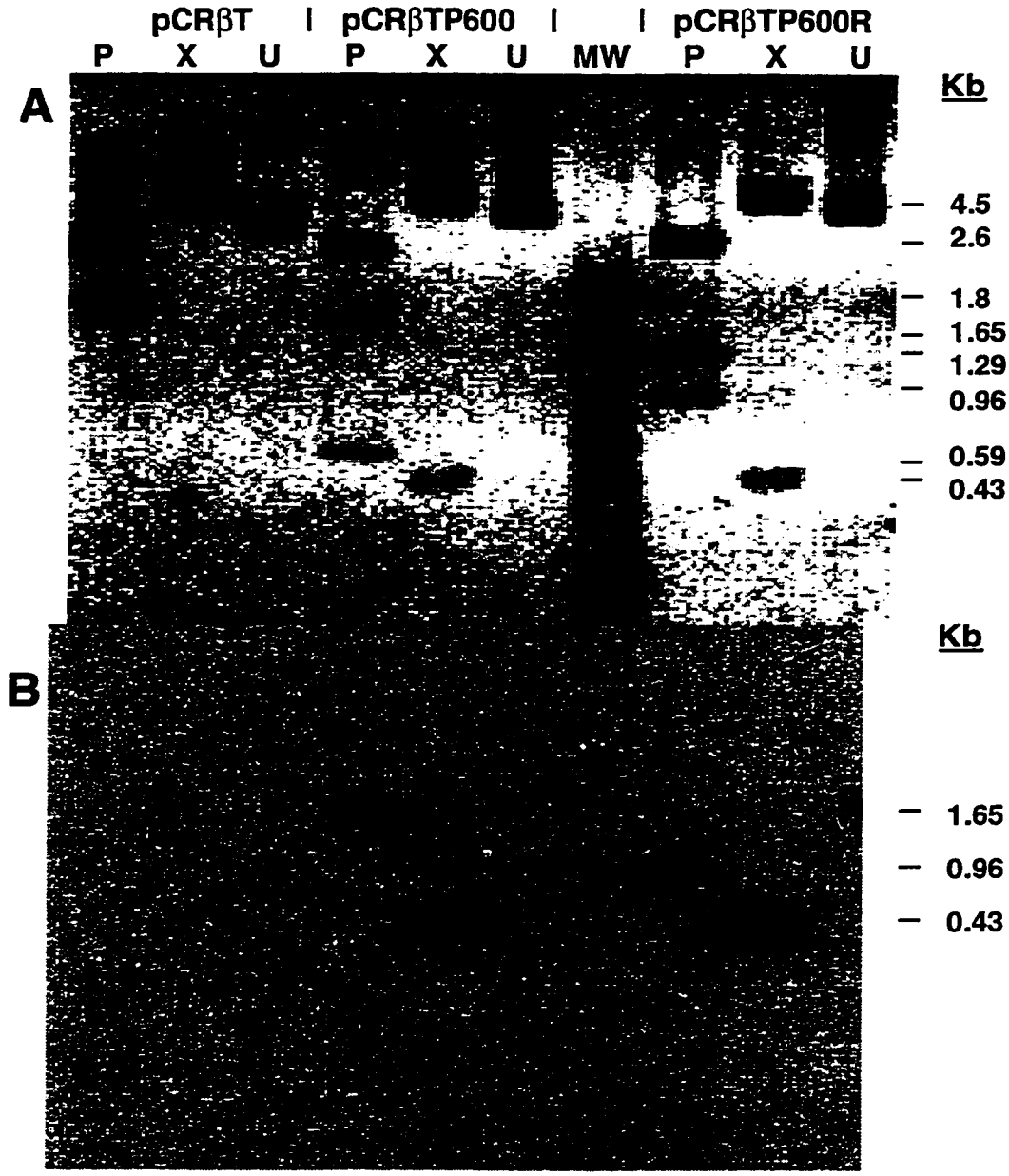


Figure 7.1. Restriction digest mapping of pCRβTP600. Plasmids containing vector alone (pCRβT, kindly provided by Dr. J. Marth) or with IL-13 cDNA inserted correctly (pCRβTP600) or in the reversed orientation (pCRβTP600R) were digested with XhoI (X) or PstI (P). The undigested (U) or digested DNA was electrophoretically resolved in 1% agarose in TBE buffer and stained with ethidium bromide. The molecular weight ladder (MW) is indicated (A). The DNA fragments from (A) were transferred to a nitrocellulose membrane for Southern blotting with radiolabelled IL-13 oligonucleotides.

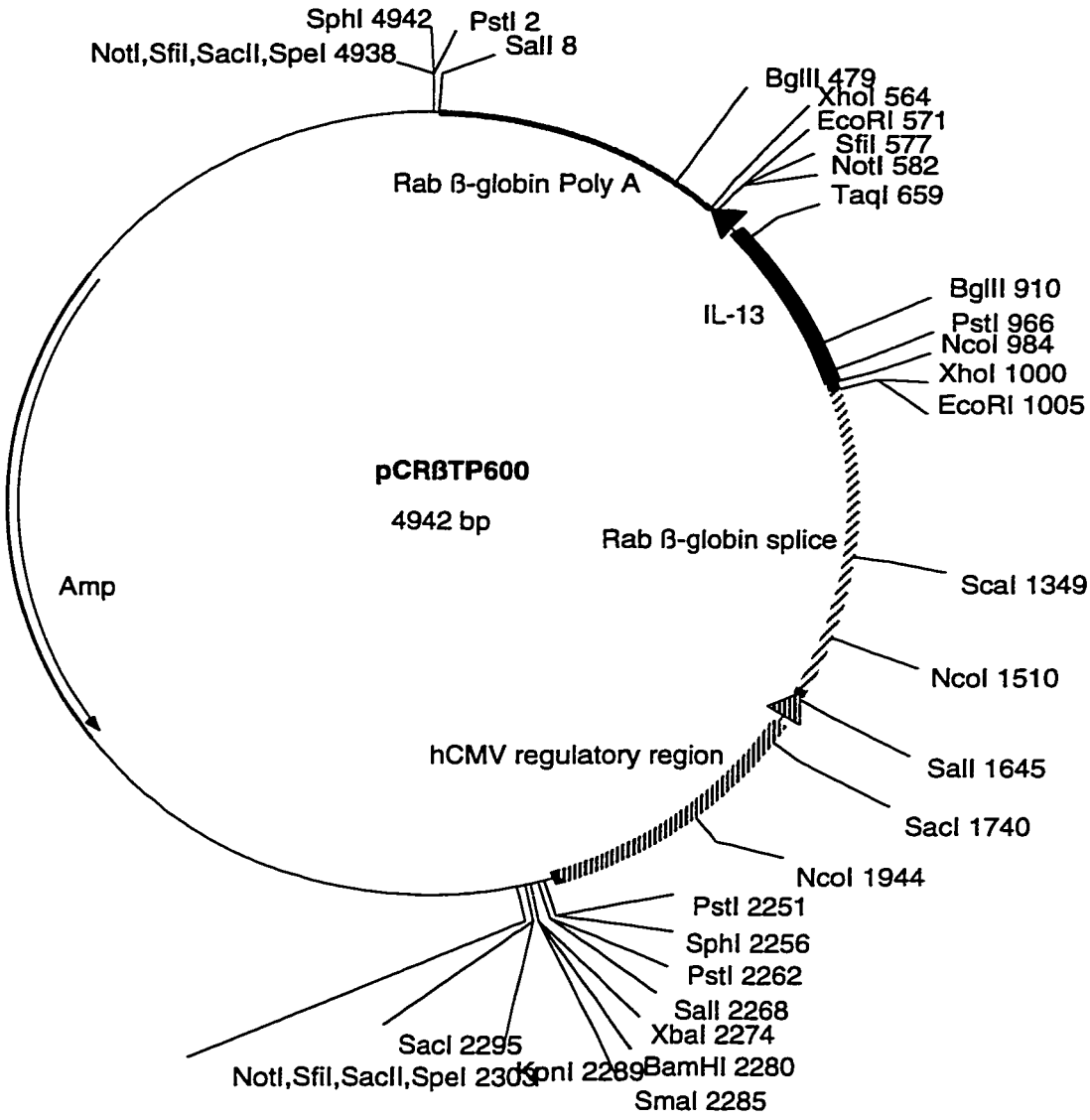


Figure 7.2. Schematic representation of pCRβTP600 plasmid with restriction digest sites.

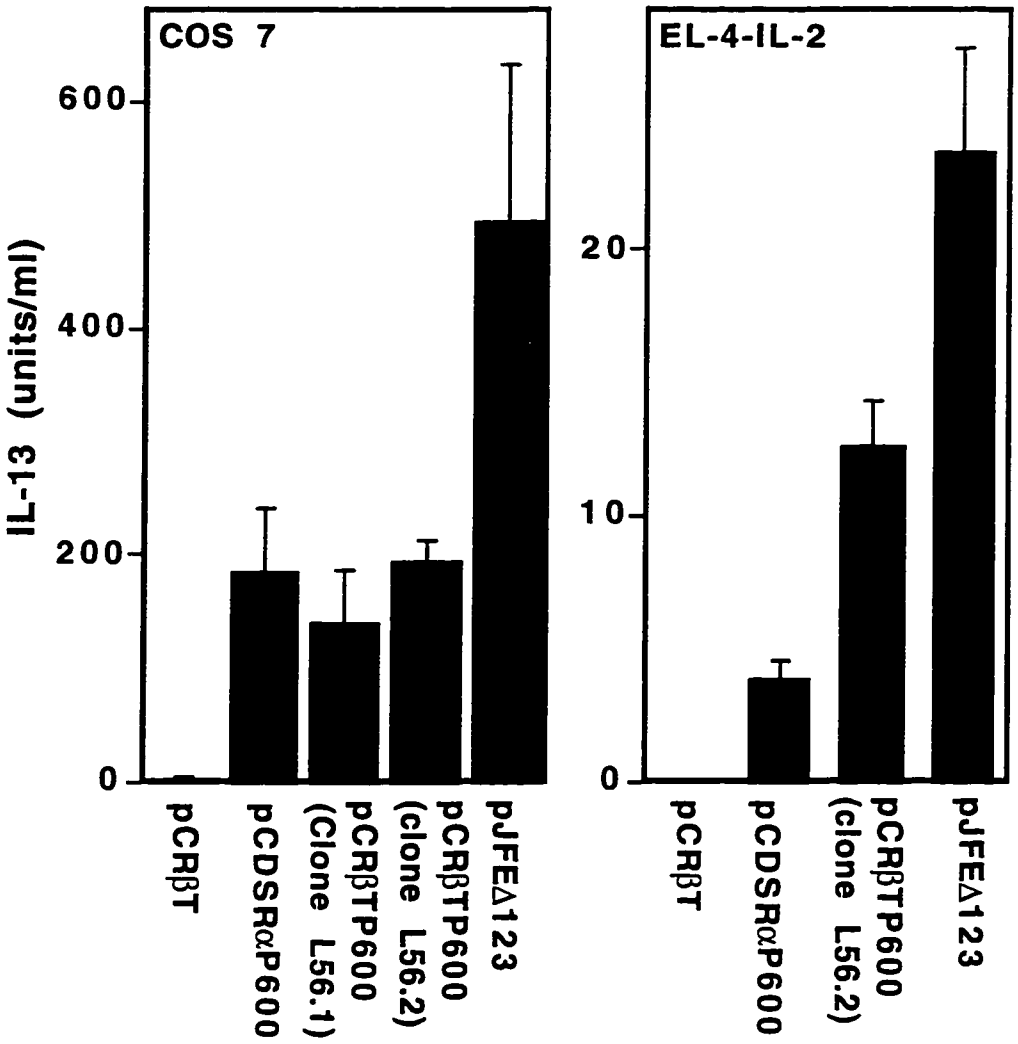


Figure 7.3. pCRβTP600 directed IL-13 production and secretion in COS 7 and EL4-IL-2 cells. Confluent COS 7 cells were transfected with 5 μg/ml of appropriate plasmids by the DEAE dextran/chloroquine method. Supernatants were harvested after 72 h of amplification or expression of the DNA or protein, respectively. Two different clones of pCRβTP600, L56.1 and L56.2, were used. The pCRβT vector alone was used as a negative control, whereas pCDSRαP600 and pJFEΔ123 plasmids were used as positive controls. EL4-IL-2 cells were transfected with 15 μg of the appropriate plasmids by the DEAE dextran method. Supernatants were harvested after 7 days of amplification or expression of the DNA or protein, respectively. IL-13 bioactivity was quantitated by TF-1 bioassay. The average IL-13 units/ml and SD from triplicate cultures are shown.

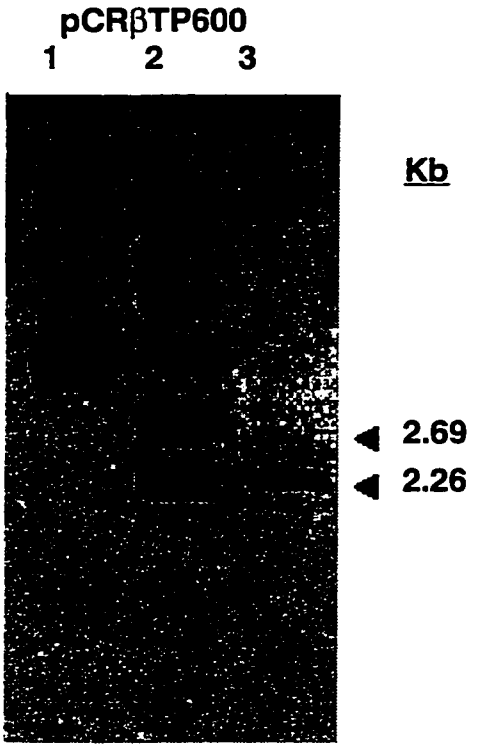


Figure 7.4. SphI-digested pCRβTP600 fragment for transgenic mice production. Lane 1, undigested pCRβTP600 (Clone L56.2); Lane 2, pCRβTP600 digested with SphI; and Lane 3, pCRβTP600 was digested with SphI, and the 2.26 kb hCMV-IL-13 transgene fragment was purified for embryo microinjection. These samples were resolved with 1% agarose and ethidium bromide staining.

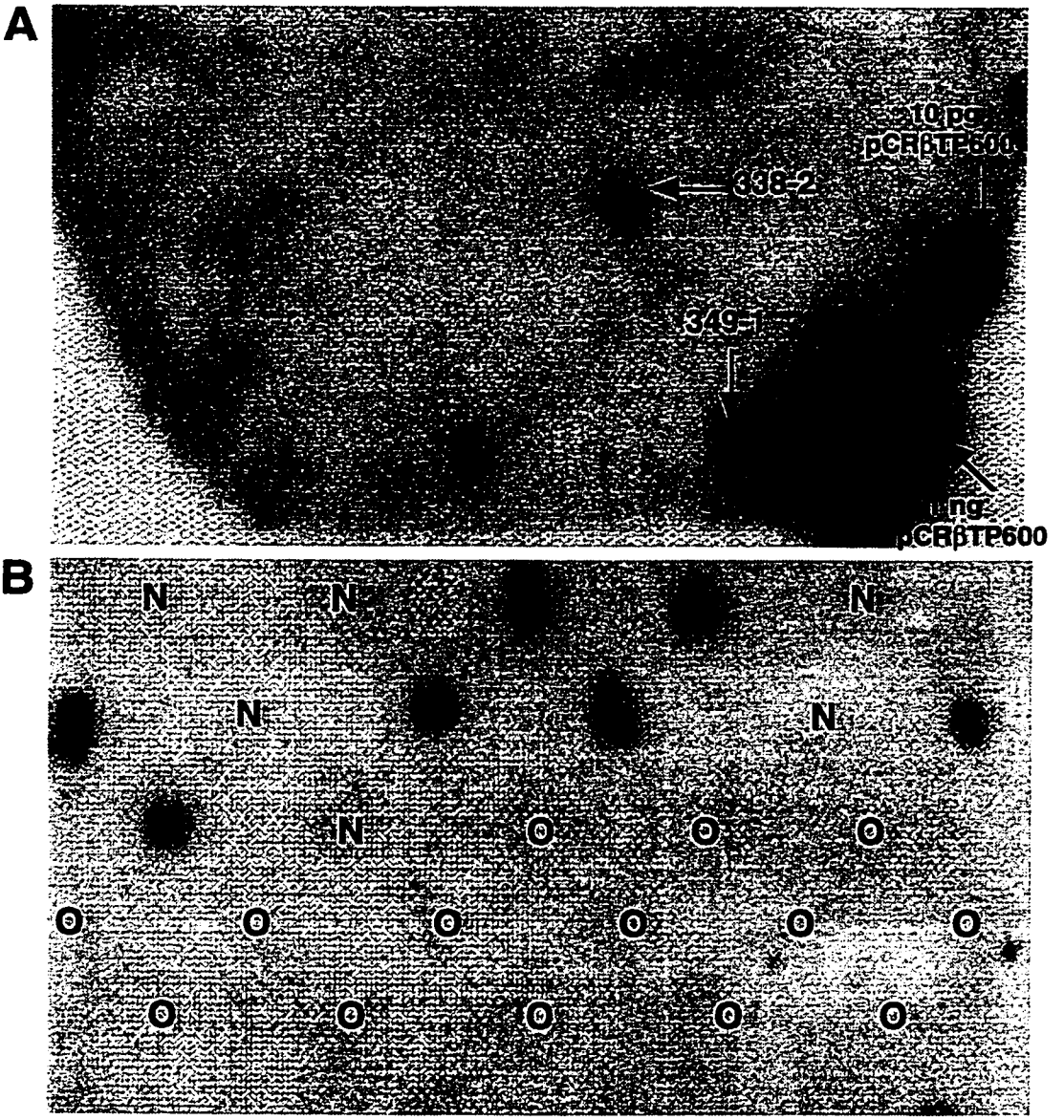


Figure 7.5. Identification of two hCMV-IL-13 transgenic founders and their offspring by dot blots. Approximately 5 μg of purified mouse tail genomic DNA samples were loaded on a nitrocellulose membrane and probed with the radiolabelled 0.6 kb hCMV fragment. After 48 h of exposure, the autoradiography film was developed. 338-2 and 349-1 founders were identified (A). 1 ng and 10 pg of pCRβT plasmid were used as high or 2-3 copy number positive controls, respectively. (B) 349-1 founder was backcrossed to C57BL/6 mice, and approximately half of his offspring hybridized to the hCMV probe. **N**, represents negative littermate controls. All the offspring from 338-2 (**O**) did not hybridize to the hCMV probe.

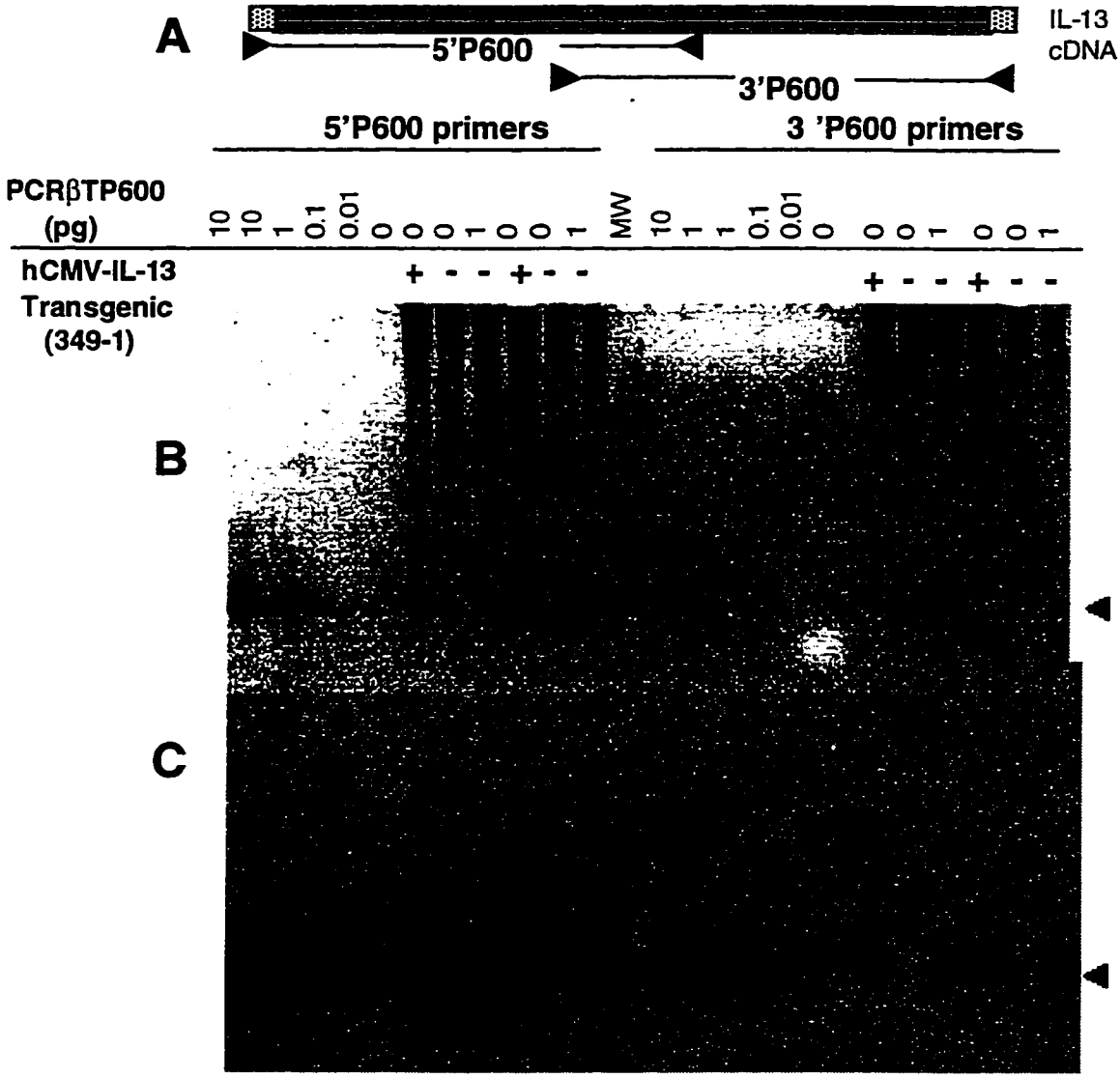


Figure 7.6. The presence of IL-13 cDNA fragment in the 349-1 hCMV-IL-13 transgenic line. (A) Schematic diagram of the locations of 5'P600 and 3'P600 primer sets for hCMV-IL-13 transgene PCR detection. (B) PCR was performed on 1 μg of purified tail genomic DNA samples from 349-1 line (+) or littermate controls (-) using 5'P600 and 3'P600 primer sets. Both sets of primers give rise to amplicons with 56 overlapping bp. 1 pg of BamHI-disgested pCRβTP600 was added to littermate control genomic DNA samples before the PCR. 100 to 0.01 pg of pCRβTP600 were used as standards (~240 bp). The PCR products were resolved with a 2% agarose gel and visualized using ethidium bromide. (C) DNA fragments from (B) were transferred to a nitrocellulose membrane and probed with radiolabelled IL-13 oligonucleotides. The expected amplicons are indicated by arrowheads.

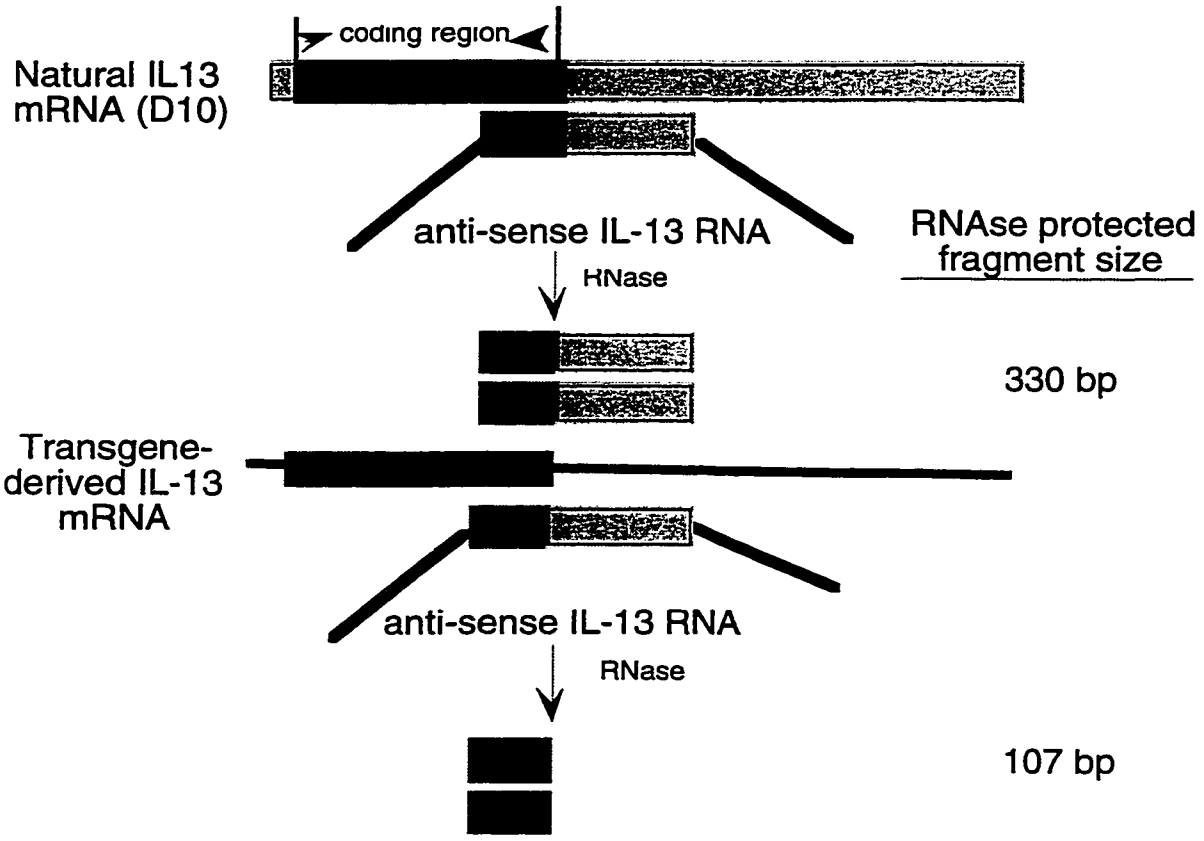


Figure 7.7. Schematic diagram of RNAse protected natural or transgene-derived IL-13 mRNA fragments. Anti-sense RNA of a 330 bp fragment spanning 107 bp of the 3' coding region and 223 bp of the 3' untranslated region was *in vitro* transcribed. After hybridization and digestion with RNAse, a 330 or 107 bp protected fragment corresponding to the natural or transgene derived IL-13 mRNA, respectively, can be observed.

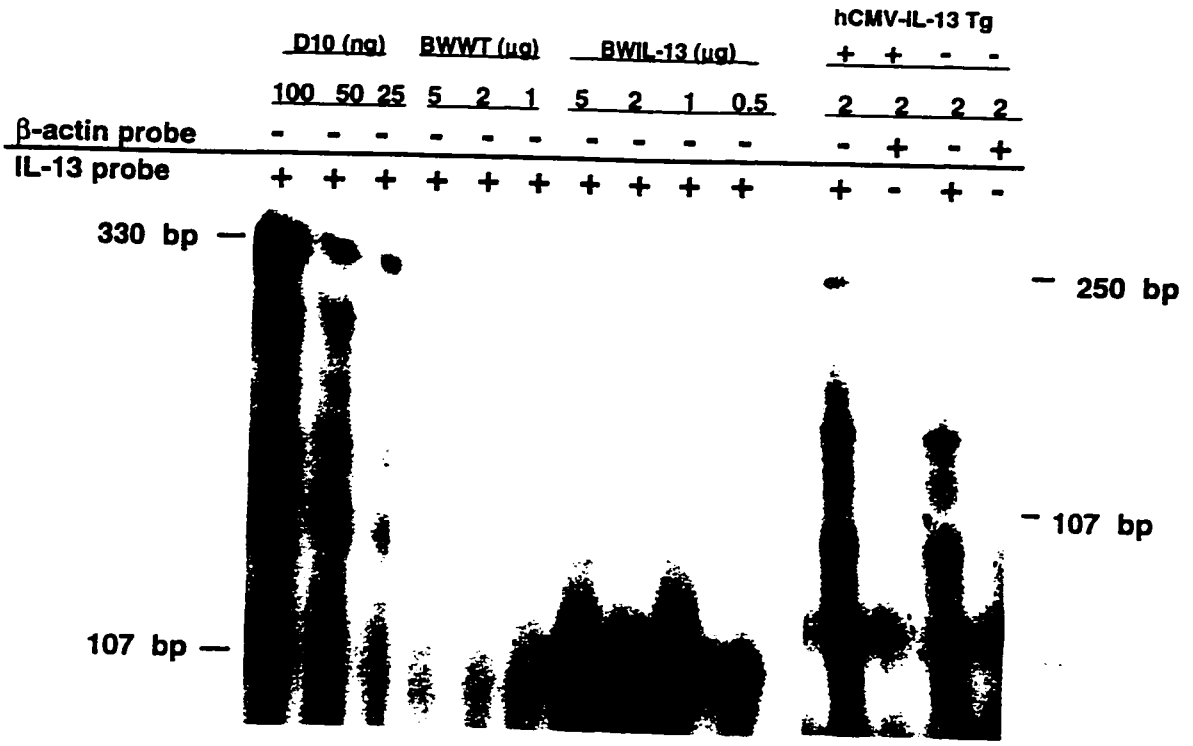


Figure 7.8. Absence of IL-13 mRNA in the spleen cells of littermate controls or hCMV-IL-13 transgenic mice (349-1line). RPA was performed on total purified RNA samples, and these samples were electrophoresed on 12% PAGE-TBE. Protected fragments were revealed by autoradiography film. 5, 2, 1, or 0.5 µg of IL-13 transfected BW5147 (BWP600) total RNA (positive controls for transgene-derived IL-13, 107 bp protected fragments) or wildtype parental cell line (BWWT) were used. 100, 50, or 25 ng of purified D10 mRNA were used as a natural IL-13 mRNA control (330 bp protected fragments). 2 µg of spleen total RNA from littermate control (-) or hCMV-IL-13 transgenic mouse (+, 349-1 line) were incubated with IL-13 or β-actin anti-sense RNA probes.

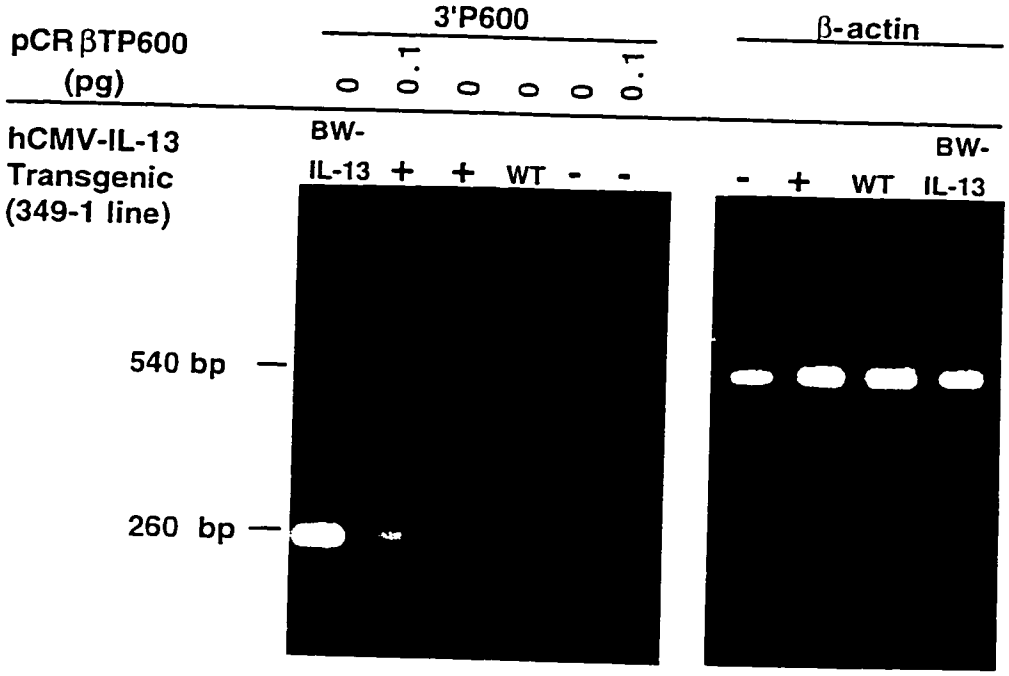


Figure 7.9. Detection of tissue transgene-derived IL-13 mRNA by RT-PCR. 2 μg of DNAse I-treated purified total RNA from littermate control (-), hCMV-IL-13 transgenic mouse (+), wild type (WT), or stable IL-13 cDNA (BWP600) transfected BW5147 cells were reverse transcribed, and 2 μl of each reverse transcribed sample, in the presence or absence of 0.1 pg of BamHI-digested pCRβTP600 plasmid, were subjected to PCR with 3'P600 primer set or β-actin primers. The amplicons were electrophoresed on 2% agarose and visualized by ethidium bromide staining.

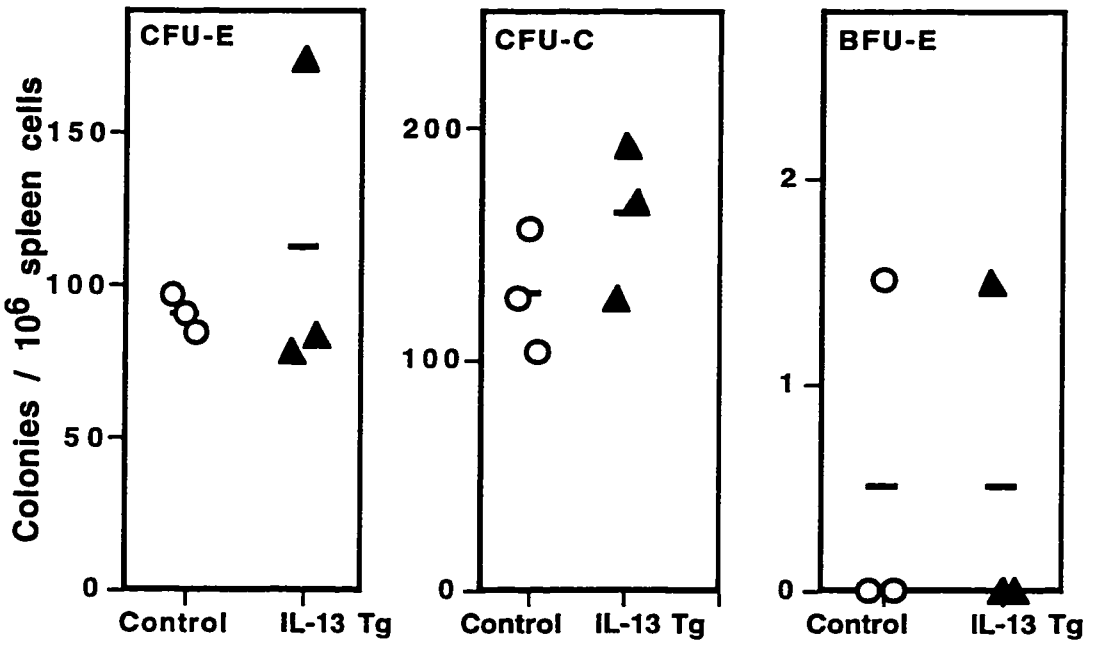


Figure 7.10. Hemopoietic precursor frequencies of the spleen cells from hCMV-IL-13 transgenic mice (349-1 line) were similar to those of the littermate controls. Duplicate cultures of spleen cells from littermate control (○) or hCMV-IL-13 transgenic mice (▲, 349-1 line) were established in methylcellulose medium. CFU-E, CFU-C, and BFU-E colonies were enumerated. Each symbol represents the average data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar.

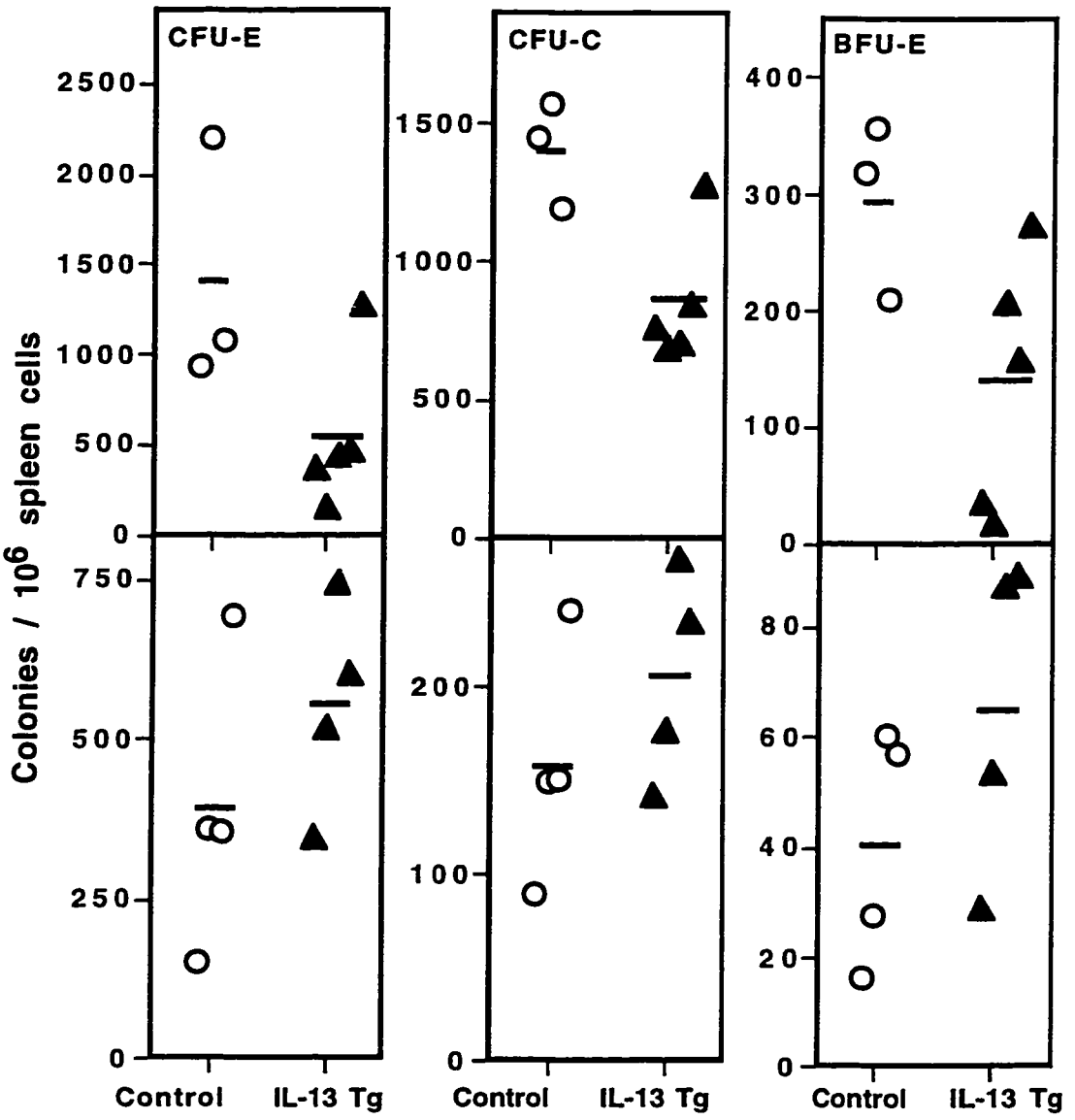


Figure 7.11. Hemopoietic precursor frequencies of the spleen cells from *N. brasiliensis*-infected or chicken red blood cells immunized hCMV-IL-13 transgenic mice (349-1 line) were similar to those of the littermate controls. Eight days after infection with 500 L3 *Nb* larvae (top three panels) or 7 days after CRBC immunization (bottom three panels), spleen cells from littermate controls (O) or 349-1 hCMV-IL-13 transgenic mice (▲, 349-1 line) were established in methylcellulose medium in duplicate. CFU-E, CFU-C, and BFU-E colonies were enumerated. Each symbol represents the average data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. CFU counts of the duplicate cultures from each mouse were within 18% of the mean of the duplicates.

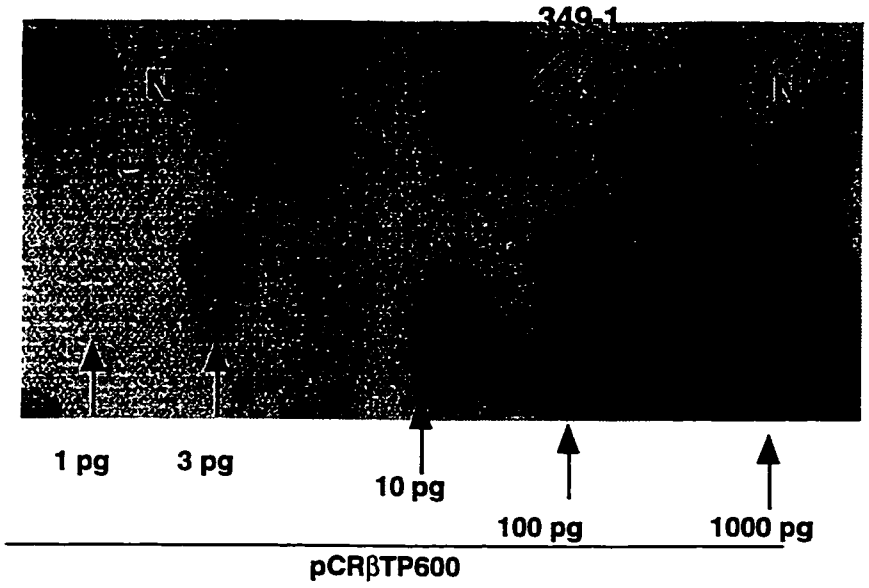


Figure 7.12. High copy number hCMV-IL-13 transgenic founder, 150, transmitted the transgene to his offspring. 150 founder was backcrossed to C57BL/6 mice. Approximately 5 μg of purified mouse tail genomic DNA samples were loaded on a nitrocellulose membrane and probed with radiolabelled 0.6 kb hCMV fragment. After 48 h of exposure, autoradiography film was developed. 1000, 100, 10, 3, and 1 pg of pCRβTP600 plasmid were used as positive controls. 3 pg of pCRβTP600 plasmid or genomic DNA from 349-1 line corresponded to 1 or 2 to 3 copy numbers, respectively. Approximately half of his offspring hybridized to the hCMV probe. **N**, represents negative littermate controls.

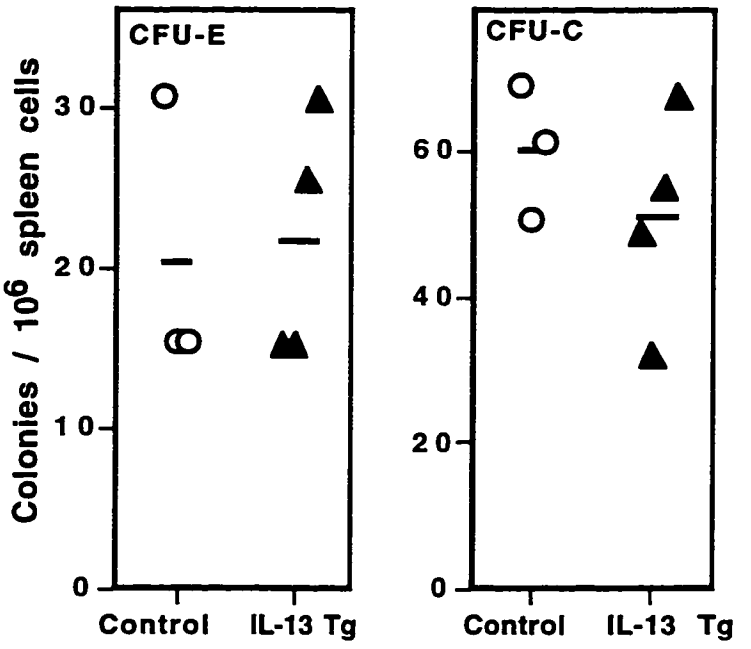


Figure 7.13. Hemopoietic precursor frequencies of the spleen cells from hCMV-IL-13 transgenic mice (150 line) were similar to those of the littermate controls. Spleen cells from littermate controls or hCMV-IL-13 transgenic mice (▲, 150 line) were established in methylcellulose medium in duplicate. CFU-E and CFU-C colonies were enumerated. Each symbol represents average data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar.

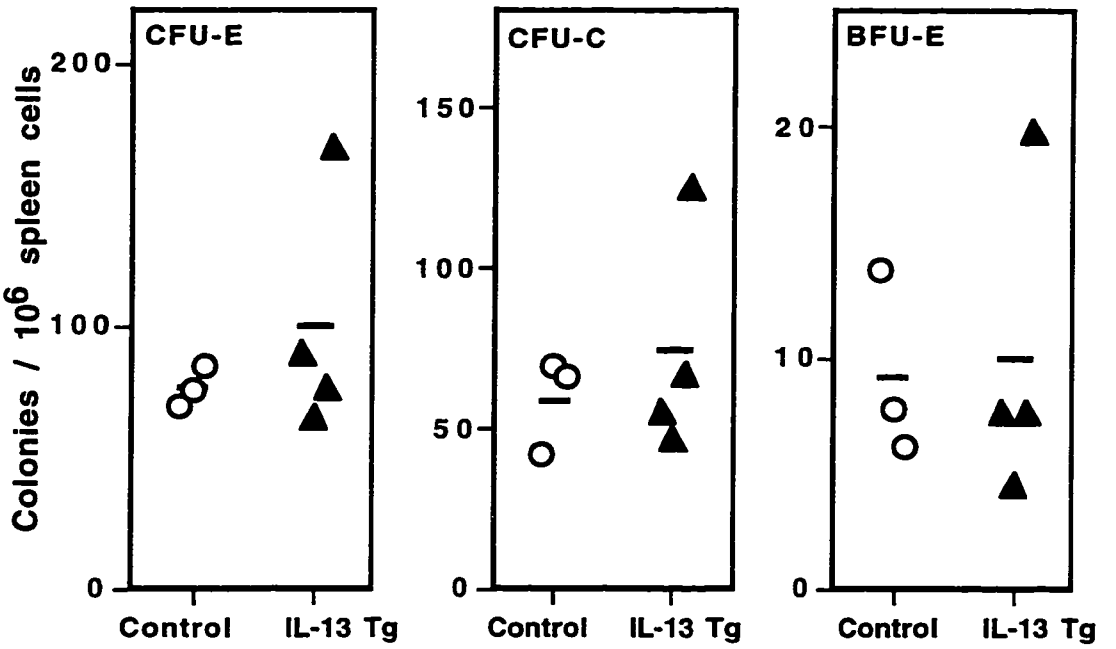


Figure 7.14. Hemopoietic precursor frequencies of the spleen cells from *N. brasiliensis*-infected hCMV-IL-13 transgenic mice (150 line) were similar to those of the littermate controls. Eight days after infection with 5000 L3 *Nb* larvae spleen cells from littermate controls (O) or hCMV-IL-13 transgenic mice (▲, 150 line) were established in methylcellulose medium in duplicate. CFU-E and CFU-C colonies were enumerated. Each symbol represents the average data obtained from an individual mouse. The average value in each group is indicated by a solid horizontal bar. CFU counts of the duplicate cultures from each mouse were within 12% of the mean of the duplicates.

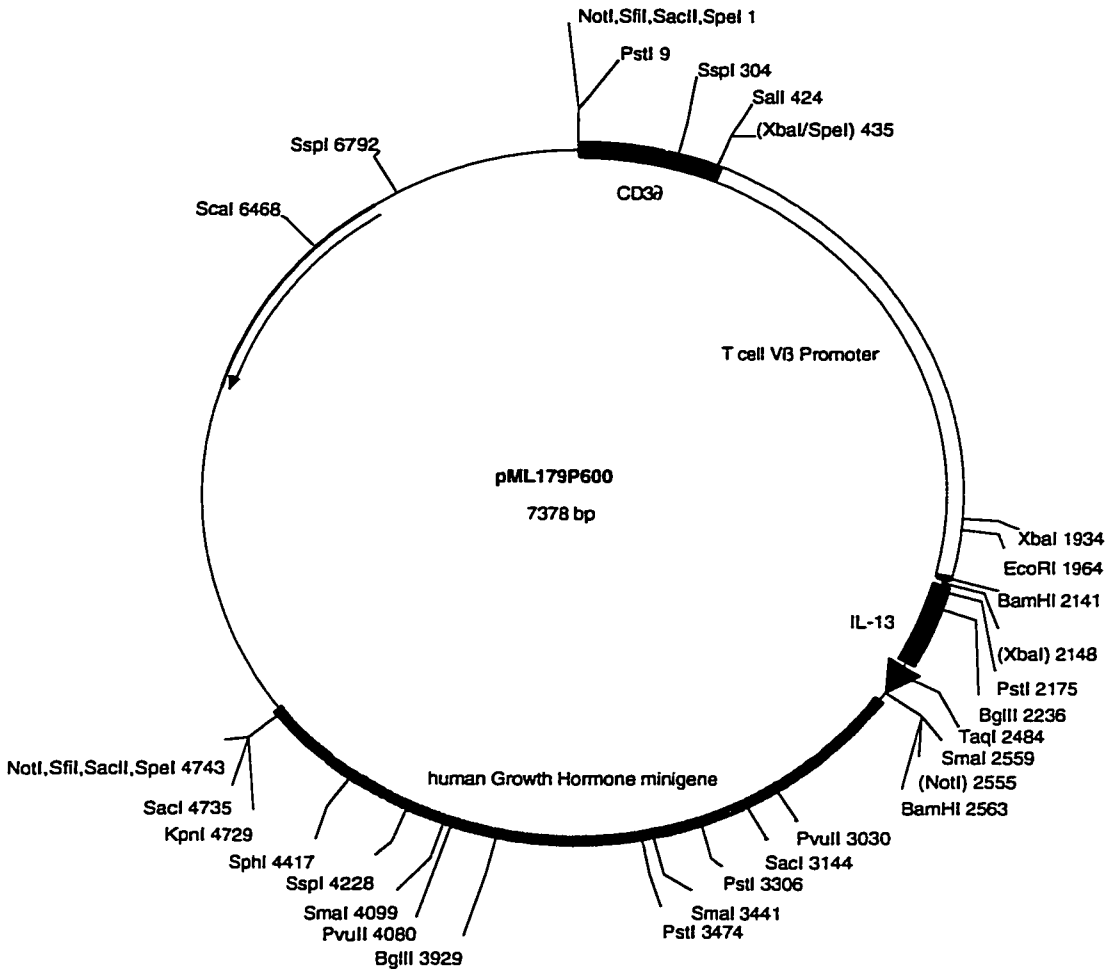


Figure 7.15. Schematic representation of pML179P600 plasmid with restriction digest sites. The EcoRI-XbaI fragment of IL-13 cDNA was excised from pJFEΔ123 and blunt-ended with Klenow, before BamHI linkers were added on both ends. After digestion with BamHI, the modified IL-13 cDNA was ligated with BamHI-digested pML179P600 vector.

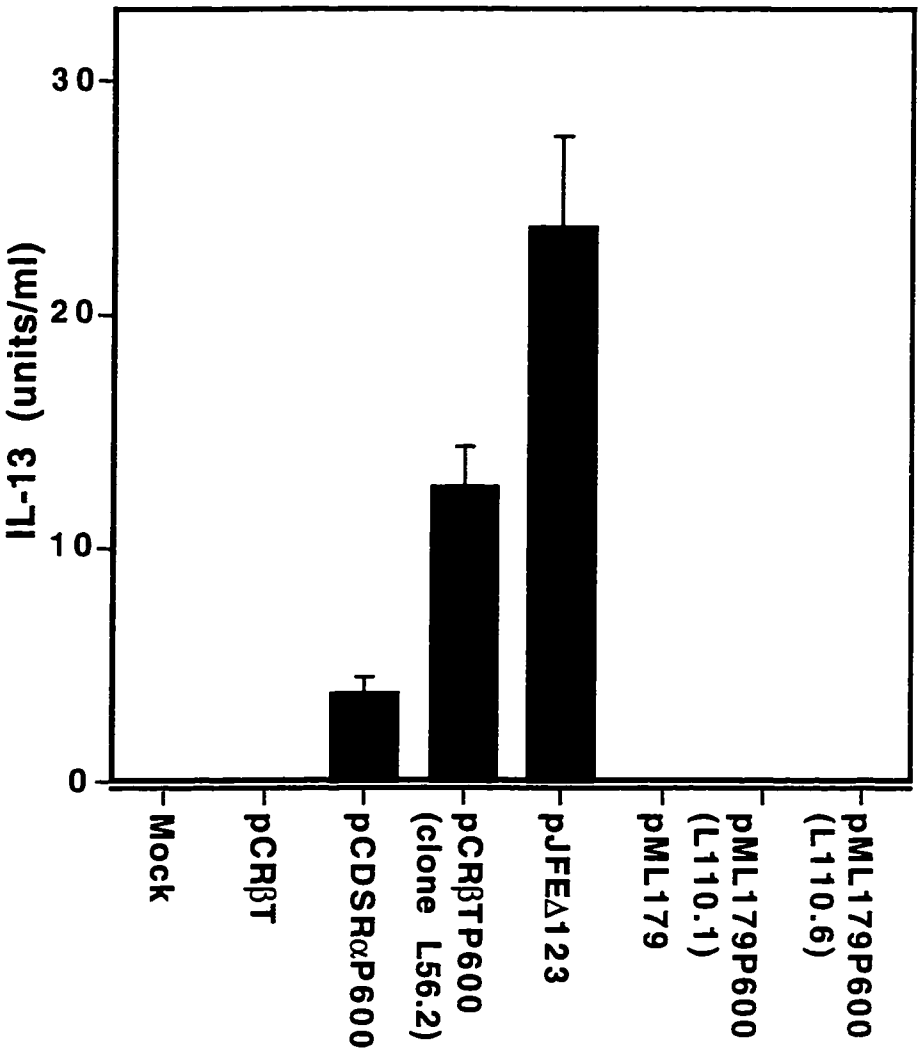


Figure 7.16. pML179P600 did not drive IL-13 production or secretion in EL4-IL-2 cells. EL4-IL-2 cells were transfected with 15 μg of appropriate plasmid by the DEAE dextran method. Supernatants were harvested after 7 days of amplification or expression of the DNA or protein, respectively. pCRβT or pML179 vectors alone were used as negative controls, whereas pCDSRαP600, pCRβTP600, and pJFEΔ123 were used as positive controls. Two different clones of pML179P600, L110.1 and L110.6, were used. IL-13 bioactivity was quantitated by TF-1 bioassay. The average IL-13 units/ml and SD from triplicate cultures are shown.

CHAPTER VIII

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Although the understanding of biomolecules has been provided by numerous valuable *in vitro* studies, the interactions between biomolecules in biological systems can be extremely complex. Hence, *in vivo* studies are vital to extend and confirm our appreciation of the complexity of biological systems. This is especially true with the discovery of new biomolecules. When IL-13 was initially identified based on *in vitro* observations (J. -M. Heslan, L. J. Guilbert, and T. R. Mosmann, unpublished) (Minty et al., 1993; McKenzie et al., 1993a), its *in vivo* functions were unknown. The current study was devoted to confirming and extending the *in vitro* functions of IL-13 in animal models and, most importantly, exploring the unexpected functions of IL-13 *in vivo*.

As the research progressed, numerous exciting findings have been identified and are described in this thesis. *In vivo* administration of IL-13 in mice results in striking monocytosis in the peripheral blood and extramedullary hemopoiesis in the spleen and liver (Lai et al., 1996) (Fig. 8.1). Part of these hemopoietic findings are consistent with the *in vitro* observations in which IL-13 stimulates the formation of macrophages from primitive hemopoietic progenitors (Jacobsen et al., 1994) and the growth of megakaryocyte progenitor cells from human cord blood (Xi et al., 1995). The *in vivo* studies described in Chapter V further reveal that IL-13 not only enhances extramedullary hemopoiesis but also the frequencies of the primitive mouse hemopoietic progenitor cells for erythropoiesis and myelopoiesis. Although it is unclear at this stage whether these *in vivo* findings are due to direct and/or indirect effects of IL-13, enhanced

extramedullary hemopoiesis in the spleen occurs as early as 3 days after *in vivo* IL-13 administration, and IL-13 stimulates mouse bone marrow cells *in vitro*, suggesting that it is likely that IL-13 directly stimulates hemopoietic progenitors. Nevertheless, further studies involving the administration of IL-13 either in the presence of mAbs against other hemopoietic cytokines or in mice deficient in certain cytokines are required to address this issue.

With the progress made from *in vitro* hemopoiesis investigations, it is natural to adapt the observations in human therapies. Before cytokines are administered to improve the rate of engraftment in humans, preclinical animal studies are also required to provide the basis for such treatment. For example, administration of G-CSF to mice increases the numbers of circulating colony forming units in the spleen (Molineux et al., 1990a). Blood from mice treated with human G-CSF successfully increase the survival rate (Molineux et al., 1990b) and accelerates the recovery of bone marrow and splenic hemopoietic progenitors frequencies of lethally irradiated mice (Tamura et al., 1991), indicating that G-CSF increased the number of hemopoietic progenitor cells in circulation. These animal model studies provide a basis for the clinical use of G-CSF to mobilize hemopoietic progenitors to rescue patients from hematologic failure following myeloablation (Craig et al., 1993; Hohaus et al., 1993; Sato et al., 1994; Khwaja et al., 1993; Steward, 1993; Barge, 1993). It will be interesting to investigate whether *in vivo* IL-13 administration can indeed accelerate the rate of bone marrow engraftment in lethally irradiated mice. Such studies can provide insights into the possibility of using IL-13 to promote bone marrow engraftment in clinical trials.

As IL-13 administration at high doses appears to exhibit hepatic and cardiac toxicities, which are consistent with the side effects observed in Phase I and II

IL-4 clinical trials (Gilleece et al., 1992; Prendiville et al., 1993; Margolin et al., 1994), further experiments to study the optimal doses of IL-13 in the induction of hemopoiesis without causing such toxicity effects are required. Alternatively, to achieve optimal results in bone marrow regeneration without causing any major side effects, IL-13 could be used in combination with other hemopoietic cytokines to achieve the desire effects.

In the presence of SCF and G-CSF, IL-13 promotes exclusively the formation of macrophages from primitive hemopoietic progenitors (Jacobsen et al., 1994). This is consistent with the *in vivo* data that showed that IL-13 administration induces striking monocytosis in the peripheral blood and peritoneal cavity. These macrophages express the activated surface marker, Mac-3. As IL-13 demonstrates overlapping functions with IL-4, and both cytokines belong to the Th2 cytokine family, it is natural to predict that these IL-13-induced macrophages may play an essential role in promoting a Th2 immune response. Interestingly, IL-13 provides protection to a characteristic Th1 cell-controlled *Listeria monocytogenes* infection by enhancing IL-12 production and NK cell cytolytic activity (Kaufmann, 1993; Flesch et al., 1997). It will be interesting to study the potential roles of IL-13 during other strong Th1 immune responses. Thus, further studies, which may provide further insights into the regulation of Th1 or Th2 immune response, are warranted to clarify the biological roles of this macrophage population.

IL-13 appears to play critical roles in the resistance to gastrointestinal helminth *N. brasiliensis* (Urban et al., 1998) or *T. muris* infection (Bancroft et al., 1998). In contrast to IL-4 deficient mice, mice treated with soluble IL-13Rα2-Fc fusion protein, or mice deficient for STAT6 or IL-4Rα (absence of IL-4 and IL-13

signaling) fail to expel the mouse-adapted strain of *N. brasiliensis* (Urban et al., 1998). Similarly, IL-13-deficient mice fail to expel *N. brasiliensis* (Urban et al., 1998). IL-4- or IL-13-deficient mice are susceptible to *T. muris* infection, which requires a Th2 response to provide protection (Bancroft et al., 1998). Both types of mice mount diminished early Th2 responses but IL-13-deficient mice are capable of generating strong Th2 responses at later time points. In contrast to IL-4-deficient mice, IL-13-deficient mice synthesize a high but unregulated level of parasite-specific IgG1, suggesting that IL-4 and IL-13 appear to play important yet different roles in mediating protection against this helminth (Bancroft et al., 1998). As extramedullary hemopoiesis in response to *N. brasiliensis* infection is accompanied by elevated IL-13 as determined by the TF-1 bioassay, it is conceivable that IL-13 could be responsible for regulating the regeneration of effector cells to expel gastrointestinal helminths. Additional studies with IL-13-deficient mice are required to provide further evidence to support this hypothesis.

Enhanced antigen specific Ig levels can be detected in IL-13-treated mice undergoing a strong immune response. Based on *in vitro* assays, IL-13 stimulates antibody production in mouse B cells, mainly by promoting their survival (Fig. 8.1). This is in sharp contrast to IL-13 effects on human B cells. Similar to human and mouse IL-4, IL-13 induces class switching in human B cells to produce IgG4 (or mouse IgG1) and IgE (McKenzie et al., 1993a; Punnonen et al., 1993). On the contrary, IL-13 does not appear to induce selectivity of antibody class switching in mouse B cells. Although mouse IL-13 stimulates IgE production in human B cells, a comparable effect has not been observed in this study, suggesting a species difference in the requirements of IgE class switching. This is rather surprising but there are other circumstances in which biological functions are not conserved

between species. Mouse bone marrow cells cultured with mouse IL-3 for over 4 weeks produce almost pure mouse mast cell populations (Saito et al., 1987), whereas basophils and eosinophils but not mast cells, can be obtained from human cord blood or bone marrow mononuclear cells cultured in the presence of IL-3 (Ishizaka et al., 1989). Moreover, mouse IL-5 is a potent B cell stimulator, but human IL-5 does not appear to be a regulatory factor for human B cells except in the presence of mitogenic stimulations, such as *Moraxella catarrhalis* (Huston et al., 1996) or staphylococcal A Cowan 1 strain (SAC) under particular culture conditions (Bertolini et al., 1993). Finally, IL-10 stimulates proliferation of SAC-stimulated human B cells (Rousset et al., 1992), but inhibits proliferation of LPS-stimulated mouse B cells (Marcelletti, 1996).

Under certain circumstances, IgE can be produced in an IL-4-independent mechanism. For example, IgE was produced in IL-4-deficient mice treated with anti-CD40 mAb (Ferlin et al., 1996) or infected with malaria (von der Weid et al., 1994) or *Leishmania major* (Noben-Trauth et al., 1996); or during the course of retrovirus-induced immunodeficiency syndrome or anti-IgD treatment (Morawetz et al., 1996), but not during *N. brasiliensis* infection (Kuhn et al., 1991; Lawrence et al., 1995; Morawetz et al., 1996). Despite the lack of evidence in this study for a selective role of IL-13 in IgE production, it remains possible that IL-13 may directly promote IgE production under selective conditions. Unlike the potent effects of IL-5 on mouse B cells, biological effects of IL-5 on human B cells can be observed under particular stimulations (Huston et al., 1996; Bertolini et al., 1993). Alternatively, IL-13 may indirectly enhance IgE levels by promoting B cell survival. IL-5 does not induce class switching but efficiently enhances antibody production in B cells that have undergone class-switching (Sonoda et al., 1989; Yokota et al., 1987; Yuan et al., 1990; Webb et al., 1991).

When mouse B cells were stimulated with anti-CD40 mAb alone, the numbers of surviving and IgM-secreting B cells were enhanced, compared to cultures without anti-CD40 mAb stimulation. Epstein-Barr virus-transformed human B cell lines express IL-13 mRNA and produce a small amount of IL-13 (de Waal Malefyt et al., 1995; Fior et al., 1994; Kindler et al., 1995). Hence, it is conceivable that mouse splenic B cells can produce IL-13 upon strong anti-CD40 activation, and IL-13 can function in an autocrine fashion in stimulating Ig production in mouse B cells. This would be consistent with the inability of exogenous IL-13 to further enhance antibody production at high anti-CD40 concentrations. This hypothesis can be addressed with the availability of B cells derived from IL-13-deficient mice. Alternatively, Ig secretion levels and survival of purified mouse B cells, which are cultured with anti-CD40 mAb and in the presence of blocking IL-13 mAb or soluble IL-13Rα2-Fc fusion protein, can be analyzed to test the above hypothesis. For many cytokines, the data obtained by blocking cytokine function are often more useful than the data obtained by treating with the cytokine itself. Hence, it is a high priority to produce blocking mAbs against IL-13. Our and other laboratories have been actively involved in trying to generate blocking IL-13 mAbs, but to no avail.

Further studies are needed to investigate IL-13 biochemical pathways involved in maintaining the survival of mouse B cells. *Bcl-2* family members are important in protecting (Vaux et al., 1988; Nunez et al., 1990; Boise et al., 1993; Hengartner et al., 1992) or accelerating (Oltvai et al., 1993; Boise et al., 1993; Yang et al., 1995; Chittenden et al., 1995) apoptosis. Consistent with this idea, IL-13 can further increase the survival of CD40L-stimulated human B cells (Lomo et al., 1997). This effect is associated with the augmentation of the expression of *Bcl-2*

homologues, *Bcl-xL* and *Mcl-1*, while the levels of *Bcl-2* and two other *Bcl-2* family members, *Bax* and *Bak*, are unaltered. Thus, *Bcl-2* family members may be preferentially regulated by IL-13 in anti-CD40 mAb stimulated-mouse B cells.

Generation of IL-13 transgenic mice which constitutively express high levels of IL-13 may provide useful and interesting insights into the roles of IL-13 during embryogenesis and development. Three IL-13 transgenic mice under the control of strong and ubiquitous hCMV regulatory elements were generated but only one high and one low copy number hCMV-IL-13 founder transmitted the transgene to their offspring. Unfortunately, no transgene-derived mRNA or protein could be detected in either of the transgenic lines. One possible explanation can be attributed to the toxicity effects of the constitutively expressed IL-13 during embryonic development, as high levels of IL-13 *in vivo* administration lead to fatality in adult mice. Similarly, the fully active form of an IL-4 transgene, under the control of the potent human Ig enhancer and mouse Ig promoter, is lethal in newborn mice (Tepper et al., 1990). Viable mice can be obtained when the potent Ig promoter/enhancer activity is reduced by inserting attenuating *Escherichia coli lac* operator sequences (Tepper et al., 1990; Burstein et al., 1991). In order to circumvent the embryonic toxicity effects, the potent hCMV-IL-13 transgene can be attenuated by *Escherichia coli lac* operator sequences, or the IL-13 transgene could be expressed under the control of a tetracycline-responsive promoter so that the transgene transcription can be abrogated upon administration of tetracycline (Furth et al., 1994).

Alternatively, the integrated hCMV-IL-13 transgenes may be gradually downregulated during embryonic development or inactivated by surrounding genomic sequences (Palmer et al., 1991; Scharfmann et al., 1991). Moreover, *de*

novo methylation inhibits hCMV-regulated transgene expression *in vivo* (Gibbs et al., 1994). Expression of the transgenes can be improved tremendously by including the natural introns in the transgenes as it appears that there are interactions between promoters and sequences in the introns that influence transcription (Brinster et al., 1988; Palmiter et al., 1991). This may explain the differences observed in the IL-4 transgenic mice. Transgenic mice generated with exogenous genomic mouse IL-4 under the control of the potent human Ig enhancer and mouse Ig promoter are non-viable (Tepper et al., 1990), whereas transgenic lines generated with IL-4 cDNA under the control of mouse Ig enhancer/promoter regions do not exhibit this lethal effect (Muller et al., 1991). Instead of using IL-13 cDNA in the transgene, genomic IL-13 DNA may be the preferred candidate to achieve efficient expression of the transgene *in vivo*.

Although none the transgenic mice in this study expressed their transgenes, the idea of generating IL-13 transgenic mice is still very attractive. With a temporally controlled transgene, IL-13 expression can be closely monitored, allowing the opportunity to study the potential toxicity effects of IL-13 during embryogenesis or in adult mice. Doing so will also provide further understanding of IL-13 as a potential therapeutic agent for hemopoietic replenishment. As *in vivo* IL-13 administration increases extramedullary hemopoiesis in the spleen, increases serum Ig levels during a strong immune response, and prolongs the survival of mouse B cells *in vitro*, it will be interesting to investigate the properties of the hemopoietic and immune systems, such as the numbers of CFUs and B cells and Ig levels in the serum of IL-13 transgenic mice, after prolonged IL-13 expression *in vivo*. Mice with the IL-4 transgene under the control of the Ig promoter/enhancer reveal significant upregulation of MHC cII surface expression on B cells and markedly elevated serum IgE and IgG1 associated with the

suppression of IgG2a, IgG2b, and IgG3 isotype repertoire (Tepper et al., 1990; Burstein et al., 1991). These phenotypes of IL-4 transgenic mice are consistent with the *in vitro* observations of IL-4 effects on B cells (Isakson et al., 1982; Howard et al., 1982; Coffman et al., 1986; Pene et al., 1988a; Pene et al., 1988b; Noelle et al., 1984; Roehm et al., 1984b; Conrad et al., 1987; Pene et al., 1988a; Shields et al., 1989). On the other hand, transgenic mice that inappropriately express IL-4 under the control of the lymphocyte-specific proximal promoter for the *lck* gene unexpectedly develop osteoporosis due to a profound decrease in osteoblast activity (Lewis et al., 1993). Unexpected findings are also observed in IL-7 transgenic mice. IL-7 plays important roles in the development of T and B cell progenitors. Surprisingly, IL-7 transgenic mice develop a progressive cutaneous disorder involving a dermal lymphoid infiltrate that eventually disrupts the skin cytoarchitecture (Rich et al., 1993). Hence, IL-13 transgenic mice may reveal unexpected but interesting findings that cannot be predicted based on *in vitro* assays. As described in Chapter VI, the short-term IL-13 administration during a CRBC immune response resulted in enhanced Ig levels, an observation that was rather unexpected based on previous *in vitro* determinations.

IL-13 appears to play important roles during various infections. IL-13 is required for the expulsion of gastrointestinal helminth *N. brasiliensis* (Urban et al., 1998) or *T. muris* infections (Bancroft et al., 1998). The mechanisms of IL-13 induced worm expulsion are still under investigation. In addition, IL-13 administration provides protection against *Listeria monocytogenes* infection (Flesch et al., 1997). IL-13 transgenic mice generated with a temporally-controlled transgene should provide an excellent model for addressing the window when IL-13 is critical for worm expulsion and determining the mechanism of action of IL-13 during various infections.

Cytokines are pleiotropic and demonstrate overlapping biological activities, mainly because of the usage of common receptor components for binding and/or signaling. Moreover, overlapping functions of cytokines can be attributed to the expression of their receptors on the same cell and common intracellular signaling pathways. These principles apply to both IL-4 and IL-13. Both cytokines are classified as Th2 cytokines, share a receptor chain (IL-4Rα), utilize a common signaling component (STAT6), and exhibit biological activities on a variety of cell types, such as monocytes/macrophages, B cells, NK cells, and hemopoietic cells. Although it seems rather puzzling to have two cytokines with similar functions, it may be an important evolutionary safeguard should either cytokine be compromised. These situations could include IL-4-deficient mice, more physiologically, expression of viral IL-10-like cytokines by EBV and equine herpes virus which may provide advantages to the virus (Baer et al., 1984; Moore et al., 1990; Hudson et al., 1985; Rode et al., 1993), or infections in which pathogens specifically neutralize a particular cytokine (McFadden, 1994). In addition, overlapping but not redundant functions of two or more cytokines may provide added advantages during immune responses such as providing synergistic biological effects at much lower concentrations or at different time points.

Despite these shared biological functions and utilization of a shared receptor component, there are major differences in IL-4 and IL-13 functions, suggesting that IL-13 may play a unique role in the immune response. IL-4 serves as a growth, differentiation, and regulatory factor for CD4 and CD8 T cells (Mosmann and Coffman, 1989; Swain et al., 1990; Le Gros et al., 1990; Mosmann and Sad, 1996a; Mosmann and Sad, 1996b). In contrast, IL-13 exhibits no detectable biological activities on mouse (J. -M. Heslan and T. R. Mosmann, unpublished) or

human T cells (Zurawski et al., 1993a; Sornasse et al., 1996). IL-13 is produced by human and mouse Th2 cells. Besides Th2 cells, human IL-13 can be detected not only in human $CD4^+$ (Th0, Th1, and Th2) but also in human $CD8^+$ T cells (McKenzie et al., 1993a; Zurawski and de Vries, 1994). In contrast, the mouse IL-13 mRNA is detected in a variety of Th2 helper cell lines, and one Th1 cell line expresses IL-13 message (Brown et al., 1989). Based on their cytokine secretion profiles, $CD8^+$ T cells can be divided into Tc1 (IL-2 and IFN-γ) or Tc2 (IL-4, IL-5, and IL-10) subsets (Croft et al., 1994; Sad et al., 1995; Coyle et al., 1995). It remains to be addressed whether either mouse or human $CD8^+$ subsets preferentially produce IL-13. Nevertheless, these data indicate that IL-13 production may not appear to be tightly regulated compared to IL-4, and both cytokines may exhibit unique roles during an immune response. This is further supported by the fact that following T cell activation, IL-13 is produced earlier and for a more extended period compared to the transient expression of IL-4 (Zurawski and de Vries, 1994; Jung et al., 1996). As IL-13 is produced early but at sustained levels for a longer period of time and IL-13 *in vivo* treatment induces striking monocytosis, it is conceivable that IL-13 may induce the formation of macrophages that are efficient in presenting antigens to T cells. This is consistent with the observations that IL-13-derived bone marrow macrophages are effective in presenting antigens to Th1 or Th2 cells (J. -M. Heslan, L. J. Guilbert, and T. R. Mosmann, unpublished).

IL-4 and IL-13 induce IgG4 and IgE antibody class switching by human B cells (Punnonen et al., 1993; McKenzie et al., 1993a; Minty et al., 1993; Punnonen and de Vries, 1994; Gauchat et al., 1993; Punnonen et al., 1995). Although IL-4 plays an important role in IgE class switching in mouse B cells (Vitetta et al., 1985; Coffman et al., 1986; Snapper and Paul, 1987; Lutzker et al., 1988; Kuhn et al.,

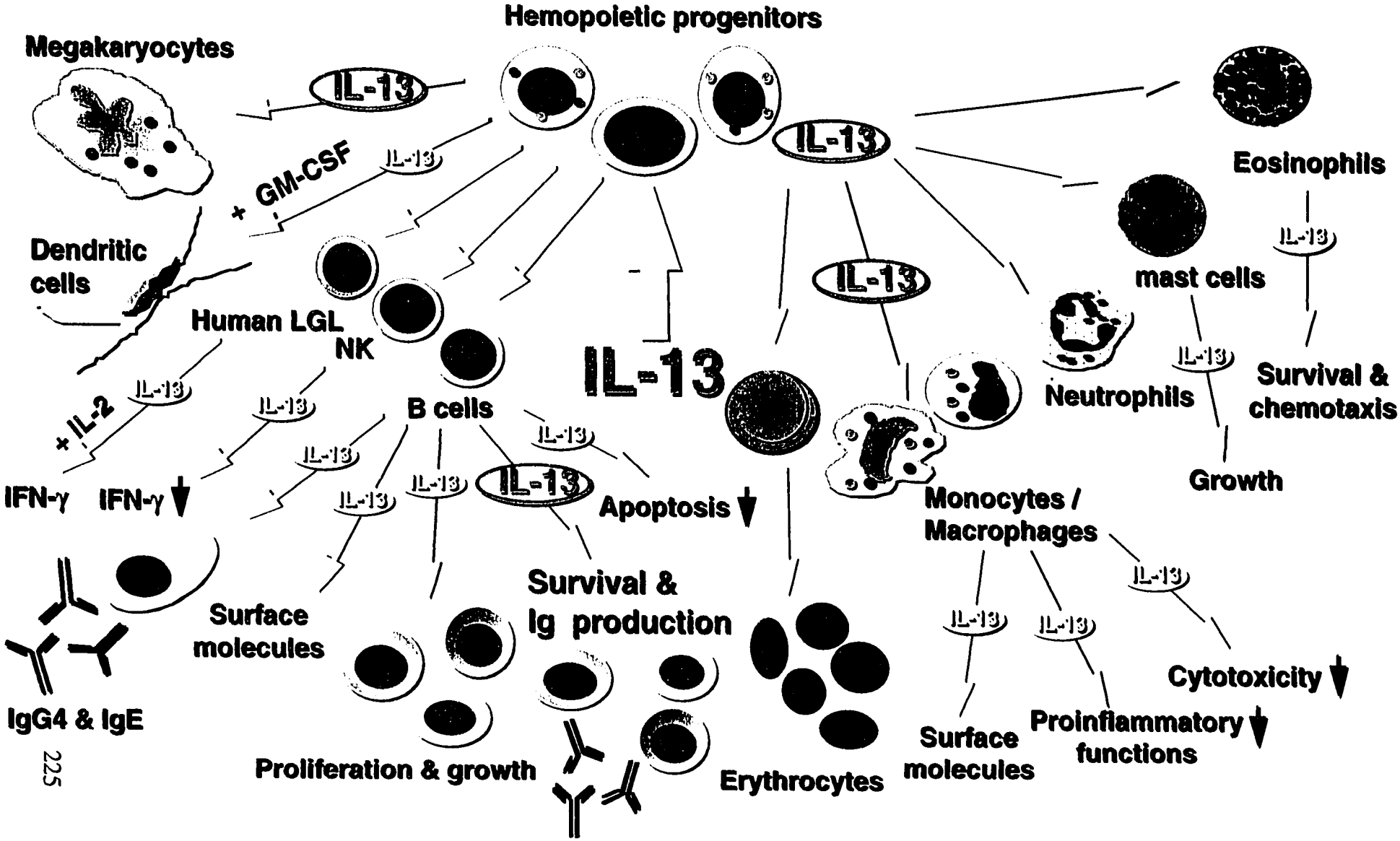
1991), IL-13 does not exhibit detectable antibody class switching effects on mouse B cells. It might appear initially that IL-13 functions on mouse and human B cells are not conserved and IL-13 possesses unique functions on human B cells and immune responses. However, the data presented in this study indicate that IL-13 prolongs the survival of mouse B cells, a phenomenon consistent with the survival effect on human B cells (Lomo et al., 1997; Billard et al., 1997; Fluckiger et al., 1994). Thus, IL-13 may be involved in maintaining the survival of activated B cells that have undergone class switching. In order to extend the *in vitro* finding that IL-13 sustains mouse B cell survival, antigen specific B cells can be tagged and introduced into IL-13 transgenic mice or mice which are treated with IL-13. The percentages of survived tagged B cells can be monitored over a period of time in the presence or absence of antigens.

Based on microculture experiments of 90 to 100 B cells (> 99.5% pure) per well, the majority of the wells should contain only B cells. IL-13 enhanced antibody levels in all replicate wells, proving that it acted directly on the B cells. This should be considered as definitive proof that mouse B cells respond to IL-13. To further support the argument that IL-13 stimulates mouse B cells directly, binding studies of IL-13 on mouse B cells appear to be an attractive proposition. This experiment has not been performed as part of this study for the following reasons. Binding studies require large numbers of sorted cells, and even at > 99.5% pure, a contaminating cell population with high receptor expression may contribute small numbers of binding sites that would be indistinguishable from very low numbers of binding sites on B cells. In addition, the understanding of IL-13R complexes are incomplete as the IL-13R complexes may involve a combination of different receptor chains, and at least four different IL-13R complexes have been proposed

(Obiri et al., 1997). The structures and expression of IL-13R complexes, especially on mouse B cells, warrant intense investigations.

Given the interesting and pleiotropic functions of IL-13, the importance of IL-13 *in vivo* functions are beginning to be appreciated. Recent studies emphasize the importance of IL-13 in rejecting the gastrointestinal nematode parasites *N. brasiliensis* (Urban et al., 1998) or *T. muris* (Bancroft et al., 1998). IL-4 is generally considered as the driving force behind a strong Th2 response, and both infections generally require mounting of Th2 immune responses for successful worm expulsion. IL-13 may be as important as, if not more important than, IL-4 in providing the necessary protection against these parasites. The exact mechanism of IL-13 involvement in parasite expulsion is unclear. Thus, *in vivo* functions of IL-13 warrant further investigations which may unravel additional *in vivo* findings that may not be predicted from the *in vitro* determinations.

Figure 8.1. *In vivo* and *in vitro* functions of IL-13.



CHAPTER IX

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IMAGE EVALUATION
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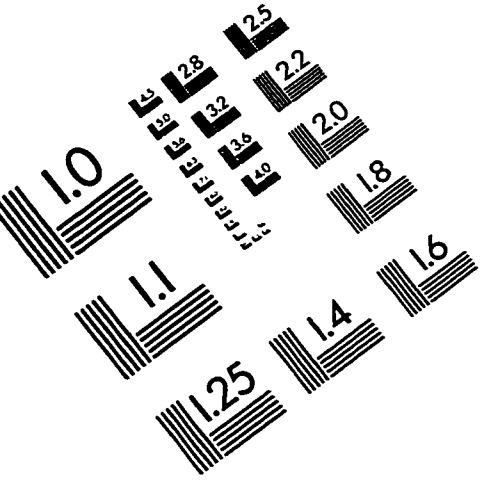

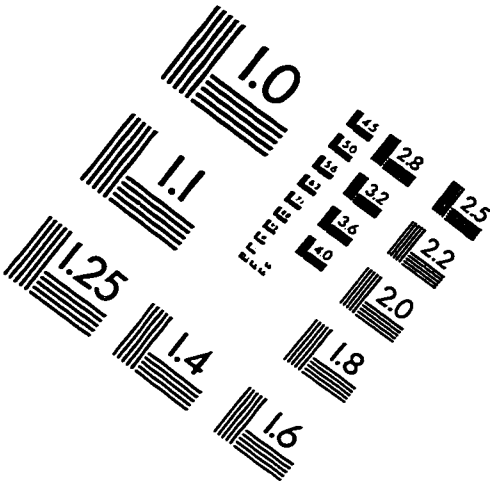

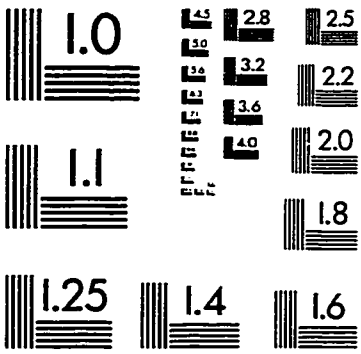

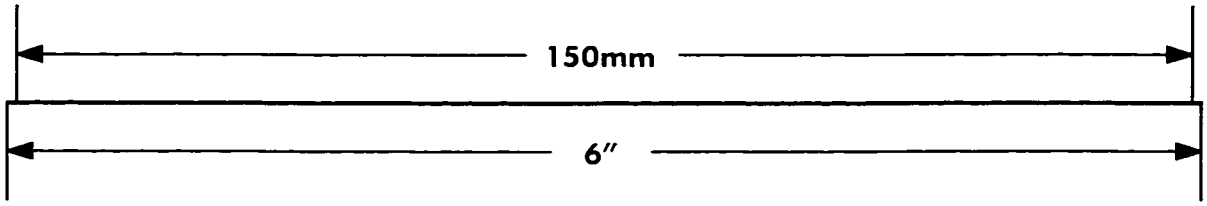

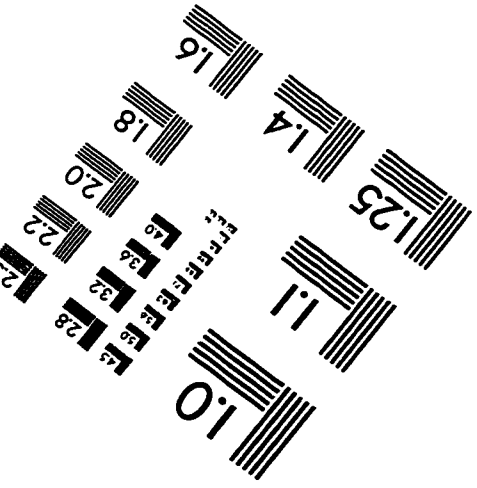

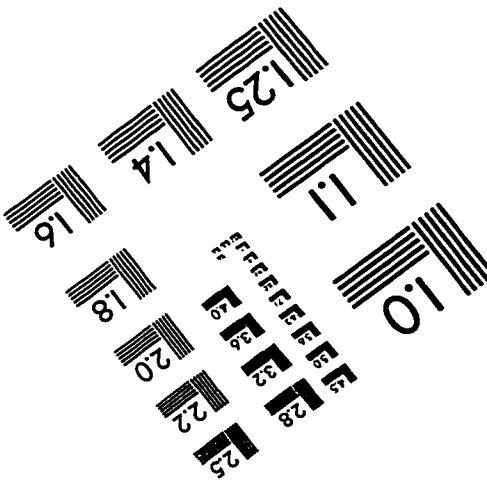

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