INTERLEUKIN-12 AND INTERLEUKIN-10 REGULATION OF ANTIBODY RESPONSES UPON PROTEIN ANTIGEN IMMUNIZATION

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A Thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of

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INTERLEUKIN-12 AND INTERLEUKIN-10 REGULATION OF ANTIBODY RESPONSES UPON PROTEIN ANTIGEN IMMUNIZATION

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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To the memories of

my father, who taught me how to work

and Norm, who showed me the strength of gentleness

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Special words of thanks go to my family and friends that allowed my dream for myself to become their dream for me.

Deo gratias pro hic incepto

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Abbreviations

Ab	- Antibody
Ag	- Antigen
AHR	- Airway hyper-responsiveness
APC	- Antigen presenting cell
BAL	- Bronchial airway lavage
B. abortus	- *Brucella abortus*
CD40 L	- CD40 ligand
CFA	- Complete Freund's adjuvant
CMI	- Cell mediated immunity
Con A	- Concanavalin A
CTL	- Cytolytic T lymphocytes
DTH	- Delayed type hypersensitivity
ELISA	- Enzyme linked immunosorbent assay
FcεR1	- Fc epsilon receptor 1, high affinity IgE receptor
FCS	- Fetal calf serum
FITC	- Fluorescein isothiocyanate
GM-CSF	- Granulocyte/macrophage-colony stimulating factor
IFN	- Interferon
Ig	- Immunoglobulin
IL	- Interleukin
i.p	- Intraperitoneal

KLH - Keyhole limpet hemocyanin

KO - Knockout

LDA - Limiting dilution analysis

LPS - Lipopolysaccharide

L. major - *Leishmania major*

L. monocytogenes - *Listeria monocytogenes*

mAb - monoclonal Ab

MHC - Major histocompatibility complex

M_r - Relative molecular mass

M. tuberculosis - *Mycobacterium tuberculosis*

N. brasiliensis - *Nippostrongylus brasiliensis*

NK - Natural killer

OA-POL - Gluteraldehyde-polymerized OVA

OVA - Ovalbumin

$(p40)_2$ - p40 homodimer

PBMC - Peripheral blood mononuclear cells

PCA - Passive cutaneous anaphylaxis

PGE_2 - Prostaglandin E_2

rIL - Recombinant interleukin

R - Receptor

SEM - Standard error of the mean

S. mansoni - *Schistosoma mansoni*

TCR - T cell receptor

Th - T helper cell

TGF - Transforming growth factor

T. gondii - *Toxoplasma gondii*

TNF - Tumor necrosis factor

T. spiralis - *Trichinella spiralis*

UPF - UltraDOMA PF (protein free)

WT - Wild type

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I. Abstract

Allergic diseases have been increasing both in incidence and severity over the past decades. Allergy is associated with type 2 immunity, typified by antigen (Ag)-specific IgE production and Th2-like cytokine synthesis. IL-4 and IL-13 promote the production of IgE, while IFNγ inhibits it. Recently, there has been much interest in the potential for IL-12 to inhibit the development or maintenance of type 2 responses in vivo. The potential value of such a strategy is suggested by (1) in vitro studies, indicating the capacity of IL-12 to inhibit IL-4 induced IgE synthesis, and (2) short-term studies in vivo demonstrating that in vivo administration of exogenous IL-12, simultaneous with immunization, can virtually prevent primary IgE responses. Here, we examine the role of IL-12 in the regulation of IgE synthesis.

We first investigated the adjuvant potential of IL-12 to induce and maintain long-term suppression of IgE synthesis upon ovalbumin (OVA) (alum) immunization (a murine model of immediate hypersensitivity). We found that IL-12 administration, simultaneous with immunization, resulted in profound inhibition of primary Ag-specific IgE, and strongly elevated IgG_{2a}, levels. However, upon secondary and tertiary immunizations, IgE synthesis was found not to differ between IL-12 treated and untreated mice. Detailed examination of short-term culture and limiting dilution analysis (LDA) following IL-12 administration revealed that enhanced IFNγ synthesis could not be attributed to an increase in IL-12 induced CD4 T cell activity. Instead, anti-NK1.1 treatment argued that this early IFNγ synthesis was affiliated with IL-12 mediated NK cell responses. This was supported by the finding that anti-

NK1.1 treatment reversed the ability of IL-12 to inhibit primary IgE levels. Taken together, the data suggested that IL-12 induced NK cell activity severely limited primary IgE synthesis, possibly through increased IFNγ production. However, this did not result in enhanced type 1 adaptive immunity, given that there was no detectable alteration in CD4 T cell cytokine profiles and no inhibition of IgE synthesis upon subsequent re-exposure to antigen.

Concurrent with these studies, we investigated the capacity of rIL-12 treatment to inhibit established IgE responses in vivo. Notwithstanding the use of a broad range of protocols, and despite the fact that Ag-specific IgG_{2a} and IFNγ levels were greatly elevated in IL-12 treated mice, exogenous IL-12 administration was consistently unsuccessful in abrogating ongoing IgE production. Direct analysis of CD4 T cell cytokine production indicated that IL-12 administration over extended time periods (up to 25 times over 61 days) decreased the frequency of CD4 T cells producing IL-4. In contrast, spleen cell culture yielded markedly elevated IL-4 synthesis following IL-12 treatment. This could be attributed to the capacity of IL-12 to (1) increase the number of non-B/non-T cells in the spleen and (2) enhance their ability to produce IL-4 in response to Ag mediated stimulation. Preliminary evidence suggests that these non-B/non-T cells are basophilic in nature and release IL-4 in response to Ag-cross linking of bound IgE.

In the third major component of the study, we examined the role of *endogenous* IL-12 production in regulation of Ab responses. Specifically, we hypothesized that endogenous IL-12 plays an essential role in shaping the type 1/type 2 balance evoked upon initial

immunization with exogenous Ag. We tested this hypothesis by examining cytokine and Ab production using conditions which routinely elicit type 2 dominated (OVA, alum) or type 1 dominated (OVA in complete Freund's adjuvant [CFA] or polymerized OVA [OA-POL] in the absence of adjuvant) responses. The experimental approaches we used were the acute neutralization of endogenous IL-12 using goat anti-IL-12 Ab and immunization of p40 and p35 KO (knockout) mice. We found that pretreatment of immunized mice with OA-POL and anti-IL-12 substantially, but incompletely, impaired the production of Ab responses normally seen, resulting in a 6 fold enhancement of Ag-specific IgE levels and reduced IgG_{2a} production. However, examination of cytokine and Ab responses in the second approach, immunization of p40 and p35 KO mice, did not result in elevated IgE synthesis or type 2 cytokine responses, independent of whether mice were immunized with OVA (alum), OVA (CFA) or *Trichinella spiralis* extract (alum). In contrast, IgG_{2a} and IFNγ levels were consistently impaired in IL-12 KO mice. These data indicate that endogenous IL-12 does not negatively regulate type 2 Ab and cytokine synthesis, but strongly promotes type 1 responses.

Given the well recognized role played by IL-10 in regulation of IL-12 synthesis, the fourth component of this thesis examined the importance of endogenous IL-10 in the initiation of type 2 responses. To do so, OVA (alum) immunized mice were treated with anti-IL-10. As a complementary approach, IL-10 KO mice were immunized. We found that both protocols resulted in depressed IgE production. IL-10 KO mice exhibited increased spleen cell IFNγ and p40 production, as well as lower IL-5 synthesis compared to wild type (WT) controls. Thus, it appears that in the absence of endogenous IL-10, type 1 cytokine levels continue to

increase following immunization, impairing IL-5 and IgE synthesis.

CD4 T cells are critical regulators of IgE responses. It also had been reported that CD8 T cells were important in the regulation of IgE levels i.e. naive CD8 T cells promoted the initiation of IgE production and activated/memory CD8 T cells inhibited established type 2 humoral responses. We used CD8 KO mice to determine if the absence of CD8 T cells affected the induction of IgE responses, or, alternatively, the capacity of a strong type 1 stimulus (OA-POL) to inhibit well-established ongoing IgE responses. Our data indicated there was some type 1 skewing of cytokine and responses in CD8 KO mice in a type 1 direction (slightly increased IFNγ synthesis; slightly increased total IgE production). Upon OA-POL treatment, CD8 KO mice exhibited increased IFNγ:IL-4 ratio, slightly increased IgG_1 and IgG_{2a} production and a minor decrease in IL-5 compared to OA-POL treated WT mice. However, while these changes are statistically significant, in most cases their lack of intensity call into question their biological relevance.

In summary, the collective data on the effect of exogenous IL-12 administration and the role of endogenous IL-12 regulation, indicate that IL-12 is an important regulator of type 1, but not type 2, Ab responses. We also found that exogenous IL-12 increased non-B/non-T cell production of IL-4 in established IgE responses, possibly contributing to stable IgE production following IL-12 administration. In addition, endogenous IL-10 appeared to influence IgE synthesis indirectly by suppressing IgE inhibitors (type 1 cytokines), and/or by promoting type 2 cytokine production i.e. IL-5. Finally, our studies suggested that CD8 T

cells do not play an essential role in the induction of IgE responses.

II. Literature review

1.0 Introduction

1.1 Demographics

This thesis examines cytokine mediated regulation of murine IgE responses. Allergy is an IgE driven hyperimmune response to a foreign Ag or allergen. The terms Ag and allergen are used interchangeably in this thesis. Allergic diseases range in degree from being a seasonal nuisance (as in hay fever) to a life threatening condition, such as bee venom sensitivity or asthma.

The incidence and severity of allergic diseases has escalated within the last couple of decades. Presently, allergic disease is the most prevalent human immunologic disorder in developed nations, affecting ~20-30% of the population. There is evidence emerging that suggests this is a consequence of pollution (as atopy is more problematic in urban centers) (Diaz-Sanchez, 1997), in addition to changes in nutrition, the intrauterine environment and genetics (reviewed in Kay, 1997). Furthermore, with an increase in the exposure to synthetic materials, the range of potential allergens has expanded considerably; evident by the recent appearance of latex allergy. However, at present the reasons underlying the marked increase in atopic disease have not been convincingly identified.

The large population of atopic individuals and the limited success attained with classical

allergen immunotherapy programs have stimulated discussion of prophylactic immunotherapy and therapeutic management of allergy and IgE responses (Barnes, 1996; Creticos, 1996; HayGlass, 1998).

Allergy comprises a collection of complex diseases in which varying degrees of cellular and humoral immunity contribute to pathology (reviewed in Barniuk, 1997). However, IgE production and excessively intense type 2 patterns of cytokine synthesis are pivotal in the pathogenesis and maintenance of most allergic diseases (Romagnani, 1994; Umetsu, 1997).

2.0 Type 1/ type 2 cytokine regulation

2.1 T cell subsets and activation

αβT cells can be divided into CD4, CD8 or natural T cells. CD4 T cells are primarily considered helper T cells. Their cognate interaction is crucial for the positive or negative regulation of B cell proliferation, isotypic class switch and CD8 T cell cytotoxicity and a range of other regulatory activities. Ag-specific stimulation of CD4 T cells requires the interaction with peptide (from exogenous Ag), usually in the context of MHC class II on Ag presenting cells (APC). Conversely, CD8 T cells are frequently cytotoxic, interacting with peptide (from endogenous Ag) in the context of MHC class I. Over the last 5-7 years, these long-held concepts of CD4 and CD8 T cell functions have been challenged. CD4 T cells can act as effector cells in delayed type hypersensitivity (DTH) reactions and display cytotoxicity;

whereas CD8 T cells exhibit cytokine regulation and can respond to exogenous Ag. It is thought that CD8 T cells can respond to exogenous Ag by leakage of small quantities of Ag (ingested in vesicles) into the cytosol of a cell where they are introduced into the MHC class I pathway for presentation to CD8 T cells. This recently was supported by the observation that OVA-specific CD8 T cells were generated after OVA (alum) immunization (MacAry, 1997). Natural T cells, while often cytotoxic, are activated by a non-classical MHC class 1 molecule: CD1. They tend to be CD4+ or double negative and carry natural killer cell markers, such as NK1.1. They may play a critical role in linking innate and adaptive immune responses upon bacterial infection (as suggested by their reactivity to CD1) or predisposing immunity towards type 2 cytokine and IgE production under certain circumstances (Yoshimoto, 1995).

γδ T cells are similar to natural T cells in that they are thought to play a critical role in innate immunity, interact with CD1 and may display NK cell markers. γδ T cells are considered a first line defense against bacterial infections and have a broad affinity for a number of bacterial Ag including phosphorylated nonpeptidic ligands (Libero, 1997). In addition, evidence suggests that CD4+ gd T cells may also induce the initial (weaker) atopic response, resulting in IL-4 production, B cell help and IgE synthesis (reviewed in Spinozzi, 1998). However, like natural T cells, there are still questions as to how γδ T cells recognize Ag (Libero, 1997).

Ag-specific ab T cell activation requires the ligation of TCR/CD3 with peptide/MHC on APC. In addition, CD80 (B7.1) and CD86 (B7.2) (on APC) interaction with CD28 (on T cells) is

important in promoting T cell proliferation, IL-2 gene transcription, cytotoxic T lymphocyte (CTL) generation and perhaps commitment to type 1 verses type 2 cytokine production. TCR ligation in the absence of CD28 stimulation results in anergy of the T cell. CD80 and CD86 can be expressed on APC in response to different signals. CD40 (on APC) and CD40 ligand (L) (on T cells), as well as adhesion molecule interactions are also important in T cell activation and in T cell help.

2.2 Th1/Th2 paradigm

A reciprocal relationship between cell mediated and humoral immunity has been recognized for more than 25 years (Bretscher, 1981; Parish, 1972).In 1986, Mosmann and colleagues reported discrete patterns of cytokine production from murine CD4 T cell clones. These polarized CD4 T cell clonal populations were defined as Th1 and Th2 on the basis of their cytokine synthesis. Th1 clones produce IL-2, IFNγ and lymphotoxin, but not IL-4, IL-5, IL-6, IL-10 and IL-13. The latter are synthesised by Th2 cells in the absence of IL-2, IFNγ and lymphotoxin. Both subsets have IL-3, GM-CSF, and TNF production in common (Coffman, 1991; Mosmann, 1986). Furthermore, Th1 clones are associated with the capacity to mediate DTH responses and activate macrophages (Cher, 1987); while Th2 cells enhance B cell activity, including the induction of IgE and IgG_1 (Coffman, 1988).

Naive T cells, upon in vitro stimulation, produce predominantly IL-2, and little IL-4 or IFNγ. The cytokine environment at the time of initial priming (in vivo or in vitro) greatly influences the cytokine production when cells are subsequently stimulated (reviewed in Muraille, 1998;

Murphy, 1998; Trinchieri, 1995; Trinchieri, 1998). Numerous in vitro studies have shown that IL-4 (produced by mast cells, basophils, T cells and natural T cells) is critical in driving Th2 clonal development of upon priming of CD4 T cell populations. Conversely, IL-12, a product of APC, strongly promotes the growth of Th1 clones. When IL-12 and IL-4 are added together during in vitro priming of transgenic T cells, the effects of IL-4 dominate such that the resulting clones produce substantial IL-4 and less IFNγ. In addition, the elimination of endogenous IL-4 in culture (in the absence of exogenous IL-12) encourages the production of IFNγ by T cell clones.

IFNγ production by T cells is a hallmark of Th1-like activity (Paludan, 1998). Furthermore, the addition of IFNγ to T cell cultures facilitates Th1 cytokine production. Unlike IL-12, IFNγ does not appear to directly promote Th1 clonal responses. Instead, IFNγ acts on T cells to counter the effects of Th2 cytokines, including Th2 clonal expansion, IL-4 induced IgE production and B cell differentiation (Fitch, 1993). In a complimentary manner to IFNγ, IL-10 supports Th2 clonal expansion by suppressing Th1 cytokine synthesis. This permits the enhanced production of endogenous IL-4 which drives Th2 clonal development.

It has been more difficult to generate polarized human CD4 T cell Th1 and Th2 clones than murine clones. However, human Th1 and Th2 clones have been derived from individuals with strongly skewed responses resulting from chronic immune stimulation. Thus, Th1 clones have been obtained from individuals with tuberculoid leprosy upon repeated stimulation with mycobacteria (Haanen, 1991). Th2 clones, such as those specific for dust mite allergen Der

p 1 (producing IL-4 and IL-5, with very low levels of IFNγ and IL-2), have been obtained from atopic individuals (Kapsenberg, 1991; Yssel, 1992). Generally, the addition of exogenous IL-4 to CD4 T cells human cultures at priming induced stronger IL-4 producing cells, while IFNγ and IL-12 resulted in mainly IFNγ producing cells. However, studies have indicated that depending on priming conditions (i.e. the strength of B7 stimulation) the addition of IL-12 to naive neonatal CD4 T cells enhanced IL-4, as well as IFNγ, production. Taken together, the data from these and related systems indicate that differences in the induction of effector cytokine production is dependent on more than just the initial cytokine environment and that at different stages of development (i.e. neonatal) CD4 T cells may be more sensitive to other factors (Delespesse, 1997; Shu, 1994). Some of these factors (dose, route of administration, etc.) are discussed below. Human clones appear to be more plastic than murine clones, displaying more diverse cytokine profiles than initially attributed to murine Th1 and Th2 clones (reviewed in Muraille, 1998; Romagnani, 1994). In contrast to mice, human Th1 and Th2 clones are reported to produce IL-6 and IL-10. In addition, exogenous IL-10 inhibited both Th1 and Th2 cytokine production and proliferation.

2.3 Beyond the paradigm

The Th1/Th2 paradigm provides a needed framework for understanding the development of cytokine production by CD4 T cells. However, the production of Th1 and Th2 associated cytokines is not limited to CD4 T cells. CD8 and natural T cells also secrete these cytokines, as do other cells of different lineages (Carter, 1996).

Furthermore, while CD4 T cell clones have allowed for specific insightful studies, the difficulty with this system is that it is artificial. Extended in vitro manipulation provides for a homogeneous system, with a very high frequency of antigenic responders that exhibit limited variation. While experimentally useful, this differs from in vivo situations where T cell networks exist as a diverse interactive group of cells which exert positive and negative influences on each other. As such, responder T cells of a given Ag specificity are usually present in vivo at a very low frequency. In addition, long-term in vitro stimulation generally modifies the nature of T cells, such that clones can have different (i.e. lower) activation requirements than their parent cells in vivo.

Because of the formal association of Th1 clones with cell mediated immunity (CMI) and Th2 clones with humoral responses, it was initially thought that Th1 and Th2 CD4 T cell populations existed in vivo. T cells stimulated immediately ex vivo, prior to long term culture with antigen, generally produced a mixed cytokine profile (termed Th0). Th0 cells were considered precursors for Th1 and Th2 cell populations (Kelso, 1988). Recently, an alternative understanding of the Th1/Th2 paradigm had been voiced (Kelso, 1995). Kelso *et al.* have shown that ex vivo, prior to extensive culture manipulation, the majority of individual CD4 T cells produced different combinations of Th1 and Th2-like cytokines. A minority of individual CD4 T cells produced strictly Th1 or Th2 cytokine patterns, but T cell populations producing restricted Th1 or Th2-like cytokines patterns were very rare. This suggests that the Th0 cells observed ex vivo are not precursors for distinct Th1 and Th2 populations in vivo, but are representative of diverse physiologic T cell populations.

Type 1 and type 2 dominated responses, however, are visible in vivo especially in diseases where there is a strong polarization of CMI or humoral responses. The terminology type 1 and type 2 is preferred to Th1 and Th2. Type 1 responses refer to a *predominance* of CMI, resulting from primarily Th1-associated cytokine and Ab (such as IgG_{2a} in mice) production. Type 2 responses refer to *stronger* Th2-associated cytokine and Ab profiles (particularly IgE synthesis) characteristic of humoral responses. It should be noted that while most CMI responses are associated with type 1 immunity, cell mediated responses involving macrophage, T cell, mast cell, basophil and eosinophil activity can be seen in type 2 associated diseases, such as allergy (discussed below).

2.4 Factors which influence the generation of type 1 verses type 2 responses

There are a number of variables in addition to the cytokine environment that can act in vitro (on clonal and heterogenous cell populations) and in vivo to skew the immune system in a type 1 or type 2 direction. Adjuvants have long been known to induce particular types of immune responses. Alum, for example, preferentially induces type 2 humoral responses such as IgE; whereas CFA or heat killed *Brucella abortus* given with Ag will induce type 1 immunity (Scott, 1997). In addition, Ag dose is also relevant, as low doses of Ag induce strong IgE responses. Low IgE levels are seen when mice are immunized with a high dose of Ag (100 μg of KLH, alum) (Arps, 1998; Levine, 1970). Similarly, it was reported that in vitro immunization of CD4 T cells with low and high doses of Ag resulted in Th2-like cytokine profiles, while "intermediate" doses produced more Th1-like profiles (Constant,

1997; Hosken, 1995). The route of administration of an Ag can also alter the type of immunity generated. Epicutaneous administration of Ag has been seen to induce IgE synthesis; whereas, i.p. injection of aqueous Ag generally had not (Wang, 1996). In addition, the nature of the Ag is important. Differences in cytokine synthesis by PBMC from atopic and normal individuals have been seen in response to polyclonal (anti-CD3 and phytohemagglutinin) and antigenic stimuli in vitro (Imada, 1995). Recently, Ag processing by APC and resulting cytokine production have been shown to be influenced by Ag size. Large carbohydrate complexes ingested by macrophages (through mannose receptor-mediated phagocytosis) stimulated stronger IFNγ and IL-12 production compared to macrophage processing of smaller structures (Shibata, 1997). Further, reports have suggested that differences in the expression of CD80 and CD86 on APC at the time of activation can influence T cell development. CD80 expression had been associated with type 1 immunity. Conversely, CD86 expression may be required for the induction of type 2 responses (Tsuyuki, 1997). Finally, it has been observed that macrophages and dendritic cells more easily activate type 1 immunity; whereas B cell presentation preferentially generate type 2 immunity (Chuang, 1996; Gajewski, 1991). Dendritic cells, the most efficient and probably the most biologically relevant APC for activation of naive CD4 T cells by exogenous Ag, are potent producers of IL-12 (Hilkens, 1997; Ohshima, 1997).

For the purposes of this thesis, the majority of discussions will be limited to cytokine regulation of type 1 and type 2 responses.

3.0 IL-12

3.1 IL-12 and IL-12 receptor

IL-12 (p70) is produced by APC in response to bacteria Ag or by T cell stimulation. IL-12 is a heterodimeric compound consisting of two disulfide linked chains termed p40 and p35. p35 is an 197 amino acid peptide with 7 cysteine residues (Gubler, 1991) possessing some homology with IL-6 and G-CSF α-helix rich structures (Merberg, 1992). While p40 displays little homology to other cytokines, it does share features with the hematopoietin receptor family which includes the IL-6 receptor (R). It is 306 amino acids long with 10 cysteine residues (Gubler, 1991). IL-12 can be secreted as a p35:p40 heterodimer, single uncomplexed p40 entities, or p40 homodimer ($(p40)_2$) (D'Andrea, 1992; Podlaski, 1992). Since p40 production has been correlated with p70 synthesis and it is produced in excess of p70, p40 detection is often used as a surrogate for p70 production. However, discordant regulation of p40 and p70 has been seen upon stimulation with IFNa (Hermann, 1998), meaning that a linear relationship between p40 and p70 can not be assumed and care must be taken in using p40 production (which is readily quantified) as a surrogate for the more difficult to measure p70.

$(p40)_2$ binds to the IL-12R. The normal physiologic consequences of this binding have not been determined. In vitro studies utilizing synthetic $(p40)_2$ argue that the homodimer (but not free p40) acts as an IL-12 antagonist by directly competing for the receptor (Gillessen, 1995). This was supported by in vivo studies which showed decreased NK cytotoxicity, DTH

responses and IFNγ:IL-4 ratios upon immunization with KLH (CFA) in p40 transgenic mice (Yoshimoto, 1998). In addition, treatment with p40 homodimer reduced IL-12 dependent IFNγ production in response to LPS (Heinzel, 1997). In contrast, $(p40)_2$ may participate in the generation of type 1 immunity under certain conditions, as p35 KO mice exhibited more resistance than p40 KO mice to *Listeria monocytogenes* and *Cryptococcus neoformans* infection (reviewed in Gately, 1998). With the recent availability of p40 and p35 KO mice, continued studies will broaden our understanding of endogenous IL-12 regulation, and of $(p40)_2$ function.

The IL-12R has been detected on NK cells and activated T cells. It consists of two heterologous subunits IL-12Rβ1 (Chua, 1995) and IL-12Rβ2, the more tightly regulated component (Presky, 1996). These molecules are highly homologous between human and mouse; conferring a combination of low and high affinity receptors. In humans, both β1 and β2 bind IL-12, while β2 has the capacity for signal transduction (Zou, 1997). In murine systems β2 is involved in signal transduction, but only β1 has binding capacity (Chua, 1995; Presky, 1996). Independent expression of human β1 and β2 results in low affinity binding sites in transfected cells, while the high affinity receptor (in human and mouse) requires co-expression of β1 and β2. Human and mouse Th1 clones express β1 and β2 and exhibit IL-12 responsiveness. Conversely, Th2 cells express only β1, rendering them unresponsive to IL-12 (Rogge, 1997; Szabo, 1995).

IL-12 can be produced by APC in either a T cell independent manner following stimulation

of APC with bacterial Ag, or in a T cell dependent manner involving CD40:CD40L interactions (DeKruyff, 1997). However, it has been suggested that naive T cells are poorer inducers of IL-12 production by dendritic cells compared to bacterial Ag (Hilkens, 1997). Prostaglandin E_2 (PGE_2), IL-4, IL-10 and TGFb all inhibit monocyte/dendritic cell production of IL-12 (Kalinski, 1997). PGE_2 suppression of APC IL-12 production is associated with impaired IFNγ and IL-2 synthesis, along with increased IL-4, IL-10, IL-13 and IL-5 production by T cells (Demeure, 1997; Kalinski, 1997). Further, there is evidence that CD40:CD40L interaction, in the absence of MHC/TCR stimulation, can result in IL-12 production by APC, which is able to stimulate T cell IFNγ synthesis (Armant, 1996). This has consequences in inflammatory diseases where chronic stimulation could activate bystander T cells, augmenting the disease process.

3.2 IL-12 functions

IL-12 is a strong stimulator of type 1 responses in vitro and in vivo. This is explicitly seen in the ability of IL-12 to promote IFNγ production by T cells and NK cells, as well as to augment CTL and NK cell growth and cytotoxicity (Muraille, 1998; Trinchieri, 1998). In vivo, exogenous IL-12 administration to naive mice or even to mice injected with a strong type 2 stimulus (such as goat-anti-mouse IgD or Ag in alum) decreases IL-4 and increases IFNγ production (McKnight, 1994; Morris, 1994). Collectively these studies demonstrated that IL-12 can act as a potent promoter of type 1 immunity and inhibitor of type 2 responses in vivo.

3.3 IL-12 regulation in parasitic disease

The ability of rIL-12, when added to in vitro cultures, to shape developing cytokine and effector responses stimulated enthusiasm for the potential of exogenous IL-12 administration to act as a biological adjuvant for prophylactic or therapeutic induction of type 1 responses (Hall, 1995; Scott, 1993). The potential pharmacologic utility of IL-12 has been examined in many systems. IL-12 can enhance CMI in many parasitic models (reviewed in Romani, 1997). However, this section (for the sake of brevity) will concentrate on IL-12 regulation of murine immunity against *Leishmania major* as a model Ag since (1) *L. major* infection in mice was one of the first models in which IL-12 was studied, (2) the dichotomy of human and mouse immune responses against intracellular parasites, often exhibited as a predominance of type 1 (and cell mediated) immunity or type 2 (and humoral) immunity, is clearly seen in the genetic distinctions of murine responses to *L. major*, and (3) it is an excellent example of the potential effects of exogenous IL-12 administration and endogenous IL-12 regulation. The effect of rIL-12 treatment on Ab responses upon immunization with protein Ag is discussed below.

C57Bl/6 and C3H mice are able to resolve *L. major* infection by generating healing type 1 dominated responses, as illustrated by enhanced IFNγ and undetectable IL-4 production in lymphoid organs. C57Bl/6 mice are slightly more susceptible to *L. major* than C3H mice possibly due to differences in endogenous IL-12 synthesis in response to infection. Within a day of infection C3H mice produced high and stable levels of p40. p40 production was not visible within the first week of infection in C57Bl/6 mice. Neutralization of endogenous IL-

12 (with anti-IL-12) resulted in increased foot pad swelling and parasite burden, and eliminated the capacity of resistant mice to contain infection. These findings were supported by the incapacity of p40 or p35 KO mice, on resistant backgrounds, to ward off infection associated with an absence of DTH responses and increased Th2 cytokine expression from lymphatic CD4 T cells (Mattner, 1996). The absence of endogenous IL-12 is accompanied by diminished IFNγ production, important in microbicidal macrophage activity, and in preventing the elevation of detrimental IL-4 synthesis (Heinzel, 1995; Scharton-Kersten, 1995). In particular, anti-IL-12 treatment inhibited NK cell cytotoxicity and IFNγ synthesis in infected C3H mice indicating the importance of early endogenous IL-12 production in inducing NK cell IFNγ production, critical to protective immunity. IL-12 induced NK cell activity was also reported to be a vital component in resistance to *L. monocytogenes, Toxoplasma gondii* (Hunter, 1997) and *Schistosoma mansoni* (Mountford, 1996) infections.

Conversely, infected BALB/c mice develop type 2 responses (low IFNγ and elevated IL-4 synthesis) usually leading to fatal disease. Treatment of BALB/c mice with rIL-12, simultaneous with inoculation, resulted in healing type 1 responses. This was associated with decreased or undetectable IL-4 and heightened IFNγ synthesis from IL-12 treated/infected mice compared to untreated/infected mice. Moreover, reduced foot pad swelling, parasite burden and mortality in IL-12 treated BALB/c mice indicated an improvement in clinical disease following IL-12 treatment (Afonso, 1994; Heinzel, 1993). Furthermore, exogenous IL-12 was able to act as an adjuvant in this system, in that treatment with rIL-12 at initial

infection prevented disease upon secondary inoculation in the absence of further IL-12 treatment (Heinzel, 1993). Unexpectedly, BALB/c mice exhibited p40 synthesis equivalent to C3H mice 24 h after infection; however, this quickly dropped with a corresponding increase in IL-4 production, followed by unresponsiveness of lymph node CD4 T cells to IL-12 (Launois, 1997). Treatment with anti-IL-4 resulted in resistance and maintained IL-12 responsiveness by T cells, indicating the critical role of endogenous IL-4 production in suppressing potentially protective type 1 immunity against *L. major* in BALB/c mice. Endogenous TGFβ and IL-10 production was also implicated in inhibiting early IFNγ production in BALB/c mice. In addition, TGFβ has been shown to inhibit NK cell activity important in generating protective immunity against *T. gondii* (Hunter, 1995). Taken together these studies suggest that in response to *L. major*, BALB/c mice initiate type 1 immunity, which is suppressed by rising type 2 associated cytokine production.

Similar observations have been made in other infectious diseases where type 1 immunity is protective including *Mycobacterium tuberculosis* (Cooper, 1995) and *S. mansoni* (Wynn, 1996) and *L. monocytogenes* (Tripp, 1994) infections. In these studies enhanced protective immunity following exogenous IL-12 administration to susceptible mice was accompanied by increased macrophage cytotoxicity, stronger type 1 cytokine profiles, and decreased parasite/bacterial growth. Moreover, upon challenge initial IL-12 treatments prevent disease. Endogenous IL-12 production had been also shown to be important in protective immunity generated by resistant mice against *M. tuberculosis* (Cooper, 1995) and *S. mansoni* (Anderson, 1998).

Collectively, parasite models indicate that exogenous IL-12 administration possesses adjuvant characteristics for the prevention of disease in susceptible mice and endogenous IL-12 production is important in the generation of protective immunity in situations where type 1 responses are beneficial. Similar findings have also been seen in cancer, where IL-12 was able to reverse the course of established tumors and induce tumor specific immunity, preventing the occurrence of new tumors (Zitvogel, 1996).

3.4 IL-12 and type 1 dominant diseases

As a powerful inducer of type 1 immunity, IL-12 can also contribute to the pathogenesis of autoimmune and inflammatory diseases. In experimental autoimmune encephalomyelitis, administration of IL-12 to rats accelerated the onset and inhibits disease remission. Treatment with anti-IL-12 mAb ameliorated clinical symptoms (Leonard, 1995). Enhanced IFNγ and IL-12 production, with lower IL-4 synthesis, was also observed in inflammatory bowel disease. Anti-IL-12 treatment after established inflammation in mice resulted in marked suppression of IFNγ production and improved disease scores. This implicated an active role for endogenous IL-12 in disease pathology (Davidson, 1998; Neurath, 1995). Excessive IL-12 and IFNγ production by macrophages and T cells respectively in Crohn's disease patients has also been reported (Parronchi, 1997). Together, these studies illustrate that excessive endogenous IL-12 production can stimulate type 1 autoimmune responses which (if left unchecked) can have highly detrimental effects. In addition, they suggest that anti-IL-12 therapy may be beneficial in the treatment of autoimmune or inflammatory disease.

3.5 Summary

Studies investigating the ability of IL-12 to promote primary Th1-like responses have been interpreted by many as strongly advocating adjuvant-like properties for this cytokine. It has also been endorsed as a powerful therapeutic agent for augmenting CMI. In addition, the in vivo neutralization of endogenous IL-12 may prove valuable in the fight against autoimmune and inflammatory diseases.

4.0 IL-10

4.1 IL-10, an anti-inflammatory cytokine

IL-10 is produced by a number of cells, including T cells, macrophages, keratinocytes, NK cells and some tumor cells. Although IL-10 is often associated with type 2 immunity, it is not strictly a type 2 cytokine. In human systems, IL-10 is made by both Th1 and Th2 clones. It also has been shown to down regulate type 1 and type 2 responses. In mice, IL-10 can also inhibit type 2 immunity (discussed below) (Pretolani, 1997). Thus, it is best known as an anti-inflammatory cytokine which influences many in vitro and in vivo systems (type 1 and type 2).

Some direct effects of IL-10 on T cells have been observed. For example, the addition of IL-10 in vitro inhibits IL-2 and IFNγ synthesis by Th1 cells. However, IL-10 appears to primarily act on T cells through modulation of APC responses. The effect of excess IL-10

on macrophage function have been demonstrated in vivo by the use of IL-10 transgenic mice which over-express IL-10 protein (Murray, 1997). *Mycobacterium bovis* infection of transgenic mice was associated with an incapacity to clear bacteria and compromised macrophage ability to stimulate T cells. In contrast, T cell proliferative responses of transgenic mice were unaffected upon stimulation with macrophages from WT mice. The absence of typical anti-mycobacterial activity by macrophages from IL-10 transgenic mice was most likely associated with the capacity of IL-10 to inhibit the effects of IFNγ on enhancing macrophage nitric oxide and TNFα mediated cytotoxicity (Flesch, 1994). The addition of exogenous IL-10 also was observed to inhibit macrophage IL-12, IL-1 and IL-6 synthesis, as well as their ability to promote the generation of Th1 cells (Fiorentino, 1991; Howard, 1992; Shibata, 1998). This was further supported by the observation that T cells from IL-10 KO mice activated with anti-CD3 produced high amounts of IFNγ. In addition, corresponding macrophages stimulated with chitin particles, bacillus Calmette-Guérin and lipopolysaccharide (LPS) exhibited increased IL-6, TNFα and IL-12 production (Kuhn, 1993; Shibata, 1998).

There is considerable evidence indicating that IL-10 is important in controlling excessive/detrimental pro-inflammatory cytokine production. In IL-10 KO mice the development of enterocolitis was affiliated with the inability to down regulate normal anti-bacterial inflammatory processes against flora in the gut (Kuhn, 1993). The removal of the regulatory functions of IL-10 could allow disease progression in IL-10 KO mice through a variety of mechanisms (including hyperactivation of macrophages). However, anti-IL-12

treatment was shown to prevent disease, indicating that unregulated IL-12 synthesis in these mice was critical in disease development (Davidson, 1998). The absence of endogenous IL-10 in *T. gondii* infected IL-10 KO mice resulted in high levels of IL-12 and IFNγ (in serum and spleen cell culture) associated with fatal systemic shock (Gazzinelli, 1996). Since *T. gondii* infection results in systemic infection, IL-10 control of pro-inflammatory cytokines in response to infection appears to prevent detrimental host immunity initiated upon *T. gondii* infection.

In other disease systems such as localized infections, the capacity of IL-10 to inhibit CMI restricts the development of protective immunity. *L. monocytogenes* infection was cleared more quickly in IL-10 deficient mice as indicated by a decrease in bacterial burden. This was associated with elevated IL-12, IFNγ, TNFa, and IL-6 production from heat killed *Listeria* stimulated spleen cells (Dai, 1997). Thus, in this case, endogenous IL-10 impairs type 1 immunity required for protection against *L. monocytogenes*.

It had been also reported that IL-10 enhanced certain aspects of CMI. IL-10 has demonstrated antitumor activity following systemic administration and local release (via transfection of tumor cells) (Berman, 1996; Giovarelli, 1995). Furthermore, IL-10 and IL-12 synergistically augmented anti-tumoral responses. IL-10 most likely initiates this effect through increased anti-tumor humoral responses, NK cell activity, and the recruitment and differentiation of CD8 T cells (Giovarelli, 1995; Jinquan, 1993). Furthermore, IL-10 was observed to up regulate NK cell IFNγ production, proliferation and cytotoxicity under certain

conditions (Shibata, 1998).

Taken together, these findings indicate that IL-10 is highly pleiotropic and can serve to both inhibit and enhance type 1 immunity, depending on the disease conditions. In addition, whether exogenous IL-10 treatment or endogenous IL-10 regulation are beneficial or detrimental depends greatly on the interactions between the host and the infectious (or other immunologically threatening) agent.

4.2 IL-10 and IL-12

It is thought that the balance between IL-10 and IL-12 determines the duration of immune responses to certain stimuli. Since IL-12 is a potent promoter of type 1 immunity, its production in response to type 2 inducing diseases is critical. However, for this reason it is equally important that IL-12 production is controlled to prevent damaging inflammatory responses (Davidson, 1998). In vitro addition and in vivo administration of IL-12 have been found to up regulate IL-10 production. This was augmented by IL-2 and other T cell stimulators (Jeannin, 1996). Since IL-10 can interfere with both T cell dependent and T cell independent production of IL-12, it is thought that the up regulation of IL-10 is a negative autoregulatory mechanism by IL-12 (Shibata, 1998; Takenaka, 1997). The production of IL-10 after clearance of infection could limit the recruitment of CD4 T cells to the site (Jinquan, 1993). IL-10 could also down regulate macrophage activity, reducing the further production of IL-12 and prevent detrimental type 1 immunity due to an excess of IL-12 production (Meyaard, 1996).

4.3 Summary

In conclusion, IL-10 inhibits pro-inflammatory cellular and cytokine responses. Depending on the host-disease interactions, endogenous IL-10 production can serve to limit type 1 immunity, be it protective or detrimental. IL-10 can also inhibit type 2 immunity (discussed below) and enhance some type 1 responses, such as NK cell activity. Taken together, this indicates that IL-10 is a highly diverse cytokine that should not necessarily be classified as either type 1 or type 2, but as a general suppressor of immunity.

5.0 The pathogenesis of allergy and cytokine regulation

5.1 The pathogenesis of allergy

Allergy is initiated when normally innocuous Ag elicits an IgE response. Allergy is a type 2 dominant disease which has both cellular and humoral components (reviewed in Maggi, 1998). The presence of allergen-specific IgE in the sera (i.e. atopy) can over time develop into a chronic inflammatory disease (i.e. allergic rhinitis or asthma). Recently, it was suggested that the production of Ag-specific IgE could be initiated upon APC presentation of allergic peptide (in the context of CD1) to natural T cells and/or γδ T cells resulting in the production of IL-4 important for the initiation of type 2 immunity (reviewed in Spinozzi, 1998). In particular, it has been reported that γδ T cell can provide B cell help for the IgE class switch (which requires IL-4, or IL-13, and CD40L as switch factors). Furthermore, atopic individuals exhibited a greater number of γδ T cells in their BAL fluid capable of storing IL-4 intracellularly compared to non-atopics, indicating that γδ T cells are important

in allergy responses. In addition, natural T cell and/or γδ T cell IL-4 production could also promote CD4 T cell (upon antigenic stimulation by APC) differentiation into Th2-like cells, enabling them to also to stimulate B cells to produce Ag specific IgE. Once IgE is circulating in the sera, it can become bound to high affinity IgE Fc receptor (FcεRI) on mast cells and basophils. Cross-linking of IgE (and FcεRI) upon subsequent exposure to allergen triggers the mast cell to release preformed granules containing histamine, chemotactic factors, heparin, platelet-activating factor, proteolytic enzymes and a variety of cytokines. These reactions are responsible for the rapid onset of symptoms in immediate hypersensitivity, allergic rhinitis, asthma and, in extreme cases, anaphylaxis. Arachidonic acid pathway metabolites such as prostaglandins, thromboxane and leukotrienes are also synthesized contributing to vasodilation and airway edema (augmenting cellular influx) and smooth muscle contraction, resulting in bronchial constriction.

The late phase reactions seen in allergic rhinitis and asthma are primarily cell mediated, beginning 3-12 hours later. They are contingent on the recruitment (partly in response to mediators released during the early reaction) of eosinophils, neutrophils, basophils and T cells (including natural and γδ T cells) into nasal secretions (in the case of allergic rhinitis) and the lung (in the case of asthma). The migration of these cells is mediated by the up regulation of adhesion molecules and chemotactic factors, such as RANTES and eotaxin which aid the recruitment of eosinophils. Recruited cells (and activated epithelial cells) produce additional mediators and cytokines amplifying the migration and differentiation of other cells, as well as producing molecules (i.e. toxins, cytokines and histamine) which directly aggravate the

allergic symptoms. IL-4 release is known to be of particular consequence. IL-4 can skew the development of uncommitted T cells that are entering the area into becoming Th2-like T cells and promote the continued production of IgE. Thus, the initial induction of Ag-specific IgE can trigger an amplification loop that can result in allergic rhinitis or asthma, a chronic and debilitating inflammatory condition.

5.2 IL-12 and IL-10 regulation in allergy and murine models of asthma

PBMC and whole blood cultures from allergic individuals have type 2 skewed cytokine profiles compared to non-allergic individuals (Esnault, 1996; van der Pouw Kraan, 1997). As such, whole blood cultures from allergic individuals produce less IL-12 and IFNγ in response to bacterial stimuli. While some studies have found IL-12 responsiveness to be intact in allergic individuals (van der Pouw Kraan, 1997), other investigators have found reduced IL-12 responsiveness relative to non-allergic controls (HayGlass, 1997; Lester, 1995). Interest in the potential therapeutic utility of exogenous rIL-12 for treatment of asthma has been stimulated by findings that (1) IL-12 is an effective inhibitor of T cell dependant human PBMC IgE secretion (Boer, 1997; Kiniwa, 1992), (2) in vitro addition of rIL-12 to memory CD4+ cells from allergic patients decreases IL-4 and increases IFNγ synthesis (Marshall, 1995), and (3) successful allergen immunotherapy is associated with enhanced endogenous IL-12 expression (Hamid, 1997).

In the most widely used animal models of human asthma (murine models of airway hyper-responsiveness, AHR), mice are sensitized i.p. with Ag, then aerosolized or intratracheally

challenged weeks later. It had been observed that IL-12 treatment at time of *sensitization* inhibited airway responsiveness, eosinophil influx and total IgE production, with little impact on bronchial airway lavage (BAL) cytokine production (Kips, 1996; Sur, 1996). Administered at time of *challenge*, IL-12 treatment inhibited airway responsiveness and eosinophil influx without altering IgE production (Gavett, 1995; Kips, 1996). This corresponded to increased BAL IFNγ production (and decreased IL-5 and IL-4 synthesis) (Gavett, 1995; Kips, 1996). Unaltered airway responsiveness upon IL-12 treatment has also been reported (Hofstra, 1998; Sur, 1996). These differences in AHR may be attributable to differences in Ag and IL-12 doses or treatment schedules which exist between the different experimental systems. However, it is interesting to note, that in one instance where exogenous IL-12 (or IL-18) alone was insufficient to reduce AHR, the combination of IL-12 and IL-18 was shown to return AHR to normal levels indicating that IL-12 and IL-18 treatment can synergize to limit AHR (Hofstra, 1998).

Decreased IL-10 responses have been observed in asthmatics. Spontaneous BAL IL-10 production was greatly diminished compared to normal individuals (Borish, 1996). Furthermore, it has been observed that in AHR mouse models, treatment with rIL-10 decreases recruitment of neutrophils, eosinophils and T cells to airways and TNFα and IL-5 synthesis in the lungs. In contrast to the benefical effects of IL-10 treatment, anti-IL-10 exacerbates symptoms (Zuany-Amorim, 1995; Zuany-Amorim, 1996). This was consistent with reported IL-10 impairment of eosinophilia by inhibiting T cell IL-5 synthesis (which is chemotactic for eosinophils and mast cells) and macrophage and mast cell IL-3 and GM-CSF

production (eosinophil growth and differentiation factors) (Pretolani, 1997). In addition, IL-10 can also directly interfere with eosinophil GM-CSF production required for eosinophil activation.

Aspergillus fumigatus is a fungus which causes lung hypersensitization that can result in asthma. IL-10 pretreatment of human monocytes incubated with *A. fumigatus* indicated that IL-10 limited lung damage by (1) inhibiting macrophage superoxide anion production in response to the fungus, and (2) exhibiting anti-fungal activity (Grunig, 1997). Furthermore, these effects were reversed by IFNγ (Roilides, 1997). Although an number of different protocols were used, IL-10 KO mice infected with *A. fumigatus* generally exhibited increased IL-5. A greater strain dependence was shown on the lack of endogenous IL-10 regulation to result in exacerbation of AHR and mortality (Grunig, 1997). This indicates that endogenous IL-10 has an important role in the regulation of lung responses, but other factors such as genetics may compensate for the absence of IL-10 upon exposure to certain stimuli.

Together these data indicate that lack of endogenous IL-12 and IL-10 production or an inappropriate balance of cytokines can exacerbate asthmatic responses. They also suggest that exogenous IL-12 and IL-10 treatment may be beneficial in the therapeutic management of asthma and allergic responses.

6.0 Cytokine regulation of humoral responses

6.1 Induction of IgE synthesis

The initial production of Ag-specific IgE in response to an allergen is considered to be a primary factor in the appearance of allergic disease.

In vivo, the importance of IL-4 in IgE synthesis has been demonstrated. Treatment with anti-IL-4 or anti-IL-4 receptor inhibited IgE production in mice injected with strong IgE inducing stimuli (such as Ag in alum, goat anti-mouse IgD or helminth) (Finkelman, 1990; Finkelman, 1991). Furthermore, IgE synthesis was virtually undetectable in IL-4 KO mice (Kuhn, 1991). Together this studies show that endogenous IL-4 synthesis promotes both primary and secondary IgE production upon immunization. During primary immunization the source of IL-4 has been shown to be predominantly T cell derived, while IL-4 synthesized at secondary Ag challenge has been mostly attributed to non-T cell sources (Finkelman, 1990; Yamashita, 1996).

In vitro experiments demonstrate the requirement of CD40 ligation on B cells (provided by the presence of CD40L on activated T cells, or by anti-CD40 or CD40L in experimental systems), in the induction of e germline transcription in addition to IL-4 (Splawski, 1993). CD40 ligation and IL-4 also induced the production of IgG_1 (in mice) and IgG_4 (in humans) (Akdis, 1997; Mandler, 1993). In a human B cell line, CD40 ligation and endogenous IL-10, or TGFβ, were sufficient for induction of γ1 and α transcripts, however, the addition of IL-4 was required to initiate the transcription of germline γ4 and ε (Cerutti, 1998).

In mice, isotype switch had been shown to occur sequentially from IgM ➡ IgG_1 ➡ IgE. This was further supported by the finding that sequential switch (upon CD40 ligation and IL-4 stimulation of resting B cells) was seen in coordination with B cell replication. As such, membrane bound IgG_1 was evident after fewer cell divisions than membrane bound IgE (Hasbold, 1998). Moreover, induction of switch was dependent on the dose of IL-4, since at lower IL-4 doses more cell divisions were required before the B cell switch to IgG_1 and then to IgE occurs. This suggests that endogenous IL-4 (from T cell sources) induction of IgG_1 and IgE is a function of cell cycle signals, expanding on the observation that DNA replication precedes isotype switch (Lundgren, 1995).

In the absence of IL-4, IL-13 is also capable of initiating e germline transcription (Punnonen, 1993b). In addition, IL-13 can synergize with IL-4 to enhance IgE production, as can IL-2, IL-5, IL-6 and TNFα (Pene, 1988b).

It was also reported that soluble CD23, upon interaction with its ligand (CD21), enhanced IgE production; whereas, membrane bound CD23 has been shown to inhibit IgE synthesis upon binding with IgE. Thus, CD23 provides another mechanism of IgE regulation. In addition, anti-CD40 and IL-4 synergistically induced soluble CD23 release from human B cell cultures, with little effect on the membrane CD23 expression (Paterson, 1996; Sarfati, 1992). IL-13 also augmented CD23 production (Punnonen, 1993b).

Collectively, these studies indicate that while there are limited switch factors (anti-CD40, IL-4

and IL-13) for IgE production, its production can be augmented through a variety of agents. Another one of these factors is IL-10.

6.2 The importance of IL-10 in IgE responses

In vitro studies indicate that IL-10 is a critical regulator of B cell growth, differentiation into memory B cells and plasma cells, and Ab secretion (Arpin, 1995). Anti-IL-10 treatment of naive or immunized mice demonstrated that IL-10 augmented IgM and IgA production, while inhibiting IgG_{2a} synthesis (Ishida, 1993). In human studies examining recombination events of germline DNA, IL-10 has been shown to be a switch factor for IgG_1, IgG_3 and IgA (Malisan, 1996).

At the beginning of this present study, the importance of IL-10 in IgE production was not well understood. Since IL-10 is produced by Th2 murine clones (and human Th0 and Th2 clones, as well as APC) and is a strong inhibitor of pro-inflammatory cytokines, it was thought to be a possible augmentor of IL-4 induced IgE production.

Studies with IL-10 KO mice or anti-IL-10 treatment indicated that basal IgE levels were unaffected by the absence of endogenous IL-10. Similar results were seen upon immunization protocols that induce weak IgE production (high Ag dose in alum, or Ag in the absence of alum) (Dobber, 1995; Ishida, 1993; Kuhn, 1993). However, these studies did not convincingly demonstrate that IL-10 was not involved in IgE synthesis due to the lack of strong positive control (i.e. the induction of high IgE levels) in normal controls.

In vitro human experiments indicate that IL-10 does not directly alter the capacity of purified B cells stimulated with IL-4 and T cells (or anti-CD40) to produce IgE. However, in the presence of monocytes, IL-10 either inhibited (Punnonen, 1993a) or enhanced (Uejima, 1996) IgE synthesis. While these findings indicate that IL-10 can influence IgE production through its actions on monocyte function, the discrepancies between these studies may relate to a recent finding showing that IL-10 exerted different affects on IgE synthesis depending on the activation status of the B cells (Jeannin, 1998). The addition of IL-10 to PBMC stimulated with IL-4 within the first 3 days (prior to the induction of IgE) inhibited IgE production, suggesting that IL-10 is antagonistic to IL-4 switching. If added to differentiated cultures, already producing IgE, the addition of IL-10 enhanced IgE synthesis. Although, Uejima *et al.* added IL-10 at the beginning of cultures, they also added anti-CD40. Anti-CD40 is a potent stimulator of IgE isotype switch, which could impair the potential of IL-10 to inhibit IL-4 induced IgE switch. Thus, the enhanced IgE synthesis reported by Uejima *et al.* may be the result of lower endogenous IFNγ or IL-12 levels due to initial addition of exogenous IL-10 or (if any exogenous IL-10 remained in culture after switch occurred) augmented memory B cell and plasma cell differentiation.

This same study (Jeannin, 1998) showed that while IL-10 was not a switch factor for IgG_4, it up regulated both IL-4 induced IgG_4 switch and the production of IgG_4 from differentiated cultures, even in the absence of IL-4. In allergy, both allergen specific IgE and IgG_4 are produced. Some immunotherapy studies suggest that IgG_4 production exerts a protective effect against IgE binding to Ag, since successful outcomes are sometimes associated with

increases in IgG_4 levels (Akdis, 1998a; Lu, 1998). This is also supported by the ability of IFNγ to enhance IgG_4 synthesis (Akdis, 1997).

6.3 The impact of IL-12 and IFNγ on development of antibody responses

This section reviews the state of knowledge regarding IL-12 and IFNγ regulation on Ab production at the beginning of this thesis work.

Exogenous IL-12 blocked IgE secretion from human PBMC by inhibiting the switch to IgE (Boer, 1997; Kiniwa, 1992). In addition, the capacity of exogenous IL-12 administration to inhibit primary IgE responses was seen in several in vivo systems (Ag in alum or goat anti-mouse IgD) (Germann, 1995b; McKnight, 1994; Morris, 1994). While this was cited as demonstration of the adjuvant capacity of IL-12, all of the data addressed primary responses. None of the studies examined the ability of IL-12 to continue inhibition of IgE upon subsequent Ag exposure. IL-12 also influences the production of other isotypes, of particular interest to this thesis, IgG_{2a} and IgG_1. Generally, it was observed that rIL-12 administration elevated IgG_{2a} and decreased IgG_1 synthesis (McKnight, 1994; Morris, 1994), although enhanced IgG_1 was also reported (Germann, 1995b). Exogenous IL-12 induced changes in Ab production are uniformly associated with some type of skewing of cytokine production in the type 1 direction (such as increased IFNγ production).

In vivo, very high doses of rIFNγ (close to those that are lethal) were found to inhibit IgE and IgG_1 in mice injected with goat-anti-mouse IgD (Finkelman, 1988). In vitro studies reported

that IFNγ inhibited CD4 T cell dependent stimulation of IgE synthesis through costimulatory signals by CD4 T cells, since IFNγ did not inhibit B cell production of IgE when B cells were stimulated with anti-CD40 and IL-4 (Gascan, 1991; Pene, 1988a). Enhanced endogenous IFNγ synthesis following IL-12 treatment has been cited as one mechanism to explain how exogenous IL-12 can inhibit IgE production. IL-12 inhibition of primary IgE synthesis exhibited some dependence on elevated IFNγ levels following IL-12 treatment, as anti-IFNγ/IL-12 treatment upon helminth infection impaired the capacity of IL-12 to inhibit IgE (Finkelman, 1994).

In human PBMC (stimulated with IL-4), rIL-12 has been shown to increase IgG_4 synthesis. The capacity of exogenous IL-12 added to IL-4 and hydrocortisone stimulated blood cord neonatal cells suppressed IgE production was not altered by the addition of anti-IFNγ treatment (Kiniwa, 1992). Furthermore, anti-IFNγ treatment was not always sufficient in reversing the effect of IL-12 treatment on IgE (goat-anti-mouse IgD/IL-12 treated mice) (Morris, 1994). Whether these studies reflect incomplete neutralization of IFNγ upon IL-12 treatment is unknown.

IFNγ strongly up regulates IgG_{2a} production. Low doses of IFNγ were able to increase IgG_{2a} synthesis, while anti-IFNγ mAb treatment reduced the typically high IgG_{2a} production seen in *B. abortus* infected mice (Finkelman, 1988) or exogenous Ag stimulated systems (HayGlass, 1991b). Neutralization of endogenous IFNγ (with anti-IFNγ mAb) simultaneous with IL-12 treatment indicated that enhanced IgG_{2a} levels following IL-12 administration

were dependent on endogenous IFNγ synthesis (Germann, 1995b; Morris, 1994). The inability of anti-IFNγ treatment to inhibit IL-12 induced IgG_{2a} production had also been seen, although (again) this may have been due to incomplete neutralization of endogenous IFNγ (McKnight, 1994).

Since IL-12 is known to promote NK cell IFNγ production, a potential role for NK cell derived IFNγ synthesis in mediating IL-12 effects is possible. In one study, anti-NK1.1/IL-12 treatment with TNP-KLH immunization decreased Ag-driven IFNγ synthesis. However, IgG_{2a} and IgG_1 responses remained unaffected, suggesting that NK cell IFNγ production did not explain IL-12 induced changes in Ab production (McKnight, 1994). The importance of NK cell IFNγ production on IL-12 inhibition of IgE synthesis or the importance of IFNγ in mediating IL-12 inhibition of IgE synthesis in response to Ag in alum were not investigated. Together, these studies indicated that exogenous IL-12 administration could inhibit IgE in vitro and primary IgE in vivo. However, these studies did not determine the capacity of IL-12 to maintain suppressed IgE levels upon repeated exposure of Ag. Exogenous IL-12 treatment was also shown to elevate IgG_{2a} synthesis. Enhanced endogenous IFNγ production appears to be one mechanism by which IL-12 inhibited IgE and increased IgG_{2a} levels. However, independently of IFNγ activity, IL-12 can either act directly on B cells (Vogel, 1996) or impact Ab production through altering the synthesis of cytokines other than IFNγ.

6.4 Summary

CD40 ligation and IL-4 (or IL-13) are critical in the initiation of IgE responses upon

immunization. IL-10 was shown to promote or inhibit IgE production depending on the stage of B cell activation. Exogenous IL-12 administration had demonstrated the ability to inhibit the strong primary IgE production (via IFNγ dependent and independent mechanisms) normally seen following immunization with alum or type 2 polyclonal activators, but the capacity of IL-12 to continue inhibition of IgE upon subsequent Ag exposure had yet to be determined.

7.0 CD8 T cell regulation of humoral responses

The critical role of CD4 T cells in IgE isotype switch by providing cognate B cell help (CD40 interactions) and producing IL-4 and IL-13, is well recognized. Recently there has been renewed interest in the significance of CD8 T cells in disease regulation, including atopy. Studies from three laboratories, in particular, suggested that CD8 T cells were also important in the regulation of IgE.

Kemeny and his colleagues have reported that CD8 T cells were required for optimal induction of immune responses. A five fold decrease in total, (but not Ag-specific) IgE was observed when rats were depleted of CD8 cells prior to immunization (Holmes, 1996). In an AHR model, CD8 T depletion prior to OVA sensitization decreased AHR, IL-5 and eosinophil responses, but total and Ag-specific IgE were not substantially altered (Hamelmann, 1996).

In contrast, it was reported that primed CD8 T cells limited IgE production. In AHR models, when CD8 depletion occurred between Ag sensitization and airway challenge, late airway responses were enhanced (Olivenstein, 1993). Furthermore, if immunized rats were treated with anti-CD8 mAb, primary Ag-specific IgE levels were elevated (Holmes, 1997). This was also supported by the suppression of IgE synthesis upon adoptive transfer of OVA primed CD8 T cells or OVA-specific CD8 T cell clones after immunization (MacAry, 1998; Renz, 1994).

Thus, these studies suggest that CD8 T cells can either positively regulate the induction or negatively regulate active immune responses associated with atopy.

There is evidence indicating that CD8 T cells can respond to exogenous Ags when exogenous Ag escape from ingesting vesicles into the cytosol of APC, where they are transferred into the endoplasmic reticulum and bound by MHC class I molecules for presentation to CD8 T cells. This was supported by a recent demonstration that OVA incubated with cationic lipid transfection reagent (thought to deposit the Ag into the cytosol) resulted in greatly increased proliferation by CD8 T cells (MacAry, 1998). This was inhibited by anti-MHC class I, but not anti-MHC class II, mAbs. Minor proliferation occurred in the absence of cationic lipid transfection reagent.

While the idea of T suppressor cells has fallen into disrepute in the absence of sufficient evidence, CD8 T cells appear to still participate as immune regulators through a variety of

mechanisms. Firstly, CD8 T cells had been shown to regulate immune events by the killing of effector lymphocytes or APCs (Rock, 1992).

Secondly, it was reported that activated CD8 T cells inhibited bystander cells, since the depletion of CD8 T cells enhanced Ag-stimulated proliferation of spleen cells from immunized mice. This was reversed by the re-introduction of activated CD8 T cells (MacAry, 1998). Furthermore, CD4 T cell IL-4 and IFNγ synthesis was enhanced and decreased respectively following CD8 T cell depletion in immunized rats (Holmes, 1997).

Thirdly, there was also indication that CD8 T cells regulate immune events through cytokine production. Enhanced IFNγ synthesis by activated CD8 T cells suggested that this may be a mechanism by which activated CD8 T cells induced the inhibition of AHR and IgE production (Renz, 1994). Increased type 2 cytokine synthesis was also seen by CD4 and CD8 T cells isolated from PBMC of allergic individuals (Meissner, 1997; Nakazawa, 1997). In addition, the percentage of IL-4 producing CD8 T cells isolated from allergic individuals was reported to be correlated with total serum IgE synthesis ($p < 0.01$); whereas, a weaker relationship was seen between the percentage of IL-4 secreting CD4 T cells and IgE production (Meissner, 1997). However, this does not indicate a causal relationship between CD8 T cell IL-4 production and IgE synthesis in atopy.

Fourthly, different CD8 T cells subsets or populations may be involved in regulating different aspects of immune responses. Inhibition of IgE upon airway sensitization in AHR models,

was reported to be mediated by gd CD8+ T cells at the mucosal surface (McMenamin, 1995). In contrast, ab CD8 T cells appeared to be involved in regulation of i.p. immunization responses (MacAry, 1998). Finally, it had been suggested that CD8 T cells positively regulate the induction of IgE synthesis, but negatively regulate the intensity or duration of the response. A dichotomy in CD8 T cell regulation was also observed in other disease models such as collagen induced arthritis. While the incidence of disease was decreased in CD8 KO mice, these same animals displayed a greater incidence and severity of disease upon secondary immunization with collagen (Tada, 1996).

Taken together, these studies suggest that CD8 T cells may be involved in the regulation of immune responses to both exogenous and endogenous antigens through a variety of mechanisms. However, the role of CD8 T cells in IgE regulation has not been extensively investigated, except for a few laboratories inviting caution on over interpreting these findings.

8.0 Allergen immunotherapy

Currently, the most effective way to manage clinical allergy is by avoiding the allergen. However, in many situations avoidance is not possible. Allergies are often managed very effectively by use of anti-histamines, corticosteriods, bronchodilators, or other drugs. The difficulty with drugs includes detrimental side effects and required accessibility, especially in anaphylactic responses. Furthermore, these drugs only manage, and do not cure allergic disease.

With the increasing incidence and severity of allergy, effective prophylactic and therapeutic immunologic strategies are required. Traditionally the major approach to allergic immunotherapy has been allergen desensitization. Since the early 1900's, allergic individuals have been treated with allergen extracts (Noon, 1911). This approach has shown success in the prevention of anaphylaxis triggered by bee venom. In some incidences, allergen desensitization has also been successful with pollen induced allergic rhinitis, but there was considerable variation between individuals. In addition, failures due to anaphylactic shock or an increase of symptoms were also reported, as was the return of symptoms after treatments had stopped. Immunotherapy has been largely unsuccessful for asthma, as meta analyses of slight short-term improvements seen in symptom and medication scores usually did not result in long-term benefits (Barnes, 1996; Creticos, 1996).

There have been a number of attempts to make immunotherapy safer by altering antigenicity of the allergen (Ishizaka, 1978; Lee, 1977), including glutaraldehyde polymerization (Johansson, 1974; Patterson, 1973). Although a reduced incidence of negative side effects allowed for higher dose and fewer treatments with altered allergens, enhanced efficacy was generally not seen in humans (Grammer, 1982).

Treatment with T cell epitope peptides appears to be more promising in immunotherapy applications. Firstly, since the peptides are small they reduce the possible induction of IgE cross-linking by treatment allergen and associated side effects (including anaphylaxis).

Secondly, while recombinant preparations of epitopes result in a more standardized product (important for clinical trials), the use of digested extracts have the advantage of accounting for many T cell epitopes. This may be important as it had been suggested that one mechanism behind successful immunotherapy was the type 1 skewing of Th0 cells specific for less dominant epitopes of the allergen (Jutel, 1995).

Thirdly, increases in the ratio of type 1 (IFNγ):type 2 (IL-4, IL-5) secretion upon antigen specific stimulation of T cells have been reported after successful immunotherapy, suggesting that alterations in the T cell responses may be critical (Jutel, 1995; Secrist, 1993). Since T cells are important regulators of immune responses, increased type 1:type 2 cytokine ratios could limit both cellular responses (eosinophil, mast cell) and immunoglobulin synthesis associated with allergy. It should be noted that some studies also saw enhanced IL-10 production. However, there was disagreement as to whether increased IL-10 synthesis represented negative regulation of heightened type 1 immunity following immunotherapy (Bellinghausen, 1997) or active inhibition of type 2 responses linked to atopy (Akdis, 1998b). Decreased IL-10 was also reported (Lu, 1998).

Fourthly, it had been reported that native and denatured allergen (i.e. bee venom phospholipase A2, PLA) were processed differently by APC and induced different cytokine and Ab patterns (Akdis, 1998a). Native PLA was ingested by both monocytes and B cells of allergic individuals, while unfolded PLA was taken up by monocytes only. Correspondingly, cultures of B cells and T cells (stimulated with PLA) produced stronger IL-

13 and lower IFNγ and IL-12 levels than did monocyte/T cell cultures (stimulated with naive or denatured PLA). B cell/T cell cultures stimulated with denatured PLA produced negligible levels of cytokines. In addition, IL-4 and CD40L stimulation of PBMC in the presence of folded PLA resulted in greater IgE synthesis; whereas, the presence of unfolded PLA led to a predominance of IgG_4 secretion. Taken together, this indicates that conformational B cell epitopes are less effective than non-conformational (T cell) epitopes at skewing immune responses associated with allergy.

Although not consistently found, decreased Ag-specific IgE and increased IgG synthesis have been associated with relief from symptoms and may be one mechanism involved in successful immunotherapy (Hendrix, 1980; Jutel, 1995). Enhanced IgG production, particularly IgG_4, has been suggested that to block interactions between allergen and IgE, although consistent increases in IgG have not been found (Akdis, 1998a; Bousquet, 1990; Lu, 1998). Recently, murine IgG (induced upon immunization with pollen allergens) had been observed to inhibit human IgE binding to identical immunodominant B cell epitopes on allergen. In addition, this binding prevented IgE cross-linking and human basophil histamine release, indicating that enhanced IgG could interfere with IgE and allergen interactions (Vrtala, 1998).

It is still not clear why successful immunotherapy works. While different studies have implicated changes in Ab production and T cell mechanisms, it is more likely that a combination of mechanisms and factors including genetics, the type of allergy and the individual's health and environment.

Thus, with a greater understanding of cytokine regulation of IgE responses, there is increasing interest in the prophylactic and therapeutic potential of type 1 inducers, such as IL-12, to prevent and "cure" allergic disease.

9.0 A murine model of immediate hypersensivity

HayGlass and colleagues have utilized a murine model of immediate hypersensitivity consisting of immunization of C57Bl/6 mice with OVA precipitated in alum. This immunization generates intense type 2 humoral responses: Ag-specific IgE and almost undetectable Ag-specific IgG_{2a} levels in all strains of mice tested. This is associated with substantial production of IL-4 and IL-10 in Ag driven spleen cell culture (Yang, 1994).

Previously, they found that these type 2-dominant responses could be inhibited by treatment with glutaraldehyde polymerized OVA (OA-POL). The treatment of mice with OA-POL prior to OVA (alum) immunization inhibits the induction of OVA-specific IgE, in conjunction with elevation of OVA-specific IgG_{2a} for 18 months after treatment (Gieni, 1993a). Moreover, OA-POL treatment abrogates established IgE responses, resulting in 90 - 95 % reductions in OVA-specific IgE synthesis upon subsequent booster immunizations. The capacity of OA-POL to both prevent and abrogate IgE production is associated with a reversal in the cytokine pattern from that normally seen upon OVA (alum) immunization,

from type 2 dominant to type 1 dominant. An increase in the frequency of CD4 T cells producing IFNγ, determined by limiting dilution analysis, is also seen. Adoptive transfer experiments directly demonstrated that OA-POL activity is CD4 T cell dependent. Hence, OA-POL is able to induce strong type 1 humoral responses in a CD4 T cell dependent manner to exogenous Ag, the same class of Ag as most inhalant allergens relevant to human immediate hypersensitivity.

The availability of these models provides an opportunity to explore cytokine regulation in type 2 and type 1 skewed humoral responses.

10.0 Scope of research

This thesis utilized models of type 2 (OVA, alum) and type 1 (OA-POL) driven Ab responses to explore cytokine regulation of Ab synthesis. Firstly, it examined the pharmacologic potential of IL-12 to inhibit and maintain inhibition of IgE synthesis. Secondly, it considered the ability of exogenous IL-12 treatment to inhibit established IgE production. Thirdly, it examined the roles of endogenous IL-12 and IL-10 in Ab production. Lastly, it investigated the importance of CD8 T cells in the induction of type 1 and type 2 Ab responses.

10.1 Hypothesis 1

It is recognized that IL-12 strongly promotes Th1 cytokine production in CD4 T cells. Both OVA (alum) induction of IgE and OA-POL inhibition (and abrogation) of IgE is CD4 T cell

dependent. In addition, IL-12 inhibits IgE production in vitro and primary IgE synthesis following exposure to a variety of type 2 inducing stimuli. Therefore, we hypothesized that administration of exogenous IL-12 would induce and maintain long-term inhibition of IgE production normally resulting from OVA (alum) immunization. To evaluate this, we treated OVA (alum) immunized mice with IL-12, at concentrations up to those which resulted in substantial toxicity in vivo. Mice also received repeated boosts with OVA (alum) in the absence of IL-12 treatment to determine if initial skewing of immunity following IL-12 administration would result in sustained inhibition of IgE production. To assess potential mechanisms of IL-12 action on Ab responses, CD4 T cells and NK cells were analyzed for changes in cytokine synthesis.

10.2 Hypothesis 2

Secondly, we hypothesized that rIL-12 administration would inhibit established IgE responses. This was determined by immunizing mice in the absence of exogenous IL-12, under conditions leading to a type 2 dominated response (strong IL-4, IL-5 and IgE; weak IFNγ and IgG_{2a}). Mice were treated with IL-12 (or not), using a variety of protocols in an attempt to inhibit type 2 Ab production upon subsequent exposure to the sensitizing Ag. Cytokine production from whole spleen cells, CD4 T cells and non-B/non-T cells was evaluated to determine underlying mechanisms influencing Ab production.

10.3 Hypothesis 3

We wanted to investigate the role of endogenous IL-12 in Ab regulation. Since OA-POL treatment induces strong type 1 immunity in naive and previously OVA (alum) immunized

mice, and IL-12 displays potential to act as a powerful inducer of type 1 immunity, we hypothesized that OA-POL induced inhibition of IgE was dependent upon enhanced endogenous IL-12 production. This hypothesis was addressed by administering anti-IL-12 (or nothing) to OA-POL treated mice, immunizing them and assessing changes in the capacity of OA-POL to induce type 1 Ab profiles. As an alternative approach, IL-12 KO mice were immunized with type 1 (OVA, CFA) or type 2 (OVA, alum or *T. spiralis* extract) inducing stimuli. Ab and cytokine synthesis was evaluated.

10.4 Hypothesis 4

IL-10 is a negative regulator of IL-12 and other inflammatory associated cytokines. In addition, it is known to augment B cell responses. We hypothesized that OVA (alum) immunization, in the absence of endogenous IL-10 synthesis, would result in enhanced type 1 cytokine production leading to decreased IgE synthesis. This hypothesis was addressed by evaluating serum Ab production in immunized mice in the absence of endogenous IL-10 (due to treatment with anti-IL-10 mAb or genetic KO of IL-10 gene). In addition, spleen cells from immunized IL-10 KO mice were analyzed for cytokine production (IL-12 p40, IFNγ, IL-4 and IL-5).

10.5 Hypothesis 5

It has been suggested that CD8 T cells are important in the regulation of IgE responses. This was supported by preliminary experiments in our laboratory. Thus, we hypothesized that in the absence of CD8 T cells Ab and cytokine responses to exogenous Ag would be skewed

in the type 1 direction; specifically, that this would result in decreased IgE and IL-4 synthesis, but higher IgG_{2a} and IFNγ levels. This was examined in CD8 KO mice by the evaluation of Ab and cytokine responses upon immunization with OVA (alum) and treatment with OA-POL.

10.6 Summary of results

We found that exogenous IL-12 administration inhibited IgE production during the primary response > 95 %. However, this inhibition was transient, with no difference in IgE levels between treated and untreated mice upon subsequent immunizations. In contrast, IL-12 induced increases in IgG_{2a} synthesis were stable. This transient IgE inhibition was associated with enhanced NK cell dependent IFNγ production, but not with altered CD4 T cell activity, suggesting that while the presence of exogenous IL-12 evokes strong type 1 effector responses, this does not translate into adaptive memory required for the maintaining the long-term inhibition of IgE synthesis.

In our examination of the therapeutic potential of IL-12 in an ongoing type 2 response, rIL-12 treatment greatly elevated type 1 immunity (strongly increased IgG_{2a} and IFNγ synthesis, and a decreased frequency of IL-4 producing CD4 cells). However, IL-12 treatment failed to alter established IgE production and markedly enhanced Ag-driven IL-4 production (~ 5 fold). This increase in IL-4 synthesis could be attributed to increased numbers of non-B/non-T cells within the spleen and their enhanced capacity to produce IL-4 following IL-12 treatment. Thus, the pharmacologic administration of IL-12 did not prove therapeutically

beneficial and may in fact be detrimental in the treatment of individuals with chronic IgE production.

To determine if the capacity of OA-POL treatment to prevent the induction of type 2 Ab and cytokine profiles normally associated with OVA (alum) immunization is affiliated with enhanced levels of endogenous IL-12, immunized mice were pretreated with OA-POL and anti-IL-12 Ab or normal goat IgG. We found that the capacity of OA-POL treatment to inhibit IgE (and enhance IgG_{2a}) synthesis was partially dependent on endogenous IL-12 production, suggesting that changes in Ab synthesis following OA-POL treatment is reliant on factors in addition to endogenous IL-12. However, taking an independent approach by using IL-12 KO models, we did not observe an anticipated general enhancement of IgE and type 2 cytokine synthesis following immunization with type 1 (OVA in CFA) or type 2 stimuli (OVA, or *T. spiralis* extract, in alum). In contrast, the absence of endogenous IL-12 was associated with decreased IgG_{2a} levels.

We explored the role of IL-10 on IgE production, by immunizing mice in the absence of endogenous IL-10. Markedly decreased IgE production was observed when immunized mice were treated with anti-IL-10 or upon the OVA (alum) immunization of IL-10 KO mice. Furthermore, this was associated with increased type 1 cytokine (IFNγ and IL-12 p40) production, suggesting that IL-10 inhibition of type 1 cytokines in vivo allows for a cytokine environment permissive of IgE production and that the induction of endogenous IL-10 may play a role in inhibiting type 1 cytokine production in immediate hypersensitivity.

In CD8 KO mice, OVA (alum) immunization and OA-POL treatment resulted in a general skewing of Ab and cytokine production in a type 1 direction (decreased total IgE synthesis, increased IgG_1 and IgG_{2a} levels, increased IFNγ:IL-4 ratio, decreased IL-5 production). However, while these changes were statistically significant, most were minor making it difficult to interpret them as being biologically relevant.

Taken together, this thesis demonstrates that (1) although the presence of pharmacological doses of exogenous IL-12 can inhibit primary IgE production, they are insufficient to skew type 2 adaptive responses, important in maintaining inhibition of IgE synthesis, (2) IL-12 administration abolishes primary IgE production through the induction of NK cell (and not CD4 T cell) IFNγ synthesis, (3) the inability of IL-12 to inhibit established IgE production is associated with enhanced non-B/non-T cell IL-4 production following IL-12 administration, (4) in spite of the inability to decrease IgE synthesis, rIL-12 treatment to mice with established Ab responses greatly elevates type 1 immunity (enhanced IgG_{2a} and IFNγ levels, decreased frequency of IL-4 producing CD4 T cells), (5) the absence of endogenous IL-10 results in the inhibition of IgE production upon OVA (alum) immunization and is associated with enhanced type 1 cytokine production, and (6) CD8 T cells do not greatly contribute to the regulation of IgE responses.

III. Methods and Materials

1.0 Mice

C57Bl/6, CD1 (outbred) and CD8 (C57Bl/6) knockout (a kind gift from Dr. T Mak, University of Toronto) mice were bred at the University of Manitoba breeding facility (Winnipeg, MB) or purchased from Charles River Canada (St. Constant, PQ). IL-10, IL-12 p35 and p40 knockout mice, all homozygous on a C57Bl/6 background, were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Manitoba breeding facility (Winnipeg, MB). Mice were used in accordance with guidelines issued by the Canadian Council on Animal Care.

2.0 Preparation of in vivo reagents

2.1 Preparation of antigen in adjuvant

To induce IgE responses associated with immediate hypersensitivity, mice were immunized with 0.2 or 2.0 μg OVA (five times recrystallized, ICN Biochemicals, Montreal, PQ) in the presence of $Al(OH)_3$ adjuvant (alum). Alum was prepared by adding 2 drops of 0.2 % phenol red to 20 ml of 10 % $AlK(SO_4)_2$. This was followed by the addition of approximately 20 ml of 0.5 N NaCl until the solution began to turn pink. After sitting for 10 min, the solution was centrifuged for 3 min at 1000 rpm (170 g). The supernatant was discarded and the precipitate was resuspended in 0.15 N NaCl (40 ml). This centrifugation procedure was repeated two

times. The resulting pellet was resuspended in 40 ml of 0.15 N NaCl to yield $Al(OH)_3$ at 10-12 mg/ml. To prepare antigen for immunization, antigen was added to alum and left for 10 min at room temperature. Two thirds volume of Hank's balanced salt solution (Flow Laboratories, Mclean, VI) was added prior to immunization to neutralize the pH of the solution. Following this procedure >99% of antigen was adsorbed to the alum crystals.

Mice were also immunized with 100 μg OVA in CFA (Gibco-BRL Products, Burlington, ON). Prior to use, CFA mycobacteria were carefully resuspended. OVA, dissolved in 0.85 % NaCl, was mixed with an equal volume of CFA and emulsified. Emulsification was considered complete when a drop of emulsion could stay cohesive upon suspension in water.

2.2 Preparation of polymerized ovalbumin

Polymerized ovalbumin (OA-POL) of average Mr 3.5×10^7 was prepared as described (HayGlass, 1991c). Briefly, 6 % glutaraldehyde (Eastman Kodak Co. Rochester, NY) in 0.15 M NaCl was added drop wise to OVA (25 mg/ml in 0.1 M acetate buffer pH 5.3, 0.5 pH units above its isoelectric point) to obtain a final molar ratio of 200:1. A reaction time of 5 hours was used. After dialysis and gel filtration (Biogel A-50m [2.5 x 90 cm in borate buffered saline, pH 8.3 effective separation range 1.0×10^5 to 5.0×10^7 da]; Bio-Rad Laboratories, Mississauga, ON; $V_o = 5 \times 10^7$), OA-POL was recovered as a single, sharp, symmetric peak with an average relative molecular weight of 3.5×10^7. This approach to glutaraldehyde modification allows for a homogenous product unlike earlier methods which were found to result in highly heterogeneous mixtures that produced various immunological effects

(HayGlass, 1984; Johansson, 1974; Patterson, 1973). OA-POL was stored at 4°C.

2.3 mAb production

B cell hybridomas were cultured in 10 % fetal calf serum (FCS), 10 nM L-glutamine, penicillin (100 units/ml), streptomycin sulfate (100) μg/ml), fungizone (0.25 μg/ml) (Flow Labs, Mississauga, ON) and 2 x 10^{-5} M 2-mercaptoethanol in RPMI 1640. This media is designated as 10 % FCS. To maximize mAb production, cultures were expanded and maintained until cells decreased to 20 % viability. Depending on the purpose, the resulting tissue culture supernatant was either concentrated in a pressure chamber (Amicon Corporation, Holland) or purified on the Pharmacia Biopilot system as previously described (HayGlass, 1991b). After purification by Biopilot, purified mAb were generally determined to be 85-95 % pure when mAb specific concentrations (determined by ELISA) were compared to total protein concentrations (determined by A_{280}, ε=1.4).

mAb was also produced in UltraDOMA-PF (UPF) media (BioWhittaker, Walkersville, MD). UPF media does not contain proteins (which are potentially immunogenic), but consists of a rich cocktail of amino acids. Hybridomas of mAb for in vivo use were initially established in 10 % FCS media. After cells were sufficiently expanded (> 90% viability), they were washed twice in serum free RPMI 1640 (serum free) and cultured in UPF media at starting concentrations of 200,000 to 400,000 cells/ml. Cultures were maintained until they decreased to 20 % viability and the supernatant was harvested. The UPF produced antibody supernatants were concentrated and used.

mAb were injected i.p. in saline to determine the impact of depletion (of cytokines or cells) on Ab production. The efficacy of depletion was determined either by functional assays i.e. anti-NK1.1 by the elimination of NK cell cytotoxic activity (Koo, 1986; Rempel, 1997) or FACS analysis of anti-CD4 or anti-CD8 mAb treated mice (unpublished observations).

3.0 Immunization and treatment schedules

3.1 Immunization protocol

Mice were immunized i.p. on day 0 (Note: all days cited are referenced against the time of the primary immunization which is consistently designated as day 0). Secondary and tertiary immunizations followed with at least 3 weeks between immunizations. In most experiments, booster immunizations (i.p) occurred on days 28 and 56 respectively. However, these times did vary, so the days for each experiment will be documented in each section. Generally, in studies focussed on the induction of Ab responses 2.0 μg of OVA (alum) or 100 μg OVA (CFA) were used. In studies attempting to abrogate established IgE production mice were also immunized with 0.2 μg of OVA (alum), the lowest dose consistently able to elicit OVA-specific IgE responses. Doses were kept constant within experiments.

3.2 OA-POL treatment

Typically two OA-POL treatment protocols were used. To inhibit the induction of IgE synthesis, one course of 3 injections of OA-POL (80 μg/day in saline i.p.) on days -14, -12 and -10 was given prior to immunization with 2.0 μg of OVA (alum) on day 0. To abrogate

IgE responses in mice primed with 0.2 μg of OVA, mice were injected with three courses of OA-POL (consisting of 3 injections on alternating days, 80 μg/injection) in weeks 4, 6 and 8 after priming. Ten to fourteen days after the final OA-POL treatment, mice received a boost of 0.2 μg OVA (alum).

3.3 IL-12 treatment

To determine the capacity of exogenous IL-12 to direct the induction of responses in naive mice, mice were treated with or without IL-12 (a gift from Dr. Maurice Gately, Hoffmann-La Roche, Nutley, NJ) from day -1 to 3 (that is the day prior to primary immunization and for the next four days). Mice were not treated with IL-12 upon secondary and tertiary immunizations. IL-12 was administered at 20 to 400 ng/day i.p. in saline containing 0.4% normal mouse serum. To minimize harmful side effects associated with the pharmacologic administration of rIL-12 (Ryffel, 1997), the majority of experiments were carried out with 200 ng/injection of IL-12.

To determine the potential of exogenous IL-12 to alter established humoral responses, mice were not treated with IL-12 at primary immunization. Instead they were treated with IL-12 (200 ng/day x 5 days) beginning the day prior to the secondary boost and for the next four days. This treatment course was repeated at the tertiary boost. Some mice were also treated with IL-12 between OVA boosts. Due to the numerous combinations evaluated in efforts to redirect established type 2 responses by in vivo administration of exogenous rIL-12, schedules will be detailed in coordination with the results obtained by each approach.

3.4 *Trichinella spiralis* extract preparation

T. spiralis extract was prepared and provided by Dr. T. A. Dick, University of Manitoba, MB. Briefly, the extract was prepared as a whole larva homogenate, which was washed throughly and sterilely filtered (Wassom, 1988). Cytokine responses could be generated from the injection of 50 μg of extract in the absence of alum (i.p.). However, alum was required to generate Ab responses (data not shown).

3.5 Anti-NK1.1 treatment

Anti-murine-NK1.1 mAb (PK136 obtained from ATCC, Rockville, MD) was produced in UPF media and concentrated by Centipep (Amicon). Mice immunized with OVA (alum) on day 0 were injected with 0.3 mg on day -1 and 1 (i.p.). Depletion of NK1.1+ cells by this protocol had been previously described (Koo, 1986) and was confirmed by cytotoxicity assay (performed by Cyndy Ellison) and flow cytometry (Wang, 1998).

3.6 Anti-IL-12 treatment

Goat-anti-murine-IL-12 and normal goat IgG was kindly provided by Drs. Fred Finkelman (University of Cincinnati) and Maurice Gately (Hoffman LaRoche). Mice were injected with 1 mg anti-IL-12 (saline, i.p.) on days -15, -7 and -1 +/- OA-POL (80 ug/day) treatment (day -14, -12, -10). Mice were OVA (alum) immunized on day 0.

3.7 Anti-IL-10 treatment

Anti-murine-IL-10 mAb (SXC1 hybridoma, a kind gift from Dr. T. Mosmann, University of Alberta) was produced in UPF media and concentrated. Mice immunized with OVA (alum) on day 0 were injected with 1.0 mg of mAb (i.p.) on day -2, -1, 0, 1, 3 and 6.

4.0 Bleeding

Mice were bled from the tail on days 0, 10 and 14 after primary immunization and 7 days after boosts to determine serum immunoglobulin levels. IgE synthesis was generally assayed on day 10, the time of the peak primary response, while IgG isotypes were assayed on day 14, the time of peak IgG_1 and IgG_{2a} levels. All isotypes were examined 7 days after boosts. Mice were also bled between day 0 and 7 for determination of serum IFNγ production. Mice were bled and analysed individually throughout. Sera were collected by centrifugation the day after bleeding and stored at -20 °C until needed.

5.0 Antibody determinations

5.1 Murine IgE

OVA-specific IgE production was determined by 48 h passive cutaneous anaphylaxis (PCA) in female Sprague-Dawley rats (Ovary, 1986). Briefly, sera were serially diluted in saline as 2 fold dilutions. Rats were injected intradermally with 0.1 ml of each dilution. Forty eight hours, later rats received an i.v. injection of 2 mg OA in Evan's blue dye. The PCA titer was

assessed as the reciprocal of the last positive dilution (>3 mm diameter). Geometric means of duplicate or triplicate analyses and SEM are presented.

5.2 Murine total IgE

Total serum IgE levels were determined by ELISA. Briefly, ELISA plates (Corning 25805, Corning Science Products, Corning, NY) were coated overnight at 4°C with 1 μg/ml of rat anti-mouse IgE (Southern Biotechnology Associates, Inc., Birmingham, AL) in bicarbonate buffer (0.05 M, pH 9.6). Plates were blocked 45 min at 37°C with a 1 % BSA, 0.05% Tween 20 solution and washed. Plates were washed in between all subsequent incubations. Eight 2 fold dilutions of serum samples (with beginning dilutions ranging from 1/50 to 1/1000), and the standard were incubated for 3 h at 37°C or overnight at 4°C. Highly purified anti-DNP mouse IgE, prepared from B cell hybridoma 2682 (a gift of Dr. A. Froese, University of Manitoba) was used as the standard. Biotinylated epsilon specific monoclonal rat anti-mouse IgE heavy chain (0.125 μg/ml) (Serotec, UK) was added overnight at 4°C or incubated for 3 h at 37°C. Detection was by incubation with streptavidin conjugated alkaline phosphatase. A color reaction was produced by the addition of p-nitrophenyl phosphate (Sigma Chemical Co., Oakville, ON). The reaction was allowed to proceed for 1 hour before plates were read at $O.D_{405\text{-}695}$ (Dynatech Instruments, Torrance, CA). This assay had a sensitivity of 1 ng/ml. For all ELISA's the reading time and Ab concentrations were optimized to give maximal sensitivity and accuracy.

5.3 Murine IgG_1, IgG_{2a}

OVA-specific murine IgG_1 and IgG_{2a} were determined in subclass specific ELISA's. Briefly, ELISA plates were coated with 20 μg/ml OVA in coating buffer. Eight 2 fold dilutions of serum samples and standards were incubated overnight. OVA-specific IgG was detected with biotin conjugated goat anti-IgG_1 (0.2 μg/ml) or IgG_{2a} (0.1 μg/ml) (Southern Biotechnology Associates Inc., Birmingham, AL) followed by streptavidin conjugated alkaline phosphatase system. The reaction was allowed to proceed for 100 min before optical density $(O.D.)_{405\text{-}695}$ of the plates were read. Titres were calculated as the reciprocal dilution yielding an O.D. of 0.5 (a value chosen as it is well within the linear portion of the standard curve) and normalized against a polyclonal murine anti-OVA standard run in every assay.

To determine total murine IgG_1 and IgG_{2a} ELISA plates were coated with sheep anti-mouse IgG (H+L) (Jackson Immuno Research, Mississauga, ON) at 2 μg/ml to capture mouse IgG. ELISA's were developed with either biotinylated goat anti-mouse IgG_1 (0.1 μg/ml) or IgG_{2a} (0.2 μg/ml) (Southern Biotechnology Associates Inc.) followed by streptavidin-alkaline phosphatase. A total IgG_{2a} standard was developed from B cell hybridoma PK136 (mouse IgG_{2a}) and calibrated against purified mouse IgG_{2a}, kappa (UPC 10) (Sigma Chemical Co.). The IgG_1 standard was a purified mouse IgG_1 anti-OVA mAb generated by Dr. G. Lang (Univ. of Manitoba, Winnipeg, MB). It was calibrated against $O.D._{280}$. The sensitivity of these assays was typically 0.5 ng/ml for IgG_1 and 0.1 ng/ml for IgG_{2a}.

5.4 Murine anti-FCS

Plates were coated with FCS at a 1/16 dilution in coating buffer. Mouse Ab in culture

supernatants, diluted 8 x, were detected by biotinylated goat anti-mouse Ig followed by streptavidin-alkaline phosphatase. Results are expressed as titers; calibrated as the reciprocal of the dilution with an O.D. of 0.5.

5.5 Bovine Ig

Plates were coated with 2 µg/ml of goat anti-bovine IgG, F(ab') fragment specific (Jackson ImmunoResearch). Bovine Ig was detected by biotinylated goat anti-bovine IgG (H+L) (0.2 µg/ml) (Jackson Immuno- Research), followed by streptavidin-alkaline phosphatase. Results were calibrated against purified bovine gamma globulin (Jackson ImmunoResearch). The detection limit was 0.15 µg/ml.

5.6 Rat IgM

Plates were coated with 5 µg/ml of goat anti-rat IgM. mAb preparations were titrated 8 x and calibrated against purified rat IgM (Chemicon, Temecula, CA). Rat mAb was detected with 1 µg/ml of alkaline phosphatase-conjugated goat anti-rat IgG and IgM (H + L) (Jackson ImmunoResearch). Detection limit was 1.25 ng/ml.

6.0 Cell culture

6.1 Preparation of spleen cells

Mice were sacrificed and spleens were removed aseptically. Glass homogenizers were used to prepare single cell suspensions in 5 % newborn calf serum (Gibco-BRL Products), RPMI

1640. Spleen cells were passed through nytex filters (B & SH Thompson & Co. Ltd, Scarborough, ON) to remove debris and centrifuged once for 4 min at 2000 rpm (300 g). Cells were resuspended in 10 % FCS. The number of cells was determined by counting in a hemocytometer after dilution in trypan blue (0.4 %) and acetic acid (40 %) (Neplean, ON).

6.2 Spleen cell culture

For bulk culture, cells were cultured at 7.5 x 10^6cells/ml (2 ml/well) alone or with OVA at 0.3 or 1.0 mg/ml, concanavalin A (Con A) (5 μg/ml) or plate-bound anti-CD3 mAb (145-2C11 hybridoma, a gift of Dr. J. Bluestone, University) in 24 well plates (Corning Science Products, Rochester, NY) at 37°C in 10%FCS. On occasion, 48 well plates with final volumes of 1 ml/well were used. Cells from a minimum of two to four mice/group/time point were cultured and analysed independently. Cultures were harvested at 24, 48, 72 or 96 hours and stored at -20°C until cytokine analysis was performed.

To determine IL-12 p40 production spleen cells were cultured at 7.5 x 10^6cells/ml (0.2 ml/well) alone or with OVA (0.3 or 1.0 mg/ml) or LPS (1, 10 or 100 ng/ml) (Sigma Chemical Co.). Cultures were harvested at 18, 24, 36, 48 and 58 hrs.

6.3 In vitro cytokine treatment

rIL-12 (100 pg/ml) and/or IL-2 (10 U/ml) were diluted in 10 % FCS/RPMI and added to some cultures as detailed at Results.

6.4 In vitro cell depletion

Anti-CD4 mAb YTS 191.1 and GK1.5 culture supernatant (grown in 10 % FCS/RPMI) was concentrated 4 to 5 x. One part YTS 191.1 and one part GK1.5 were combined and filtered sterilized. This was added to culture at a dilution of 1/5, a titration previously found to block CD4 T cell dependent cytokine synthesis.

6.5 Enrichment and culture of CD4 T cells

Spleen cell suspensions were panned on goat anti-mouse Ig (500 μg/plate) coated petri plates to deplete adherent and sIg+ cells. Nonadherent cells were harvested and passed through a CD4 T cell negative selection column (Biotex Laboratories Inc., Edmonton, AB), yielding 92 to 96% CD4 positive cells with less than 0.7% contamination with B cells or CD8+ T cells as assessed by subsequent flow cytometry (Dr. Ed Rector, University of Manitoba). Culture was carried out using 10^6 responder cells per 200 μl well, 3 x 10^5 irradiated naive spleen cells as a source of APC and 1 mg/ml of OVA in preference to polyclonal activators.

6.6 Limiting dilution analysis (LDA)

Five days after final immunization, mice were sacrificed. Enriched CD4 cells were serially diluted (40000 to 312 cells/well; 36 replicate wells per dilution) and cultured in round bottom 96 well culture plates (Corning Science Products) with 3 x 10^5 irradiated spleen cells (1500 rad.), 20 U/ml rIL-2 (Chiron Corporation, Seattle, WA) and OVA (1 mg/ml). Each plate also included 12 wells of irradiated naive spleen cells in the absence of responder cells as negative controls. To evaluate potential spontaneous cytokine production, duplicate cultures were

established for high responder cell concentrations (10,000 to 40,000 cells/well) with IL-2 in the absence of OVA. Cultures were incubated 14 days, at which time proliferation was visually determined under low level magnification (10x). All cells were then washed and restimulated with fresh irradiated APC (3×10^5) and OVA (1 mg/ml). Supernatants were harvested at 48 h and assayed for IFNγ, IL-10 and IL-4 production. In each assay, wells were considered positive for cytokine production if the absorbance obtained in a given well was more than three SD above the mean value obtained in wells containing APC, rIL-2 and Ag in the absence of CD4 cells (negative control on each plate).

6.7 Enrichment and culture of non-B/non-T cells

Non-B/non-T populations were isolated by negative selection using a protocol based on that previously reported (Aoki, 1995). Briefly, spleen cells were depleted of red blood cells (with NH_4Cl), incubated on ice with FITC-conjugated rat IgG_{2a} anti-mouse CD4, -CD8, and/or -CD19 (PharMingen) were added directly to the appropriate wells at final Ab concentrations of 1.5, 1.5 and 3.0 ug/million cells respectively. Control samples were incubated with FITC-conjugated rat anti-mouse IgG_{2a} at 1.5 ug/0.5 million cells (PharMingen). After 45 minutes at 4°C, cells were washed twice. The unstained cells were negatively selected by flow cytometry (Dr. Ed Rector). The resulting, negatively selected population contained less than 5 % contaminating B and T cells. Culture of negatively selected non-B/non-T cells was carried out under the same conditions in 200 µl wells using 500,000 flow cytometry selected cells/well, also in the presence of OVA. Culture supernatants were harvested for analysis of IL-4, IFNg and IL-10 production at 24, 48 and 96 h respectively, time points previously

found to correspond to maximum cytokine levels.

7.0 Cell staining

Spleen cells were stained to determine the impact of IL-12 administration on the proportion of different cell populations within the spleen. 5×10^5 spleen cells were added to round bottom wells, centrifuged and washed 2x in buffer (0.5% BSA/PBS). FITC-conjugated rat IgG_{2a} anti-mouse CD4, -CD8, -CD19 and control Ab (PharMingen) were added as stated above. After 45 minutes at 4°C, cells were washed and fixed with 50 ul of paraformaldehyde or analysed immediately (Dr. Ed Rector). The proportion of CD4, CD8 and CD19 positive cells was determined. The proportion of non-B/non-T cells was determined by subtraction.

8.0 Murine cytokine determinations

8.1 IL-4

IL-4 levels were determined in an MTS assay using CT.4S cells (a kind gift of Dr. W. Paul, NIAID, NIH, Bethesda, MD) as previously described from supernatants harvested at 24 h, unless otherwise indicated (Gieni, 1993b). Briefly, supernatants were titrated four times in 50 ul of 10 % FCS. The laboratory standard (produced by injecting 10 % FCS into mice and culturing in 10 % FCS five days later) was calibrated against an rIL-4 standard (a gift from Dr. W. Paul). 7000 CT.4S cells/well were added and incubated at 37 °C for 24 hrs. MTS (3-(4,5-dimehtylthialzol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,

Promega Corporation, Madison, WI; 1 mg/ml), PMS (N-methyldibenzopyrazine methyl sulfate salt, Promega Corporation; 0.92 mg/ml) and FCS media were added together at a ratio of 1:0.005:2. Plates were incubated for an additional 18 hours and read at $O.D._{495-696}$. Detection limits were typically 0.5 U/ml for IL-4.

8.2 IFNγ

IFNγ was captured with anti-IFNγ mAb XMG 1.2 (a gift of Dr. T. Mosmann) coated at 3 μg/ml and detected with biotinylated mAb R4-6A2 (American Type Culture Collection, Rockville, ML) in conjunction with streptavidin-alkaline phosphatase. Biotinylated R4-6A2 was made in house. Due to the variations between lots, the usage optimal dilution was determined for independent lots. IFNγ standard (produced by stimulating spleen cells in the presence of Con A and IL-12) was calibrated against WHO-NIAID IFNγ reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, NIH, Bethesda, MD). Detection limits were typically 0.2 U/ml for IFNγ.

8.3 IL-10

IL-10 was captured with anti-IL-10 mAb SXC1 (3 μg/ml) and detected with biotinylated SXC2 (both hybridomas a gift of Dr. T. Mosmann, biotinylated by Bill Stefura, University of Manitoba) in conjunction with streptavidin-alkaline phosphatase. Due to the variations between lots, the usage optimal dilution was determined for independent lots. An IL-10 standard was produced by culturing spleen cells from mice injected with 10 % FCS in 10 % FCS on day 5. Standards were calibrated against rIL-10 standard provided by Dr. T.

Mosmann. The lower limit of detection was 0.5 U/ml.

8.4 IL-5

IL-5 was captured with anti-IL-5 mAb (PharMingen) (0.5 μg/ml) and detected with biotinylated anti-IL-5 (PharMingen) (0.3 μg/ml) in conjunction with streptavidin-alkaline phosphatase. Samples were diluted 2 fold 4 times and calculated against rmIL-5 standard (PharMingen). Detection limit was 15 pg.ml.

8.5 IL-13

IL-13 was captured with anti-IL-13 mAb (PharMingen) (1 μg/ml) and detected with biotinylated anti-IL-13 (PharMingen) (0.5 μg/ml) in conjunction with streptavidin-alkaline phosphatase. Samples were diluted 2 fold 4 times and calculated against rmIL-13 standard (PharMingen). Detection limit was typically 5 pg/ml.

8.6 IL-12 p40

IL-12 p70 was captured with anti-IL-12 p40/p70 mAb (PharMingen) (1 μg/ml) and detected with biotinylated anti-IL-12 p40/p70 (PharMingen) (0.5 μg/ml) in conjunction with streptavidin-alkaline phosphatase. Samples were diluted 2 fold 4 times and calculated against rmIL-12 standard (PharMingen). Detection limit was 30 pg/ml.

9.0 Lipopolysaccharide determination

The concentration of LPS in OVA was determined using the Limulus Amebocyte Lysate Pyrochrome Chromogenic Test Kit by Associates of Cape Cod, Inc. (Woods Hole, MA). Detection limit 0.0005 ng/ml LPS.

10.0 Statistical analysis

For each ELISA assay described, data were obtained from a titration of at least four dilutions against the standard curve run in every assay. OVA-specific IgE data were $\log_2$ transformed and analysed as geometric means. Generally, the comparison of two means was determined by unpaired two-tailed Student's t-test. The exception was that outbred (CD1) mice required evaluation by a non-parametric test (Mann-Whitney U) for Ab and serum IFNg levels. To compare multiple means, ANOVA and Tukey's tests were performed. Estimates of the precursor frequency of CD4 T cells reactive to OVA were obtained by both the maximum likelihood and minimum X^2 methods based on the Poisson distribution relationship between the number of responding cells and the logarithm of the fraction of negative cultures. Software was generously provided by Dr. C. Orosz (Ohio State Univ., Columbus, USA). The results obtained were similar using either method. Maximum likelihood results are presented.

IV. Results

1.0 Introduction

The primary focus of this thesis is to examine cytokine regulation of humoral responses within the context of immediate hypersensitivity. Immunization of C57Bl/6 with low doses of OVA in the presence of alum is a murine model which generates characteristic immediate hypersensitivity associated responses (strong Ag-specific IgE levels, low Ag-specific IgG_{2a}).

2.0 Production of depleting monoclonal antibodies for in vivo use

In this study, various mouse specific mAb were required for in vivo depletion of either cytokines or cells. mAb used were grown in the lab from hybridomas. Originally mAb were grown in 10% FCS and Biopilot purified. The Biopilot system separates proteins based on ionic charge and molecular weight. This procedure worked well when we used depleting mAb to examine the impact these depletions had on serum Ab levels in acute studies. However, we became increasingly interested on the impact in vivo neutralization of cells or cytokines with depleting mAb had on spleen cell cytokine production. In these studies, it became apparent that cultures from mice injected with either Biopilot purified, or raw culture supernatant preparations of mAb, produced spontaneous and substantial non-OVA driven IL-10 and IL-4 upon in vitro culture of spleen cells in FCS containing media (Table 1). This was seen to be independent of which mAb isotype was injected or its relevance to the mouse

Table 1. Raw and Biopilot purified mAb tissue culture supernatant causes spontaneous cytokine production

Exp.	Treatment[a]	IL-10 (U/ml)[e]		IL-4 (U/ml)	
		No OA	OA	No OA	OA
1	None	0	10	0	4
	Rat IgG2b anti-mouse CD4 BP[b]	6	3.5	11	2
2	None	0	12	1	3
	Mouse IgG1 anti-human MHC II BP[c]	34	37	21	35
	Mouse IgG1 anti-human MHC II TC[c]	25	24	27	60
3	None	0	16	0	7
	Mouse IgE anti-DNP BP[c]	57	69	22	47
4	None	1	16	3	26
	Rat IgM anti-mouse IL-10 BP[c]	62	83	38	60
	Rat IgM anti-mouse IL-10 BP[d]	109	101	26	29

[a]Mice were immunized with 2.0 µg OVA (alum) on d. 0. Mice were either injected with mAb preparations TC or BP on days [b] -2 and 0 or [c] -1 and 1. TC and BP mAb preparations were made as follows: B cell hybridomas were grown in 10 % FCS media until they reached 20 % viability. Supernatants were harvested and frozen until analysis as raw tissue culture supernatants (TC) or purified and concentrated by Biopilot system (BP).

[d] Some mice were also treated with mAb (on days -1 and 1) in the absence of OVA (alum) immunization.

[e] On day five mice were sacrificed and spleen cells were cultured in the presence and absence of OVA. Supernatants were harvested at 24 and 96 h for IL-4 and IL-10 determination respectively. Means (n = 2 or 4 mice) are shown.

system indicating that the cytokine production observed (in the absence of OVA stimulation) was due to a common contaminant in the Ab preparations. IFNγ (data not shown) was also spontaneously produced, but at much lower levels (refer to Table 3). Therefore, we hypothesized that in addition to injecting the mAb of interest, we were also sensitizing mice to bovine Ig co-purified (according to ionic charge and molecular weight) in the final antibody preparation. When spleen cells were subsequently cultured in vitro, they reacted against bovine Ig in the FCS media.

To prevent non-specific cytokine production after mAb treatment and in vitro culture in FCS containing media, mAb were produced in UPF media. UPF is a protein free media containing a rich cocktail of amino acids. Spleen cell culture supernatants from mice injected with mAb purified on the Biopilot and from UPF preparations were tested to see if they contained mouse Ab against FCS containing media. While neither the supernatants from mice that received nothing or UPF produced mAb generated a detectable response against FCS, Biopilot purified mAb had mouse-anti-bovine titres of approximately 150 (Fig 1). This was independent of whether the cultures were stimulated with OVA or not.

The bovine Ig content of Ab tissue culture supernatant, Biopilot and UPF preparations were assayed (Table 2). The relative percentage of bovine Ig was the same in the mAb tissue culture preparation and the Biopilot preparation. However, bovine Ig was not detected in the UPF produced mAb (detection limit 1 μg/ml).

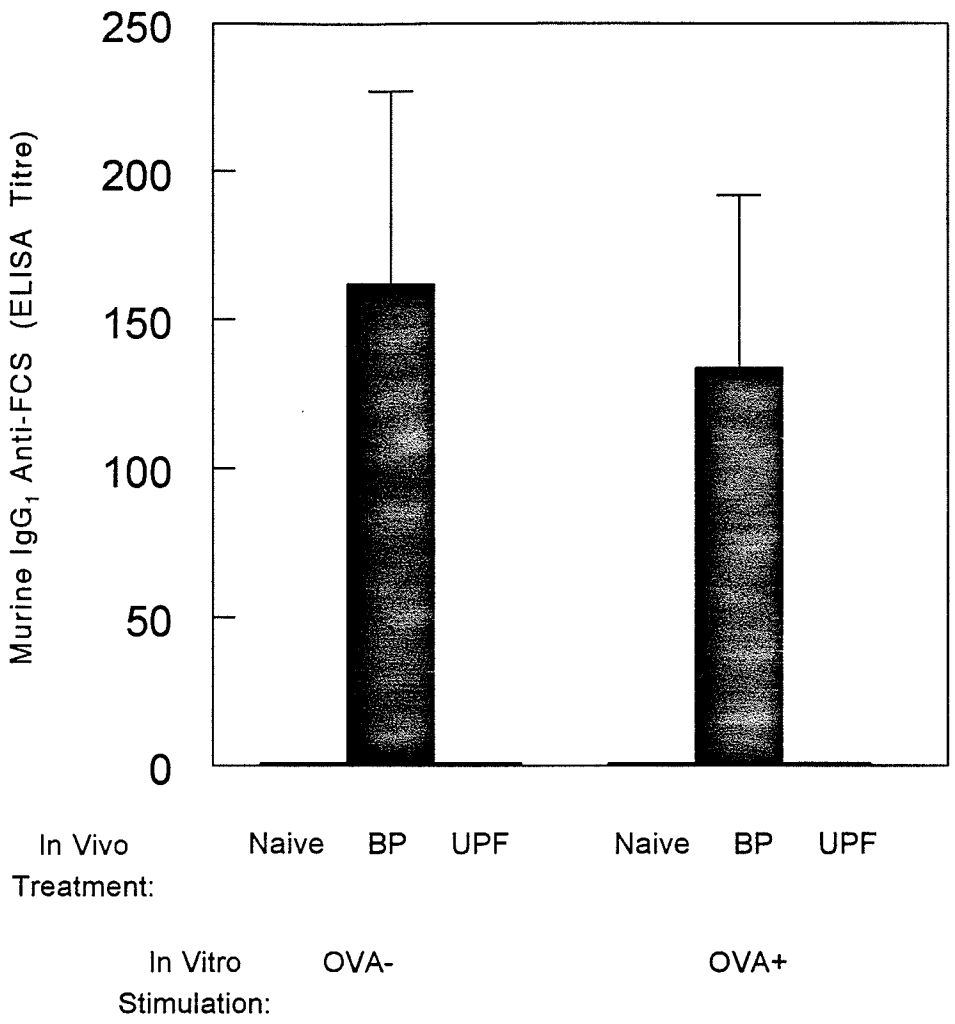

Figure 1. Injection of Ab preparation contaminated with FCS results in mouse anti-FCS responses upon culture of spleen cells in 10% FCS media. Immunized (d. 0) mice were treated on d. -1 and 1 with nothing, Ab (anti-IL-10) BP preparation or Ab UPF preparation as per Table 1. Mice were sacrificed on d. 5. Spleen cells (15 million/2 ml) were stimulated in the presence and absence of OVA (1 mg/ml). Supernatants were harvested on day 4 and analyzed for mouse IgG_1 anti-FCS by ELISA. Means ± SEM (n = 4 mice) are shown.

Table 2. Bovine Ig is not present in UPF antibody preparation

mAb preparation[a]	Rat IgM (μg/ml)	Bovine Ig (μg/ml)	Bovine Ig in mAb preparation (%) [b]
mAb TC	126	10	7.3
mAb BP	1600	185	10.4
mAb UPF	1994	<0.3	$<1 \times 10^{-4}$

[a] mAb (rat IgM anti-mouse-IL-10) was prepared from B cell hybridoma (SXC-1) cultures. TC and BP preparations were made as stated in Table 1. UPF preparation was made as follows: B cell hybridoma was grown up in 10 % FCS until cells had reached a concentration of 200,000 to 400,000 cells/ml. Cells were washed 2 times in RPMI (to remove FCS) and transferred into UPF media. Once cells reached 20 % viability, supernatants were harvested and concentrated (approximately 100 fold) in pressure chamber (100, 000 d cut off).

[b] Rat IgM and bovine Ig concentrations were determined by ELISA. The percent of bovine Ig was calculated as (bovine Ig)/(rat IgM + bovine Ig).

Table 3. mAb UPF supernatant does not induce spontaneous cytokine expression

In vivo treatment[a]	In vitro stimulation and cytokine production			
	IFNγ (U/ml)		IL-4 (U/ml)	
	No OA	OA	No OA	OA
None	0	34	0	35
mAb BP	3	4	55	59
mAb UPF	2	19	2	30

[a]Mice were immunized with 2.0 μg OVA (alum) on d. 0 and treated on d.-1 and d. 1 with mAb preparations as indicated. The mAb preparations are from an anti-IL-10 hybridoma. At this dose anti-IL-10 did not affect Ab responses. Refer to Tables 1 and 2 for description. On day five, mice were sacrificed and spleen cells were cultured in the presence and absence of OVA. Supernatants were harvested and cytokine production was determined. Means (n = 3 mice) are shown.

To determine whether the injection of mAb grown in UPF media would induce bovine specific immunological effects in vitro, mice were injected with mAb grown in UPF or 10 % FCS. It was evident that injection with mAb grown in UPF, unlike mAb produced in 10 % FCS, did not stimulate cytokine production from spleen cells in the absence of in vitro Ag when cultured in 10 % FCS (Table 3).

Collectively, this argues that bovine Ig is co-purified with the hybridoma Ig when it is grown in FCS media and Biopilot purified. Contaminating bovine proteins injected into mice prime lymphatic cells, such that when they are cultured in FCS containing media they are stimulated to produce cytokines and Ab. Preparing mAb in UPF media, in the absence of bovine proteins, eliminates this problem.

3.0 Alum alone did not induce IgE synthesis

It has been demonstrated in our lab, and in others, that alum is required for the induction of substantive exogenous Ag-specific IgE responses upon protein immunization (unpublished observations). However, to assess concerns that alum might contribute non-specifically to IgE production, mice were injected with OVA (alum), alum alone or nothing. OVA (alum) immunization produced serum Ag-specific IgE titers and total IgE levels as previously. Sera from mice injected with alum alone or nothing, had undetectable levels of Ag-specific IgE (data not shown). Likewise, total serum IgE production from these cohorts were comparable (Fig. 2) and did not increase following injection of alum alone. This implies that the majority

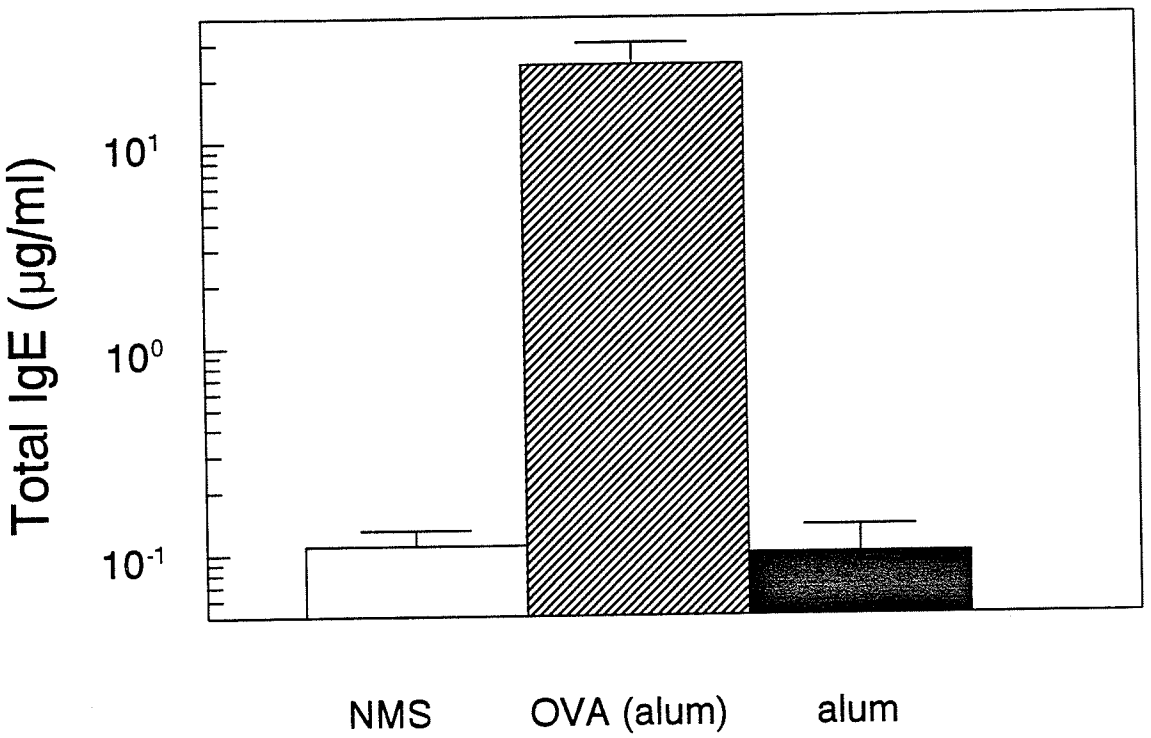

Figure 2. Alum alone does not induce nonspecific IgE responses. Mice were immunized with 2 µg OVA (2 mg alum), or 2 mg alum. Normal mouse sera (NMS) was also evaluated. Mice were bled 10 days after immunization for determination of IgE production. Means ± SEM (n = 8 mice) are shown.

IgE produced upon immunization with OVA in alum is OVA-specific and that the presence of adjuvant does not result in detectable non-OVA specific IgE synthesis.

This is supported by a correlation value of r = 0.84 between Ag-specific and total IgE production (Fig. 3). Mice were immunized and treated under a variety of immunization conditions yielding (Ag-specific) PCA titres ranging from 10 to 8000. The strong correlation between Ag-specific IgE and total IgE strongly argues that the total IgE observed upon protein immunization with alum is predominately Ag-specific IgE. In contrast, Ag-specific and total IgG_{2a} production was not correlated (r = 0.26).

Together this data indicates that while alum is required for the generation of measurable IgE responses, it does not does result in substantial polyclonal IgE production.

4.0 Exogenous rIL-12 enhanced IFNγ production in vitro

IL-12 is a strong promoter of type 1 associated immunity in vitro as demonstrated by the induction of IFNγ synthesis from naive T cells, primed CD4 T cells, Th1 clones and NK cells. We wanted to confirm the capacity of IL-12 to enhance IFNγ production in our in vitro culture systems. Naive and OVA (alum) immunized mice were sacrificed. Spleen cells were stimulated in vitro in the presence and absence of OVA and co-cultured with rIL-12, rIL-2, or IL-12 and IL-2 together. Supernatants were analyzed for changes in IFNγ, IL-10 and IL-4 synthesis.

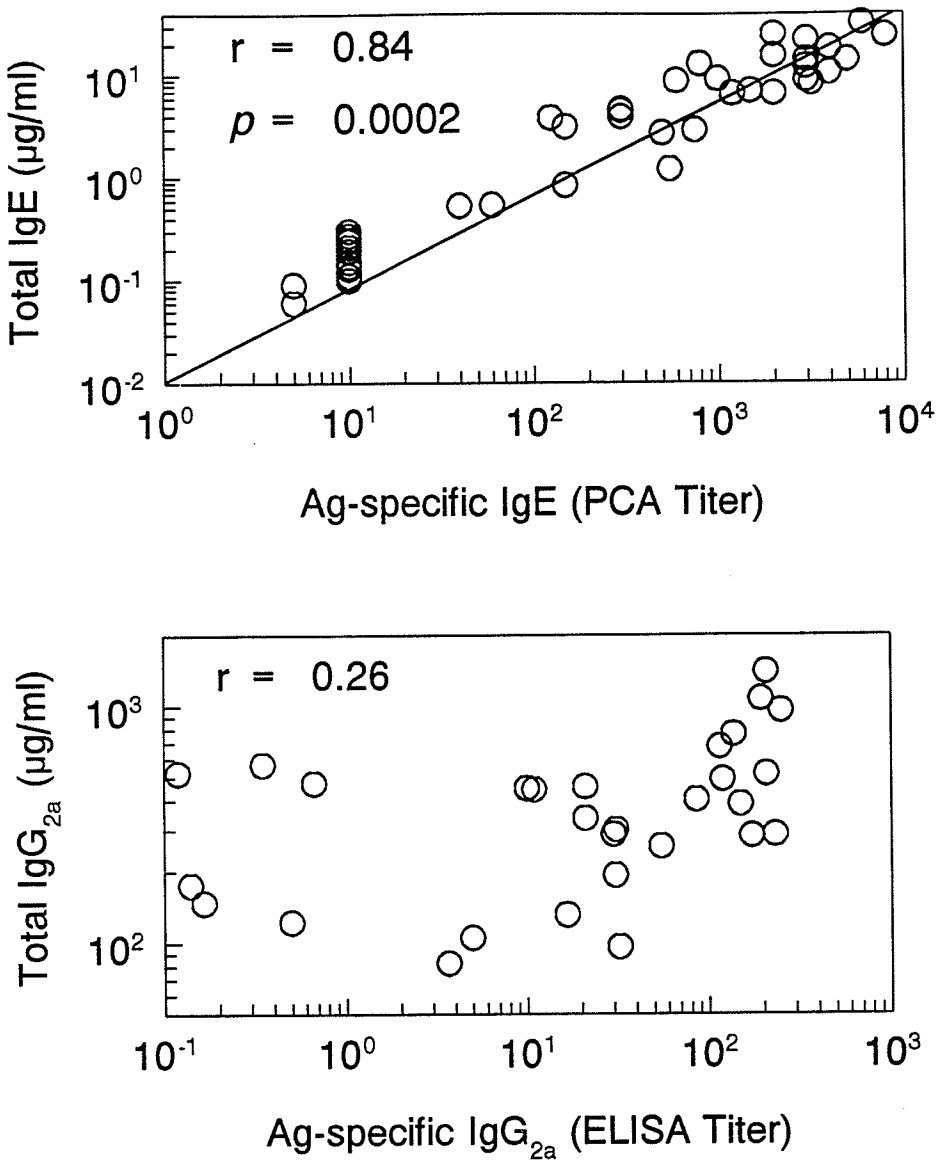

Figure 3. Ag-specific and total IgE synthesis is highly correlated upon OVA (alum) immunization. Mice were immunized under a variety of conditions to ensure a broad range of Ag-specific IgE production. Mice (n = 24 mice; 48 samples) were bled on days 10 and 35 for determination of total and Ag-specific IgE and IgG_{2a} levels.

Naive cells (Fig. 4) stimulated with media alone or OVA alone, and primed cells (Fig. 5) stimulated with media alone, produced negligible amounts of IL-4, IFNγ and IL-10. OVA stimulated cultures from primed cells exhibited typical IL-4, IFNγ and IL-10 levels. The addition of IL-12 to culture greatly elevated IFNγ production regardless of whether cells had been primed in vivo or in vitro stimulated. IL-2 alone did not impact cytokine secretion, but consistently enhanced the capacity of IL-12 to elevate IFNγ synthesis.

Aside from slightly enhanced IL-10 production by naive cells in the absence of Ag, IL-12 did not affect IL-10 or IL-4 synthesis when added to cultures.

Taken together, these data indicate that exogenous IL-12 can enhance IFNγ production from spleen cells regardless of immunization or in vitro Ag-stimulation. This also demonstrates that IL-12 can promote type 1 immunity in populations that are primed to produce a type 2 dominated cytokine response by greatly elevating IFNγ production.

5.0 In vivo rIL-12 administration, in the absence of immunization, did not alter Ag-driven cytokine production

Prior to assessing the therapeutic utility of IL-12, we wanted to first evaluate the impact of IL-12 treatment in vivo on serum IFNγ, splenomegaly and OVA stimulated spleen cell culture in the absence of immunization. As described above, in vitro addition of exogenous IL-12 prominently elevated IFNγ production in an Ag-independent manner. In addition, previous

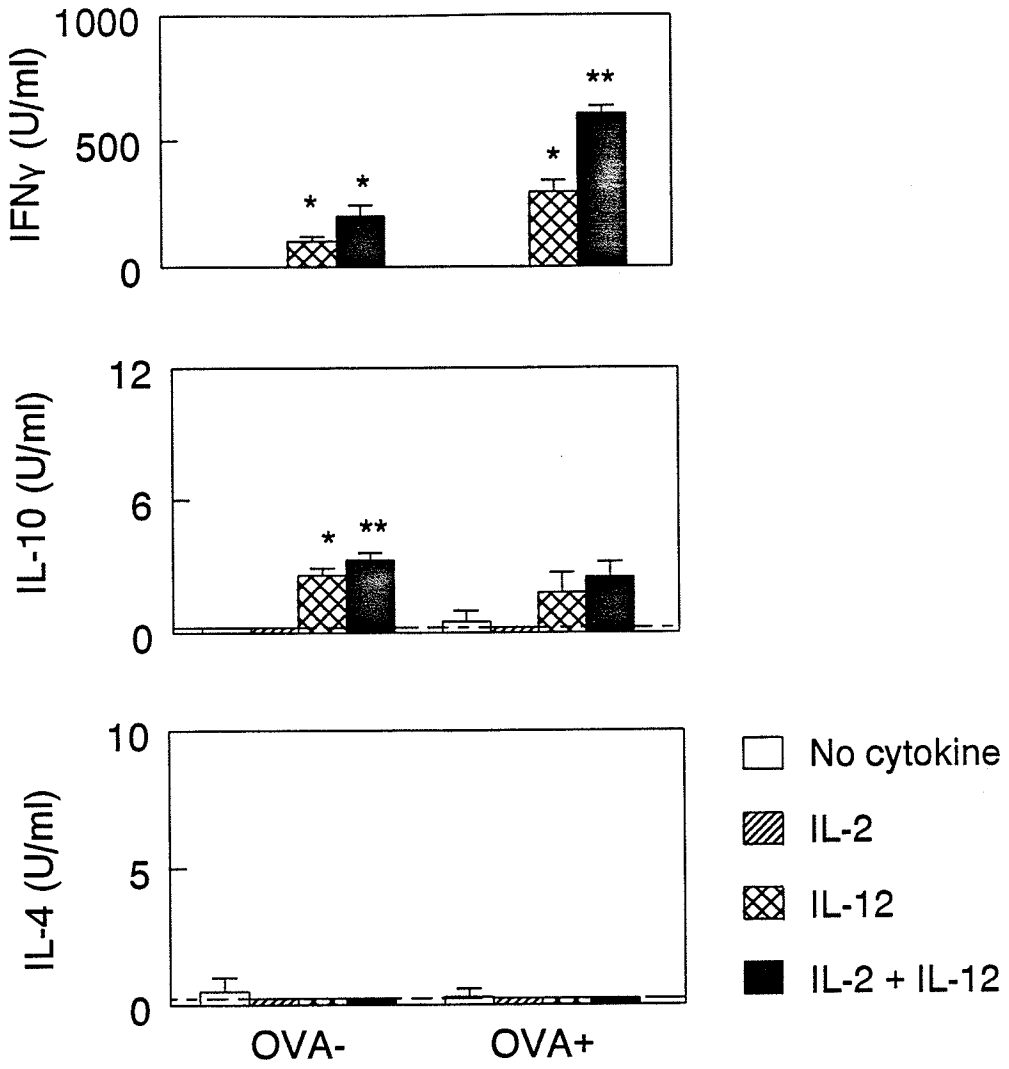

Figure 4. In vitro addition of exogenous IL-12 enhances IFNγ synthesis from naive spleen cells. Spleen cells from naive mice were cultured (15 million cells/2 ml) with and without 1 mg/ml of OVA. No exogenous cytokine or IL-2 (10 U/ml) and/or IL-12 (100 pg/ml) were added to the cultures. Supernatants were harvested at 24, 48 and 96 h for analysis of IL-4, IFNγ and IL-10 production respectively. Means ± SEM (n = 3 mice) from one representative experiment of two are shown. Significant differences to values from cultures without exogenous cytokine added are indicated (*p* values $<0.05^{*}$, and 0.005^{**}).

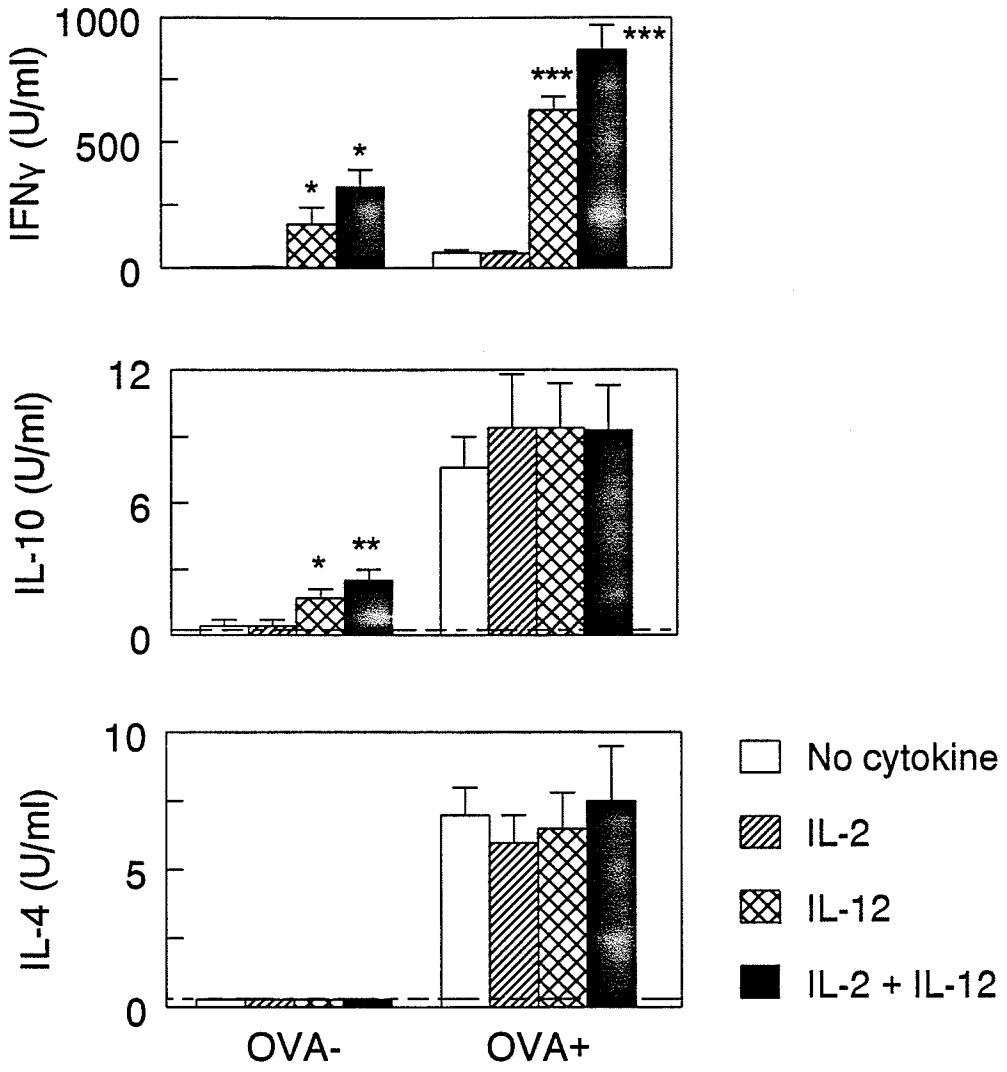

Figure 5. In vitro addition of exogenous IL-12 to spleen cells from immunized mice enhances IFNγ synthesis but fails to influence OVA-specific IL-4 or IL-10 synthesis. Mice were immunized with 2 μg OVA (alum). Five days later spleen cells were cultured with and without OVA (1 mg/ml). No exogenous cytokine or IL-2 (10 U/ml) and/or IL-12 (100 pg/ml) were added to the cultures. Means ± SEM from eight experiments are shown. Significant differences to values from cultures without exogenous cytokine added are indicated (p values $<0.05^{*}$, 0.001^{**} and 0.0001^{***}).

reports observed that IL-12, administered in vivo at high doses (1000 ng/injection x 5 injections), caused detrimental side effects including splenomegaly and abnormal CD3- and B220- cellular proliferation (McKnight, 1994; Ryffel, 1997).

Naive mice were treated with IL-12 and bled to determine the impact of IL-12 administration on serum IFNγ levels (Fig. 6). In the absence of, and prior to, IL-12 treatment, mice had undetectable serum IFNγ levels. Mice treated with IL-12 generated heightened IFNγ synthesis (12 U/ml compared to <1 U/ml in untreated mice). When IL-12 administration was stopped these values fell rapidly. By day 6 (2 days after the last IL-12 injection) the serum IFNγ levels in IL-12 treated mice were not significantly different from those seen in untreated mice. IL-12 treatment also caused enlarged spleens (determined by cell counts), confirming earlier reports that IL-12 caused proliferation of spleen cells (Fig 7). We did not examine the impact of IL-12 at 1000 ng/ml (5 x) reported in most literature, since a large portion of mice treated with this dose died in preliminary experiments.

Important for our investigations, however, was the finding that in vivo IL-12 treatment of non-immunized mice did not induce cytokine production in media or Ag-stimulated spleen cell culture (data not shown). This argued that the evaluation of Ag-driven cytokine production by spleen cells from immunized/IL-12 treated mice reflected the influence of IL-12 on spleen cell cytokine response to OVA immunization and not a generalized alteration of cytokine responses following IL-12 treatment.

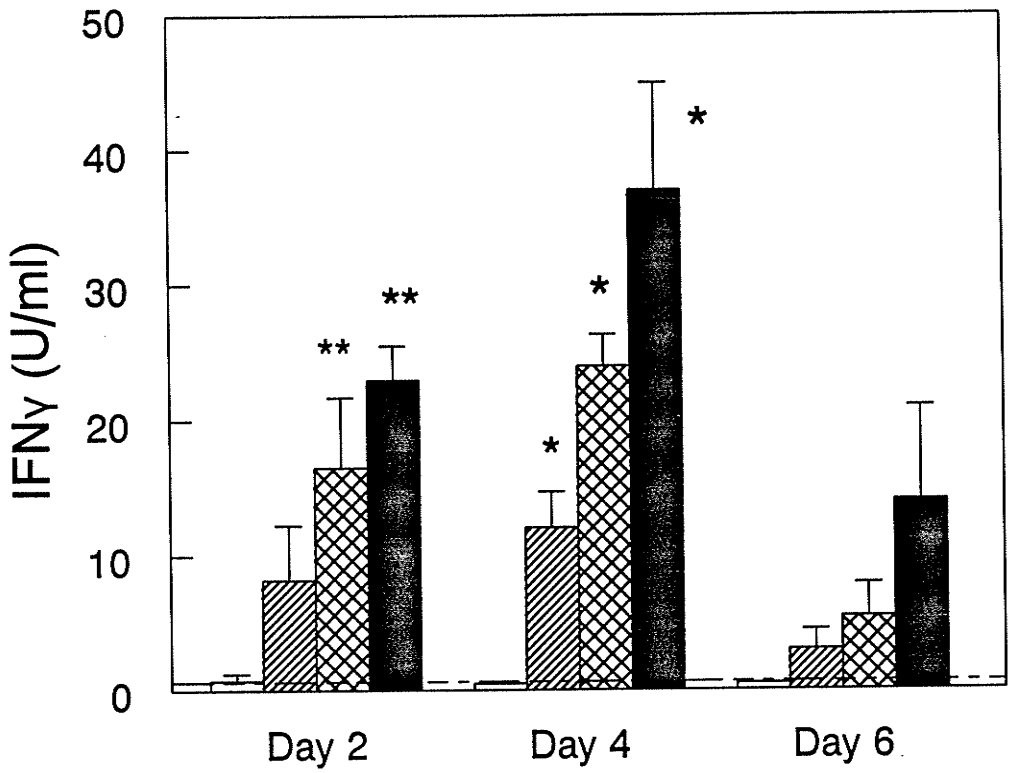

Figure 6. In vivo treatment with rIL-12 enhances serum IFNg in naive mice. Mice were treated with 0, (☐), 100 (▨), 200 (▩) or 400 (■) ng/day of IL-12 i.p.in 0.5 % NMS/saline. Treatments were given on days 0 to 4. Mice were bled on indicated days. Mean serum IFNγ levels (± SEM) (n = 4 mice) are shown. Significant differences compared to untreated cohorts are indicated (p values $<0.05^{*}$, and 0.005^{**}).

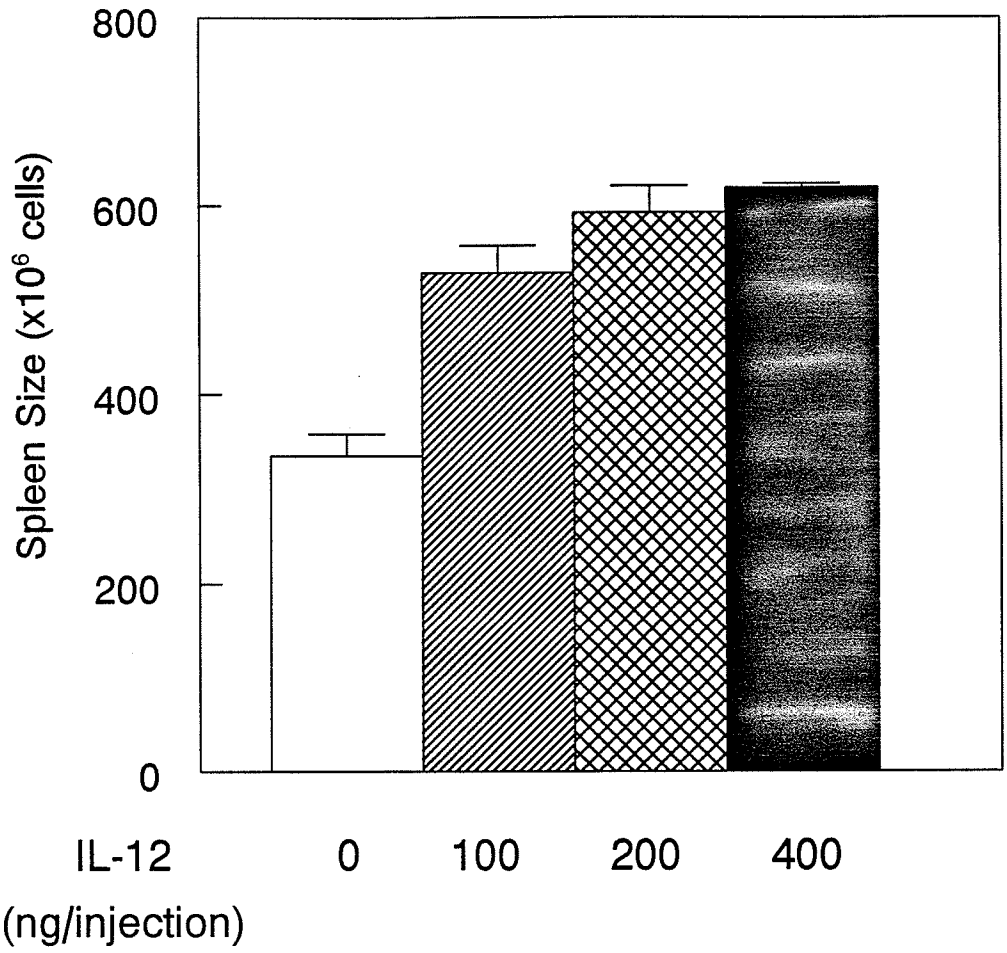

Figure 7. In vivo IL-12 administration causes spleen enlargement. Naive mice were treated with 0, (☐), 100 (☑), 200 (☒) or 400 (■) ng/day of IL-12 i.p.in 0.5 % NMS/saline. Treatments were given on days 0 to 4. Mice were sacrificed on day 7. Mean spleen counts (± SEM) (n = 2) from one representative experiment are shown.

Therefore, while IL-12 administration (in the absence of immunization) increased serum IFNγ production, Ag-driven cytokine synthesis by spleen cells in the absence of immunization was not detectably influenced by IL-12 treatments under any of the conditions examined.

6.0 In vivo administration of rIL-12, simultaneous with immunization, profoundly, but transiently, induced type 1-like cytokine and antibody responses

6.1 In vivo administration of rIL-12 inhibited primary, but not secondary, IgE responses

We hypothesized that IL-12 administration at primary immunization would be able to redirect the Ag-specific type 2 Ab production normally associated with OVA (alum) immunization (high IgE, low IgG_{2a}) to a type 1 dominated profile (low IgE, high IgG_{2a}). To assess the amount of IL-12 required to inhibit IgE production, preliminary experiments were performed utilizing 0, 20, 100, 200 and 400 ng/injection of IL-12 (Table 4). Mice were bled for evaluation of serum IgE and IgG levels. A dose dependent capacity to inhibit IgE production was seen with 100, 200, and 400 ng/injection yielding similar inhibition of IgE production. In an effort to minimize side effects, we decided to continue our investigations using 200 ng of IL-12 per injection.

The true utility of an adjuvant is determined by its capacity to induce the desired long term immunological response towards a given stimulus. Thus, we evaluated the capacity of IL-12 to maintain IgE suppression upon subsequent Ag-exposure. Mice were OVA (alum)

Table 4. IL-12 inhibits primary IgE production.

	IL-12 (ng/day)[a]				
	0	20	100	200	400
Ag-specific IgE (PCA Titer)	2080 ± 82	836 ± 180	20 ± 24	12 ± 2	10 ± 0

[a] Mice were immunized with 2.0 μg OVA (alum) on day 0 and treated with IL-12 from days -1 to 3 inclusive. On day 10 mice were bled for Ab determination. Geometric means ± SEM (n = 3 mice) from one representative experiment are shown.

immunized and treated with 0 or 200 ng of IL-12, then OVA boosted at 4 week intervals in the absence of additional IL-12. As previously, administration of IL-12 at 200 ng/day (x 5 d.) resulted in virtual abolition of primary Ag-specific IgE responses (Table 4, Fig. 8). Ag-specific IgG_{2a} production was enhanced 17-fold. However, in spite of the extreme inhibition rIL-12 treatment had on primary Ab responses, over eight independent experiments secondary and tertiary IgE responses did not differ from untreated animals. IgG_{2a} production remained somewhat enhanced.

Therefore, given together with primary immunization, rIL-12 can redirect an inherently type 2 Ab response to a type 1 direction both by inhibiting type 2 associated Ab production and elevating type 1 associated Ab synthesis. Notwithstanding the capacity of IL-12 to inhibit IgE synthesis by > 99 % shortly after administration, exogenous IL-12 was not capable of inducing Ag-specific memory required to sustain that inhibition upon re-exposure to the sensitizing Ag.

6.2 Exogenous IL-12 administration transiently enhanced serum IFNγ production in immunized mice

Cytokines have a large influence on humoral responses. IL-12 treatment in the absence of priming/immunization enhanced IFNγ production in vitro and in vivo. We examined changes in IL-12 induced IFNγ production in immunized mice to determine cytokine responses which may be responsible for the changes we observed in Ab production.

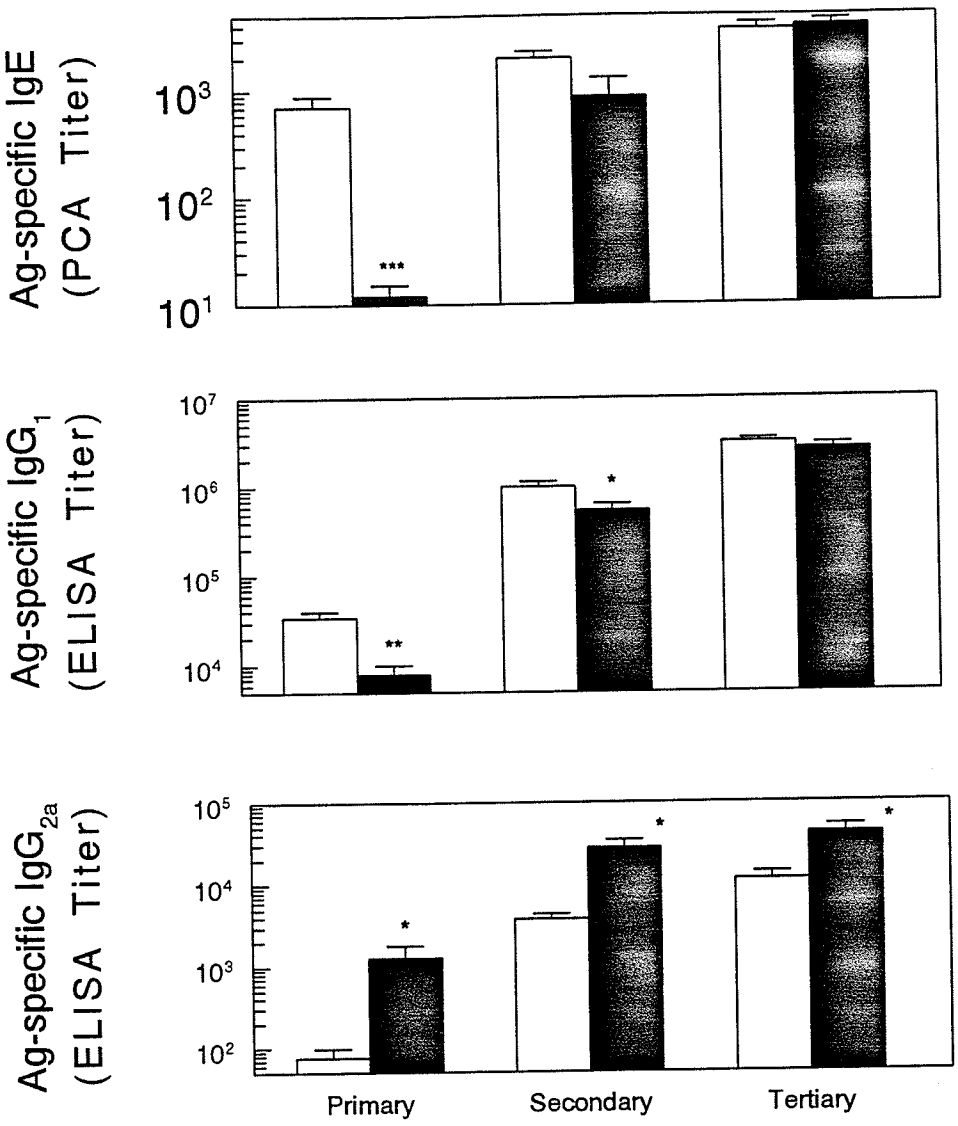

Figure 8. In vivo administration of rIL-12 profoundly, but transiently, inhibits Ag-specific IgE synthesis while enhancing IgG_{2a} production. IL-12 was administered at 0 (□), or 200 ng/day (■) for five days beginning the day prior to primary OVA immunization. Mice were bled on day 10 and 14 for determination of Ag-specific IgE and IgG's respectively. Mice were boosted on day 28 and 56 and bled 7 days after each boost. Geometric means (IgE) or means (IgG_1, IgG_{2a}) ± SEM of eight experiments are shown. Significant differences ($p<0.05^{*}$, 0.001^{**} and 0.0001^{***}) from the immunized untreated control are indicated.

To examine serum IFNγ, mice were OVA immunized and treated with 0 or 200 ng/day of IL-12 and bled (Fig. 9). Serum IFNγ production peaked on the last day of IL-12 administration and dropped in its absence, such that by day 7 levels returned to normal. The intensity and kinetics of serum IFNγ levels of OVA-immunized/IL-12 treated mice was very similar to that of non-immunized/IL-12 treated mice.

This suggested that IL-12 driven serum IFNγ production in immunized mice was independent of adaptive immunity, involving innate immune mechanisms.

6.3 Treatment with rIL-12 induced early Ag-driven IFNγ synthesis

Secondly, we examined the impact of exogenous IL-12 treatment on short-term, Ag-driven, spleen cell culture cytokine production from immunized mice. OVA immunized mice were injected with 0 or 200 ng/day of IL-12 beginning one day prior to OVA immunization and sacrificed. Spleen cells were stimulated in the presence and absence of OVA. IL-4, IL-10 and IFNγ were inhibited in IL-12 treated mice (Fig. 10). Considering the propensity of IL-12 to induce IFNγ, the depressed Ag-stimulated IFNγ synthesis was surprising. Since IL-12 induced highly transient serum IFNγ production, we carried out a detailed experiment, sacrificing mice 1, 3, and 5 days after immunization, to determine the time course of Ag-driven IFNγ production after IL-12 treatment (Fig. 11). IL-10 and IL-4 were not detected in cultures from either treated or untreated mice on days 1 and 3 (data not shown). In addition, IFNγ from immunized/untreated mice could not be observed until day 5. In marked contrast, IFNγ production in OVA immunized/IL-12 treated mice was strongly elevated by

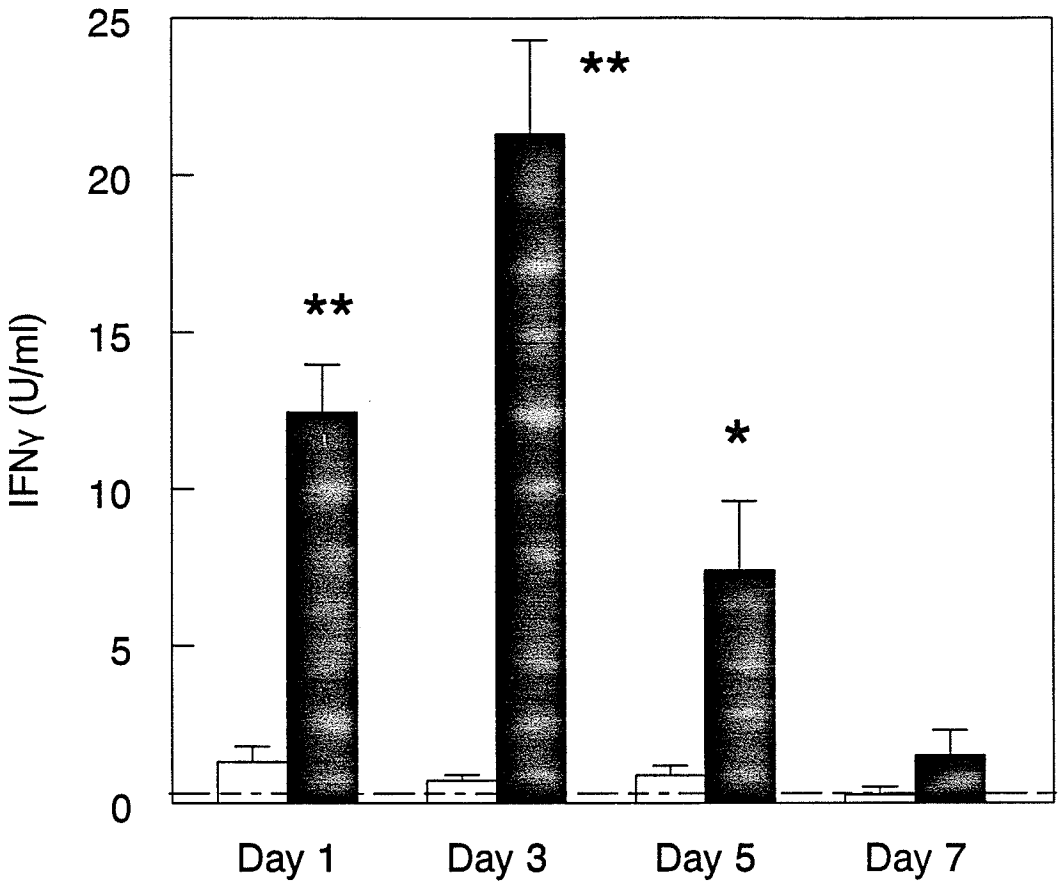

Figure 9. rIL-12 transiently elicits intense serum IFNγ production in immunized mice. Mice were immunized with 2 μg of OVA (alum). IL-12 was administered at 0 (□) or 200 ng/day (■) from days -1 to 3. Mean serum IFNγ values (± SEM) observed in nine experiments are shown. Significant differences ($p<0.05^{*}$ and 0.0001^{**}) from the immunized untreated control are indicated.

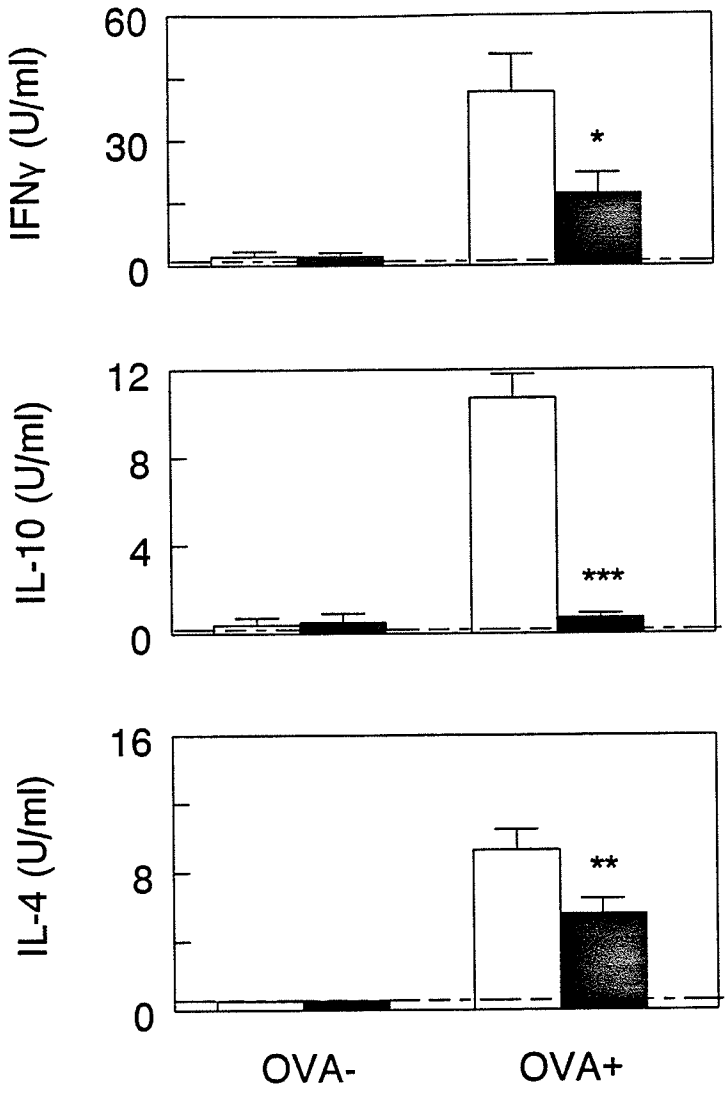

Figure 10. rIL-12 inhibits peak primary Ag-dependent cytokine production. IL-12 was administered, at 0 (□) or 200 ng/day (■), for 5 days beginning the day prior to 2 μg OVA (alum) immunization. Mice were sacrificed 5 days after immunization. Spleen cells were cultured (15 million cells/2 ml) with or without OVA (1 mg/ml). Supernatants were harvested for analysis of IL-4 (24 h), IFNγ (48 h), and IL-10 (96 h). Means ± SEM of 4 experiments are shown. Significant differences ($p<0.05^{*}$, 0.01^{**} and 0.0001^{***}) from the immunized, untreated control are indicated.

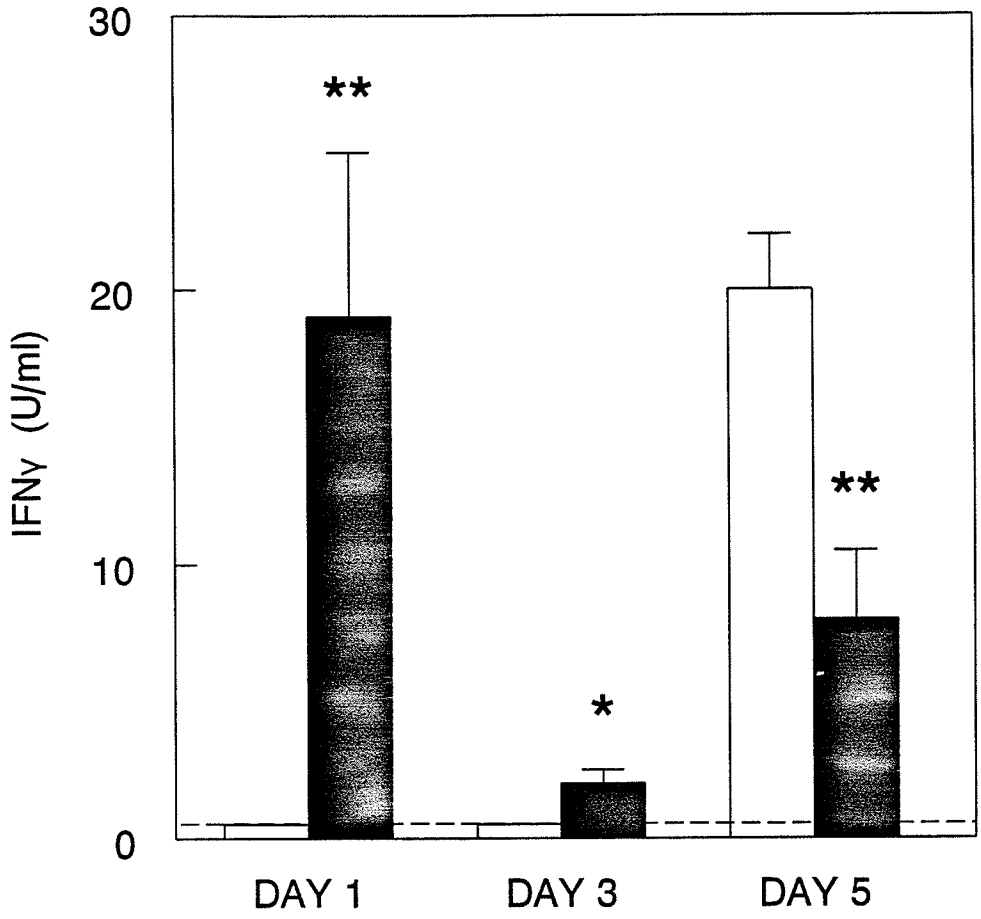

Figure 11. In vivo administration of IL-12 transiently enhances IFNγ production by spleen cells. Mice were immunized with 2 μg OVA (alum) on day 0. IL-12 was administered i.p. days -1 to 3 at 0 (□) or 200 ng/day (■). Mice were sacrificed and spleen cells cultured on the indicated days. Supernants were harvested at 48 hr. The means ± SEM of three mice within one representative experiment of two are shown. Significant differences ($p<0.05^{*}$ and 0.001^{**}) from the immunized, untreated control are indicated.

on day 1 and was significantly lower than that of mice immunized in the absence of IL-12 by day 5.

Therefore, Ag-driven spleen cell IFNγ production in response to IL-12 administration peaked 4 days prior to the height of OVA-specific CD4 T cell-mediated cytokine response. This suggested the participation of innate mechanisms such as NK cells.

6.4 IL-12 induced IFNγ production was largely NK cell, not CD4 T cell, derived

Since IL-12 is known to promote IFNγ production from NK cells, we examined the possibility that NK1.1+ cells may be contributing to the induction of type 1 responses upon IL-12 treatment. NK1.1+ cells were depleted in vivo with anti-NK1.1 mAb simultaneously with IL-12 administration. In IL-12 treated mice, NK1.1 depletion eliminated approximately 50 % of the serum IFNγ response (Fig. 12) and 80% of the IFNγ production observed in culture.

IL-12 is clearly important in T cell activation in vitro. Hence, we also assessed the impact of in vivo IL-12 administration on OVA-specific CD4 T cell IFNγ production by limiting dilution analysis and bulk culture of CD4-enriched spleen cell populations. Although decreased IL-4 was observed between OVA-immunized and OVA-immunized/IL-12 treated mice in short term Ag-driven culture, this was not significant due to a high standard error (n = 3 experiments). With more experiments this result may have been more significant. IL-10 and IFNγ were unaffected (Fig. 13). In limiting dilution analysis the precursor frequency of

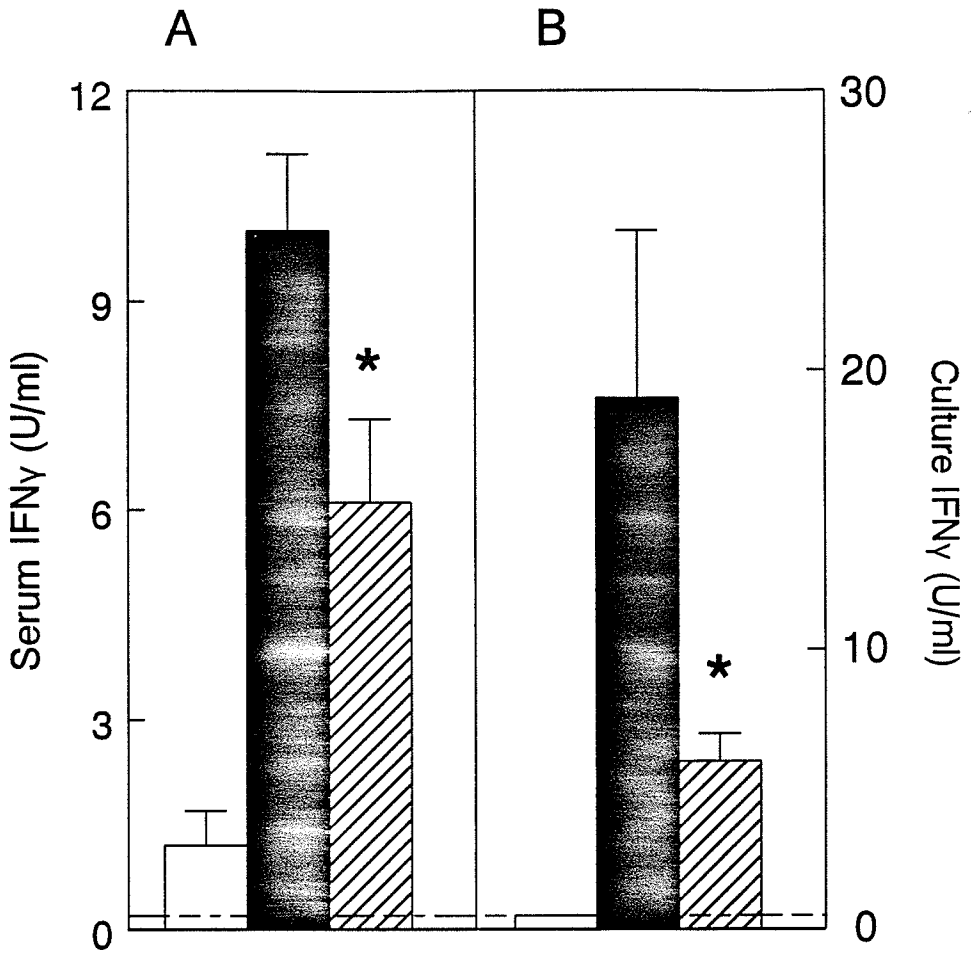

Figure 12. NK cells contribute to the elevation of IFNγ production following IL-12 treatment. IL-12 was administered i.p. from the day prior to OVA (alum) immunization and for the next 4 days at 0 (□) or 200 ng (■). Anti-NK 1.1 mAb was given at 300 μg i.p. the day prior to and the day after immunization in mice treated with IL-12 (▨). This dose depletes NK cells within 18 hrs for a minimum of 7 days as determined by cytotoxicity assays and flow cytometry (Rempel, 1997). Mean (± SEM) IFNγ responses in serum obtained at day 3 after immunization (panel A) and day 1 after establishing spleen cell cultures (panel B) from one experiment of three performed are shown. Significant differences between IL-12 treatment and IL-12 + anti-NK treatments ($p<0.05^{*}$) are indicated.

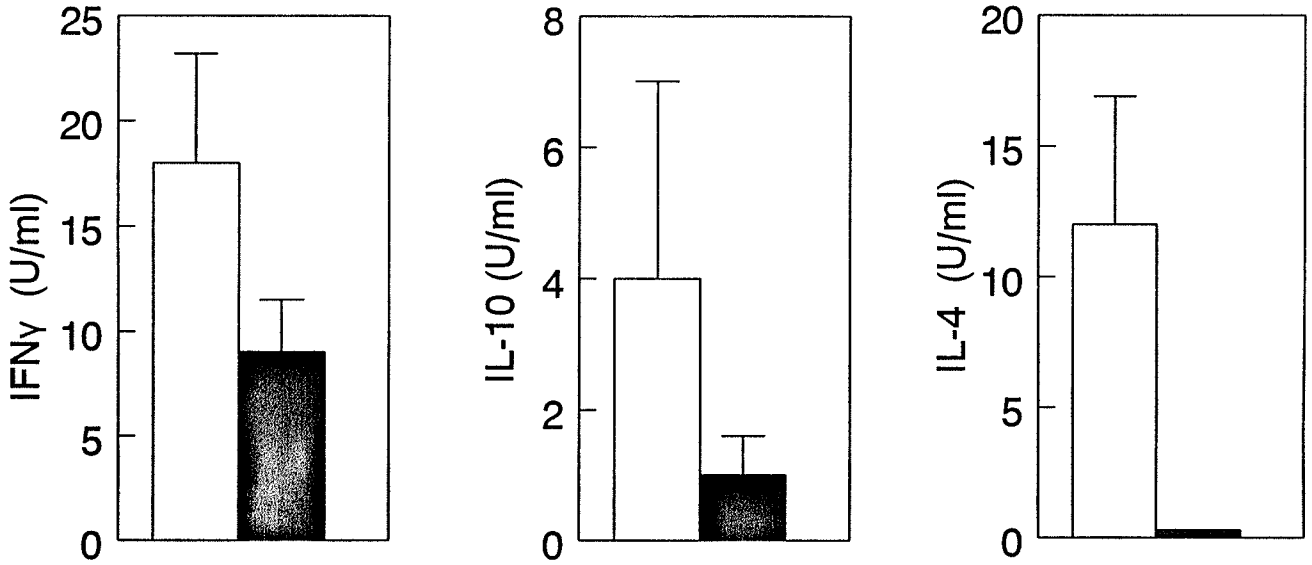

Figure 13. The impact of rIL-12 treatment on OVA-driven CD4 T cell cytokine production. Mice were immunized with 2 μg of OVA (alum). IL-12 was administered at 0 (□) or 100 ng/day (■) from days -1 to 3. Enriched CD4 T cells (1 million cells/200 ul; >92% purity) were cultured with irradiated APC (300,000 cells/200 μl) and ± OVA (1 mg/ml). In the absence of OVA stimulation, negligible cytokine was produced (data not shown). Mean (± SEM) from 3 experiments are shown (note: $p>0.05$).

cellular proliferation did not differ significantly between treated and untreated mice (1 in 11,900 vs 1 in 16,600 and 1 in 5,900 vs 1 in 8,900 respectively; in both experiments $p > 0.05$). A decrease in the frequency of CD4 T cells producing IL-10 ($p < 0.05$) was observed in two out of three experiments. Most importantly, in three independent experiments IL-12 administration did not increase the frequency of CD4 T cells producing IFNγ or decrease the frequency of CD4 T cells producing IL-4 (Table 5). The lack of decrease in the frequency of IL-4 producing CD4 T cells may suggest that the difference in IL-4 production seen in short term culture is actually not biologically significant. Alternatively, it may indicate that IL-12 decreased the amount of IL-4 produced by CD4 T cells, without altering the number of cells capable of producing IL-4. This would support the idea that exogenous IL-12 administration induces a transient effect, without long term alteration of adaptive responses.

Thus, while some decreases in CD4 T cell type 2 cytokine responses were observed in IL-12 treated mice, the great majority of increased IFNγ production was attributed to the impact of IL-12 on NK cells.

6.5 IL-12 activation of NK cells contributed to suppressed IgE production

To verify whether IL-12 induction of NK cell IFNγ production directly impacted IgE synthesis, immunized mice were treated with anti-NK1.1 mAb, IL-12 or anti-NK1.1 mAb plus IL-12. While IgE levels following IL-12 treatment were undetectable, anti-NK1.1 mAb almost fully reversed this inhibition (Fig. 14).

Table 5. IL-12 does not substantially alter the frequency of CD4+ cells producing IFNγ or IL-4.

Cytokine	Exp.[a]	Frequency cells/10^7 (95% confidence limits)		Ratio OVA: OVA + IL-12
		OVA	OVA + rIL-12	
IFNγ	1	8.8 (12.4-5.2)	6.5 (7.7-3.4)	*1.3*
	2	37.9 (43.4-28.4)	16.6 (20.5-11.4)	*2.2*
	3	19.0 (24.6-13.5)	14.1 (18.9-9.3)	*1.3*
IL-10	1	7.9 (11.4-4.4)	3.7 (6.0-1.4)	*2.1*
	2	21.1 (27.0-15.2)	5.3 (8.0-2.5)	*3.7*
	3	26.4 (33.5-19.2)	4.8 (7.4-2.2)	*5.5*
IL-4	1	12.6 (17.0-8.3)	12.7 (17.1-8.3)	*1.0*
	2	47.8 (58.1-37.4)	38.6 (47.2-30.0)	*1.2*
	3	86.9 (117.6-73.5)	27.5 (37.1-17.9)	*3.1*

[a] IL-12 (100 ng) or saline was administered (i.p. for 5 days) beginning one day prior to OVA immunization (2.0 μg in alum). Five days later spleen cells were highly enriched for CD4+ cells and cultured using 40,000 to 312 cells/well in the presence of IL-2 (20 U/ml), OVA (1 mg/ml) and irradiated naive APC. Cultures were restimulated with OVA (1 mg/ml) in the absence of IL-2 two weeks later and harvested at 48 hours. The frequency of cells proliferating in response to OVA (see text) or producing the indicated cytokines in these limiting dilution analyses was calculated as described in Materials and Methods.

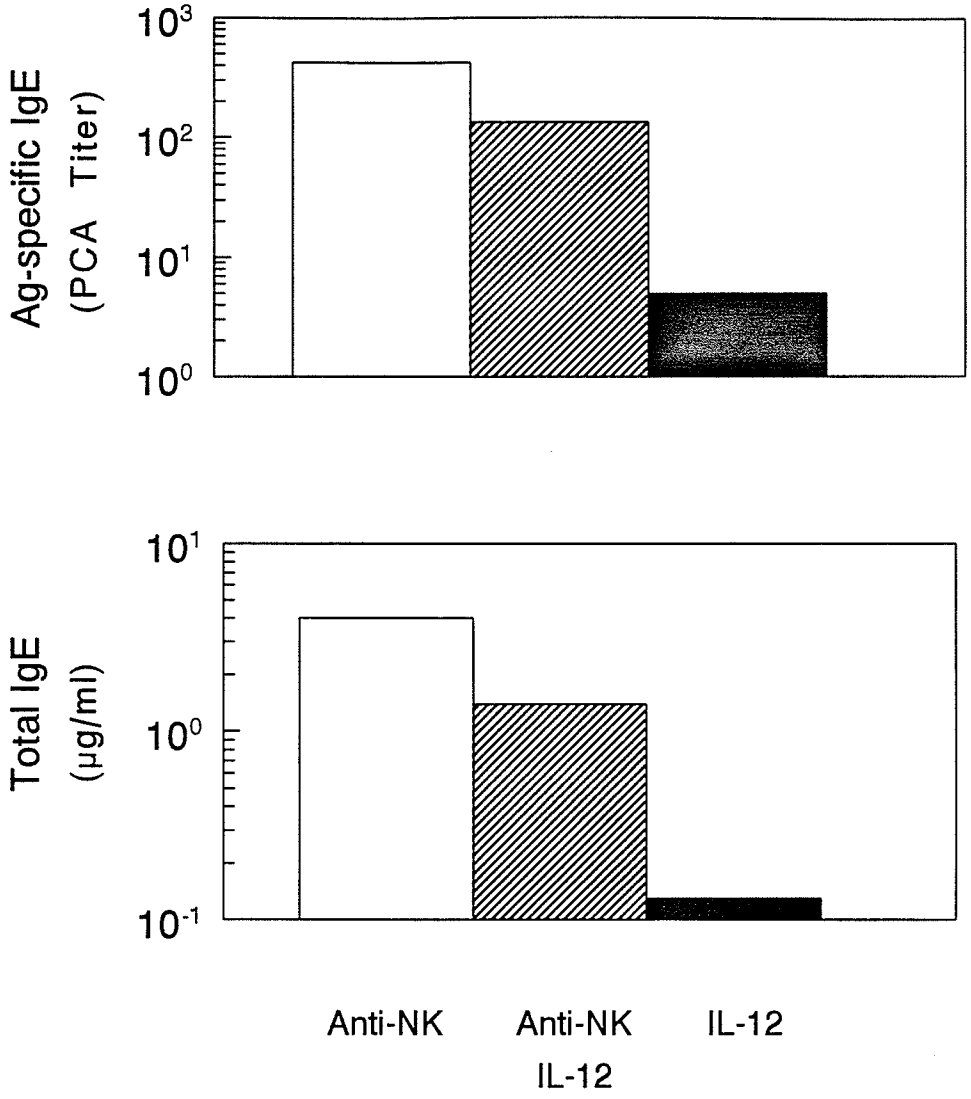

Figure 14. The capacity of exogenous IL-12 to inhibit serum IgE is reversed by anti-NK1.1 treatment. Mice were immunized with 2.0 μg of OVA (alum). IL-12 was administered i.p. from the day prior to immunization and for the next 4 days at 0 (□) or 200 ng (■). Anti-NK 1.1 mAb was given at 300 μg i.p. the day prior to and the day after immunization in mice treated with IL-12 (▨). Mice were bled on day 10 for analysis of Ag-specific and total IgE levels. Means (n = 2 mice; 1 of 1 experiment) are shown.

In summary, this examination of the adjuvant potential of IL-12 strongly argues that IL-12 administration can elicit intense type 1 antibody and cytokine responses in OVA immunized mice. However, these responses are short-lived since they are apparently driven by IL-12 induced NK cell IFNγ production, without effective alteration of CD4 T cell function. As a result, the data calls into question the ability of IL-12 to be a suitable adjuvant for the redirection of humoral responses as it does not elicit the long term skewing of isotype production in response to soluble protein Ag.

7.0 The failure of exogenous rIL-12 to inhibit established IgE responses was associated with enhanced IL-4 production by non-B/non-T cells

7.1 IL-12 administration in mice with established IgE responses greatly enhanced antigen-specific IgG_{2a}

Simultaneous with our attempt to determine the adjuvant potential of IL-12, we investigated the therapeutic benefit of IL-12 in mice with established IgE responses. C57Bl/6 mice were immunized with 0.2 μg or 2.0 μg of OVA in the absence of IL-12 and boosted in the presence of IL-12 (Table 6 and 7). To investigate if responses were strain dependent, outbred CD1 mice were similarly treated (Table 8). To allow for direct comparison, results from both strains of mice were analyzed by Mann-Whitney, a non-parametric test. Independent of the approach selected, IL-12 administration consistently failed to inhibit recall OVA-specific IgE synthesis. Total serum IgE levels were parallel between treated and untreated CD1 mice, and were in fact increased two to three fold in C57Bl/6 mice. Most striking, Ag-specific IgG_{2a}

Table 6. Immunization and treatment schedules for assessing the impact of IL-12 treatment on established type 2 humoral responses in C57Bl/6 mice.

OVA (alum)[a]	Experiment	OVA (alum) boosts (day)	IL-12 (day)
0.2 μg (n = 10)	1	39, 89[b]	88[c]
	2	50	49
	3	39, 225	168 182 196 210 224
2.0 μg (n = 17)	4	28, 53	52
	5 6	28	27

[a] C57Bl/6 mice were immunized with either 0.2 or 2.0 μg of OVA (alum) and treated with 200 ng of IL-12 as indicated. Mice were bled five days after final boost for Ab analysis (results in Table 7).

[b] Days after primary immunization (d. 0).

[c] Day after primary immunization representing the first day of a five day IL-12 treatment course.

Table 7. IL-12 administration given during established type 2 responses enhances OVA-specific IgG_{2a} in C57Bl/6 mice, but fails to inhibit Ag-specific or total IgE production upon Ag re-exposure.

Ab Isotype		0.2 μg OVA[a]		2.0 μg OVA	
		IL-12 (ng/day)		IL-12 (ng/day)	
		0	200	0	200
IgE	Specific (PCA titer)	5000	5000	2000	4000
	Total (μg/ml)	*13*	*28.0**	*10.5*	*28.8**
IgG_1	Specific (ELISA titer)	289	493	851	3,014**
	Total (μg/ml)	*849*	*879*	*1285*	*2,235**
IgG_{2a}	Specific (ELISA titer)	484	5,208**	2095	112,255**
	Total (μg/ml)	*814*	*808*	*274*	*220*

[a] C57Bl/6 mice were immunized and treated as stated in Table 6. Seven days after the final boost mice were bled for OVA-specific and total Ab analysis. Medians are shown. Significant differences from the immunized, untreated mice are indicated (Mann Whitney: $p<0.05$*, 0.005**, and 0.0005***).

Table 8. IL-12 administration given during established type 2 responses enhances OVA-specific IgG_{2a} in CD1 outbred mice, but fails to inhibit Ag-specific IgE production upon Ag re-exposure.

Ab Isotype		0.2 μg OVA[a]		2.0 μg OVA	
		IL-12 (ng/day)		IL-12 (ng/day)	
		0	200	0	200
IgE	Specific (PCA titer)	1500	1750	1500	1500
	Total (μg/ml)	*1.7*	*1*	*1.5*	*1.7*
IgG_1	Specific (ELISA titer)	1642	1563	3146	2249
	Total (μg/ml)	*1600*	*1325*	*2300*	*1790*
IgG_{2a}	Specific (ELISA titer)	3858	58,311**	20025	117,500*
	Total (μg/ml)	*276*	*104**	*380*	*164*

[a] CD1 mice were immunized with 0.2 or 2.0 μg of OVA (alum) on days 0 and 31. IL-12 was administered from days 30 to 34. Mice were bled seven days after final boost for Ab analysis. Medians are shown. Significant differences from the immunized, untreated mice (n = 10) are indicated (Mann Whitney: $p<0.05$*, 0.01**).

synthesis was elevated 10-50 fold in both strains.

Therefore, IL-12 administration in mice with established IgE responses selectively enhanced the Ag-specific component of the type 1 humoral responses without greatly altering the type 2 Ab responses.

7.2 rIL-12 treatment of immunized mice strongly elevated IFNγ levels

IL-12 strongly elevated Ag-specific IgG_{2a} production. Thus we expected elevated IFNγ synthesis since IFNγ is the switch factor for IgG_{2a} production. Administration of rIL-12 in previously immunized mice induced serum IFNγ up to 77 and 105 U/ml in C57Bl/6 and outbred mice strains respectively (Fig 15). Where IFNγ levels peaked at day 4-5 and returned to pre-immunization levels by day 7 (<3 U/ml, data not shown) following OVA booster immunization.

Similarly, when cohorts were sacrificed at day 5 after OVA boost, spleen cell cytokine analysis revealed that Ag-driven IFNγ production was elevated approximately 13 fold in OVA boosted/IL-12 treated animals (Fig. 16).

Thus, as in primary Ab and cytokine responses following in vivo IL-12 administration, administration of rIL-12 to mice with well established type 2 responses led to elevated IgG_{2a} and IFNγ production.

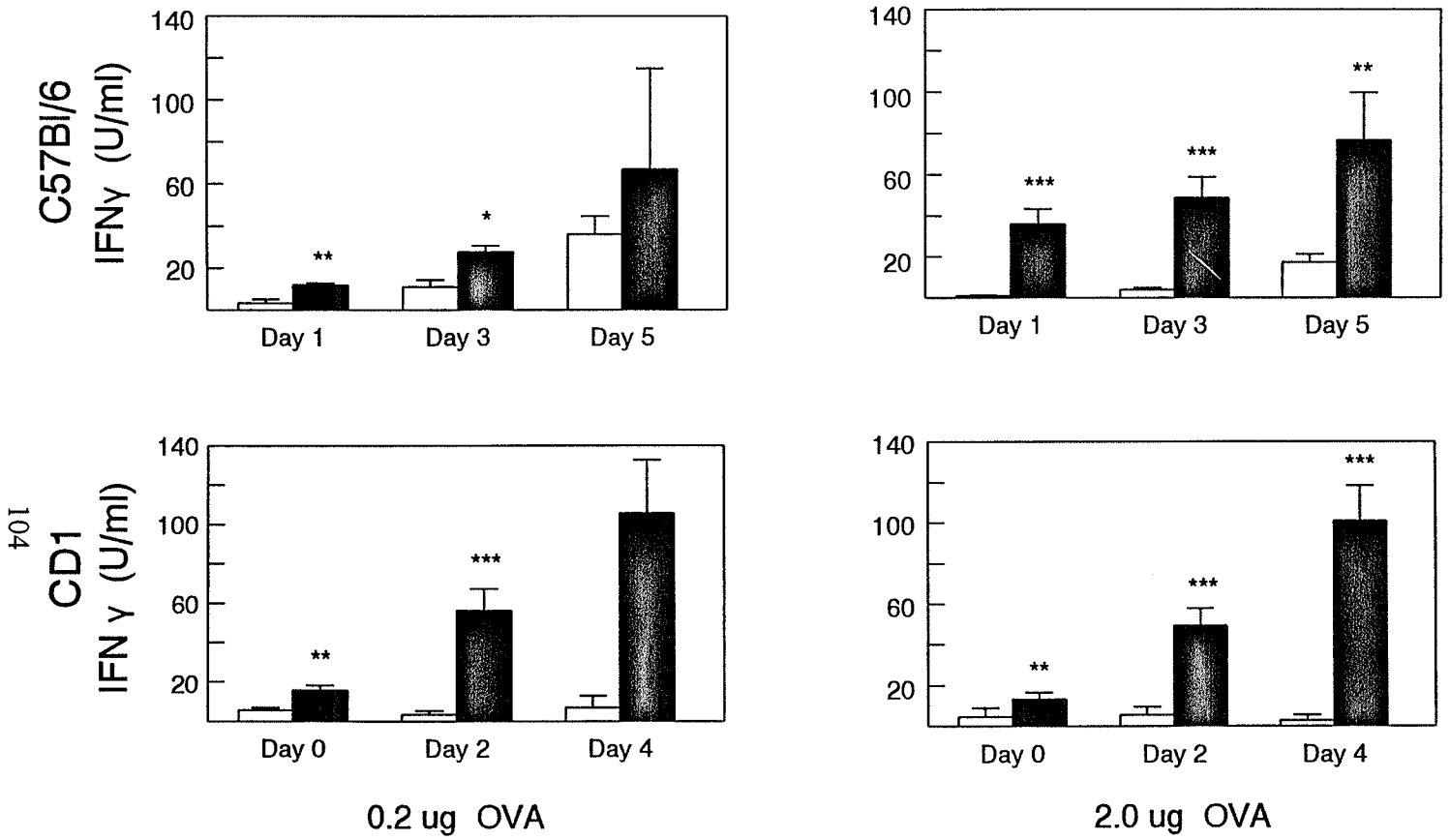

Figure 15. rIL-12 enhances serum IFNg in mice with ongoing IgE responses. Mice were treated with 0 (□) or 200 ng/day (■) of IL-12 (x 5 times) starting the day prior to 2° immunization. Mice were bled after 2° immunization. Mean serum cytokine levels (± SEM) are shown. Significant differences compared to immunized untreated cohorts are indicated (Mann Whitney: $p<0.05$*, 0.005** and 0.0005***).

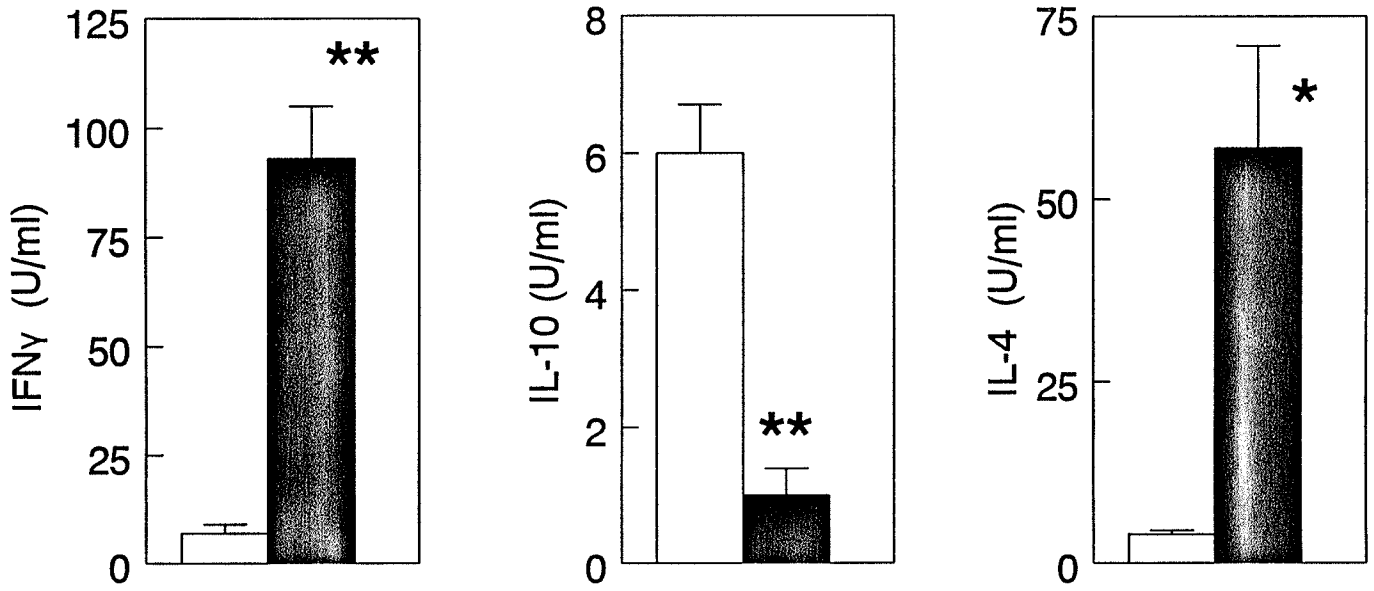

Figure 16. IL-12 administration elevates spleen cell IFNg and IL-4 production in mice with established IgE responses. C57Bl/6 mice were immunized with 0.2 μg of OVA (alum) and treated with 0 (□) and 200 ng/day (■) of IL-12 the day prior to secondary immunization and for the next 5 days. Mice were sacrificed 5 days after OVA boost and spleen cells were cultured (15 million cells in 2 mls) in the presence and absence of OVA. In cultures carried out without OVA restimulation, IFNγ responses of <0.15 vs 3.8 U/ml and IL-4 of 1.8 vs. 4.0 U/ml in immunized/untreated vs. immunized/rIL-12 treated groups were obtained (n = 42 mice cultured independently over 7 experiments). Mean cytokine responses (± SEM) are shown from the seven experiments performed. Significant differences are indicated (p<0.0005* and 5.0 x 10^{-7}**).

7.3 Exogenous IL-12 administration to immunized mice markedly increased antigen-driven IL-4 synthesis

In notable contrast to known IL-12 activity on cytokine production in primary culture, IL-12 treatment of mice with established IgE responses elevated Ag-driven IL-4 production (in short term culture) a remarkable 15 fold (Fig. 16). IL-10 synthesis was inhibited in these same cultures, whereas IL-5 synthesis was not significantly altered (data not shown).

This indicates that in established type 2 associated diseases, IL-12 has the capacity to enhance both type 1 and type 2 arms of the immune system.

7.4 rIL-12 treatment reduced the frequency of CD4 T cells producing IL-4

It had previously been reported that in animals with ongoing disease, IL-12 administration could enhance IL-4 mRNA and protein expression (Bliss, 1996; Wang, 1994). To investigate the cellular source of IL-4 production, we began by evaluating Ag-specific CD4 cells responses from treated and untreated mice. CD4 T cells were stimulated with OVA or not (Fig. 17). Unlike cytokine production observed in whole spleen cell culture, CD4 T cell cultures consistently failed to show substantial differences in IL-4, IFNγ and IL-10 production between IL-12 treated and untreated mice.

When the impact of IL-12 on the frequency of CD4 T cells was examined by LDA (Table 9),

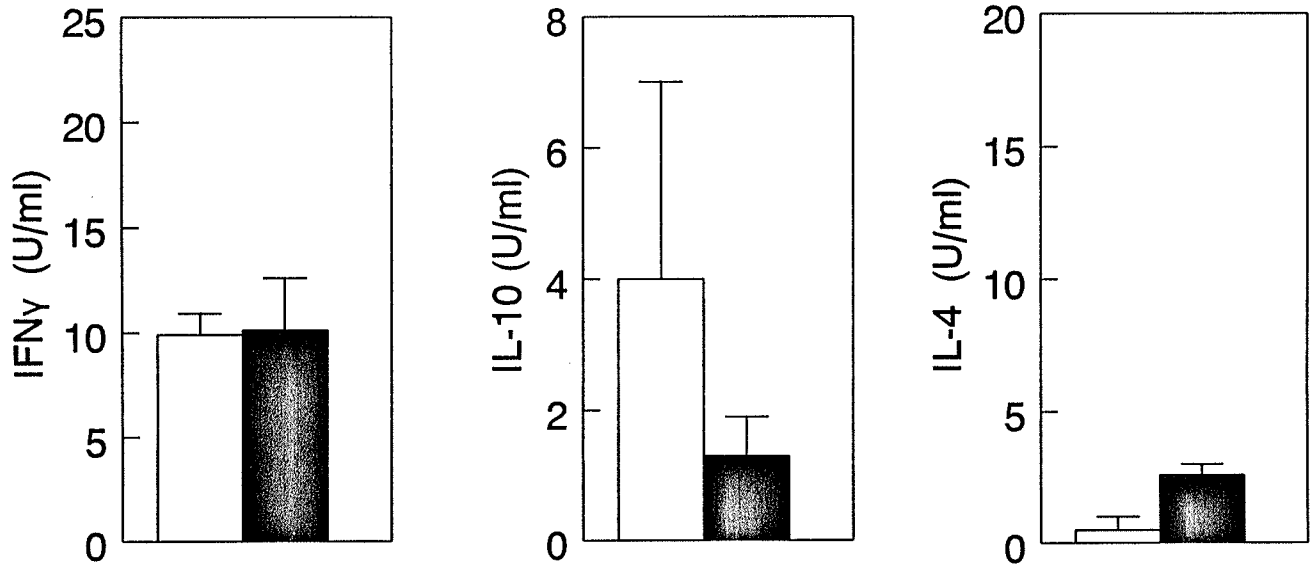

Figure 17. rIL-12 administration in mice with established IgE responses does not substantially alter CD4 T cell cytokine production. Mice received treatments of 0 (□) or 200 ng/day (■) of IL-12. Three courses of rIL-12 (5 days x 200 ng/day) were given beginning the day prior the secondary boost (0.2 μg OVA, alum), two weeks later and four weeks later (the day prior to the tertiary boost). Five days after the tertiary boost spleen cells were highly enriched for CD4+ T cells and cultured (1 million cells/200 μl) with OVA (1 mg/ml) and irradiated APC (300,000 cells/200 μl). In the absence of OVA stimulation, negligible cytokine was produced (data not shown). Mean (± SEM) from 3 experiments are shown (note: $p>0.05$).

Table 9. rIL-12 administration reduces the frequency of CD4 T cells producing IL-4 in mice with established IgE responses.

Cytokine	Exp.	Frequency cells/10^7 (95% confidence limits)		Ratio OVA: OVA + IL-12
		OVA	OVA + IL-12	
IFNγ	1	187 (245-129)	177 (235-122)	*1.1*
	2	38 (65-12)	43 (71-14)	*0.9*
	3	115 (164-69)	147 (201-94)	*0.8*
IL-10	1	97 (135-58)	18 (33-2)	*5.3*
	2	315 (405-227)	29 (52-6)	*10.0*
	3	29 (52-6)	19 (38-14)	*1.5*
IL-4	1	347 (434-261)	60 (89-31)	*5.7*
	2	374 (478-271)	61 (96-26)	*6.2*
	3	833 (1000-625)	92 (135-49)	*9.0*

[a] Mice were immunized with 0.2 μg of OVA (alum). Mice received either 0 or 200 ng/day of rIL-12. Three courses of IL-12 (5 days x 200 ng/day) were given beginning the day prior the secondary boost (0.2 μg OVA, alum), two weeks later and four weeks later (the day prior to the tertiary boost). Five days after the tertiary boost spleen cells were highly enriched for CD4+ cells and cultured from 40,000 to 312 cells/well in the presence of IL-2 (20 U/ml), OVA (1 mg/ml) and irradiated naive APC. Cultures were restimulated with OVA (1 mg/ml) in the absence of IL-2 two weeks later and harvested at 48 hours. The frequency of cells producing the indicated cytokines in these limiting dilution analyses was calculated as described in Materials and Methods.

rIL-12 consistently failed to modify the frequency of OVA-specific CD4 T cells producing IFNγ. The decreasing frequency of IL-10 producing CD4 T cells did reflect the decline in IL-10 production in whole spleen and CD4 T cell cultures due to administration of exogenous IL-12. However, in contrast to the marked increase in IL-4 production by whole spleen culture following IL-12 treatment, LDA of separated CD4 T cells (from the spleens of IL-12 treated mice) demonstrated a clear reduction in the frequency of OVA-specific CD4 T cells secreting IL-4. This decrease in the frequency of IL-4 producing CD4 cells following IL-12 administration lead to a change in the Th1:Th2 balance of Ag-specific CD4 cells from a Th2 dominance (median IL-4:IFNγ ratio of 7.2) in the absence of IL-12 to a Th1 dominance (median IL-4:IFNγ ratio of 0.6) in IL-12 treated mice. The events in whole spleen culture (IL-12 administration resulting in high IFNγ and IL-4 production) resulted in no net change in the type 1:type 2 balance of OVA-specific cytokine responses ($p > 0.05$).

Thus, IL-12 administration in mice with established IgE responses reduced the frequency of CD4 T cells producing IL-4, but this treatment was also accompanied by high OVA-driven IL-4 production observed in whole spleen culture (i.e. not CD4 enriched culture).

7.5 IL-12 administration to immunized mice enhanced the frequency and activity of non-B/non-T cells

Since the increased number or activity of OVA specific CD4 T cells were not responsible for the 20 fold enhancement in IL-4 production seen following administration of exogenous IL-12, we examined the impact of in vivo IL-12 treatment on non-B/non-T cells. It had been

reported in both human and murine systems of established immediate hypersensitivity that non-B/non-T cells were potent producers of IL-4, presumably as a consequence of Ag binding toFcεRI bound IgE, resulting in the cross-linking of the receptors and activation of the cells (Kasaian, 1996). Since a side effect of exogenous IL-12 treatment was enhanced numbers of CD3- and B220- cells within the spleen, we investigated the possibility that IL-12 was inducing this expanded population of non-B/non-T cells to produce high levels of IL-4 upon Ag-specific reactivation.

We first examined the ability of IL-12 to increase the proportion of non-B/non-T cells in the spleen. Spleen cells from OVA boosted/IL-12 treated and boosted mice were stained with FITC conjugated rat IgG_{2a} anti-mouse CD4, anti-CD8 and anti-CD19. The proportion of CD4, CD8 and CD19 positive cells was determined by flow cytometry. The proportion of non-B/non-T cells was calculated by subtraction as shown in Figure 18. The proportion of CD4 cells was reduced upon IL-12 treatment, but when assessed against the total number of spleen cells, the absolute number of CD4 cells did not change compared to untreated control mice. In contrast, both the proportion and total number of non-B/non-T cells was markedly enhanced in the spleens of mice receiving IL-12 compared to untreated controls.

The capacity of IL-12 to induce non-B/non-T cell IL-4 secretion in OVA-stimulated culture was then directly evaluated (Fig. 19). Spleen cells were stained with FITC conjugated anti-CD4, anti-CD8 and anti-CD19. Non-B/non-T cells were isolated by negative selection via flow cytometry and cultured with OVA. Supernatants were harvested at 24 hr and assayed

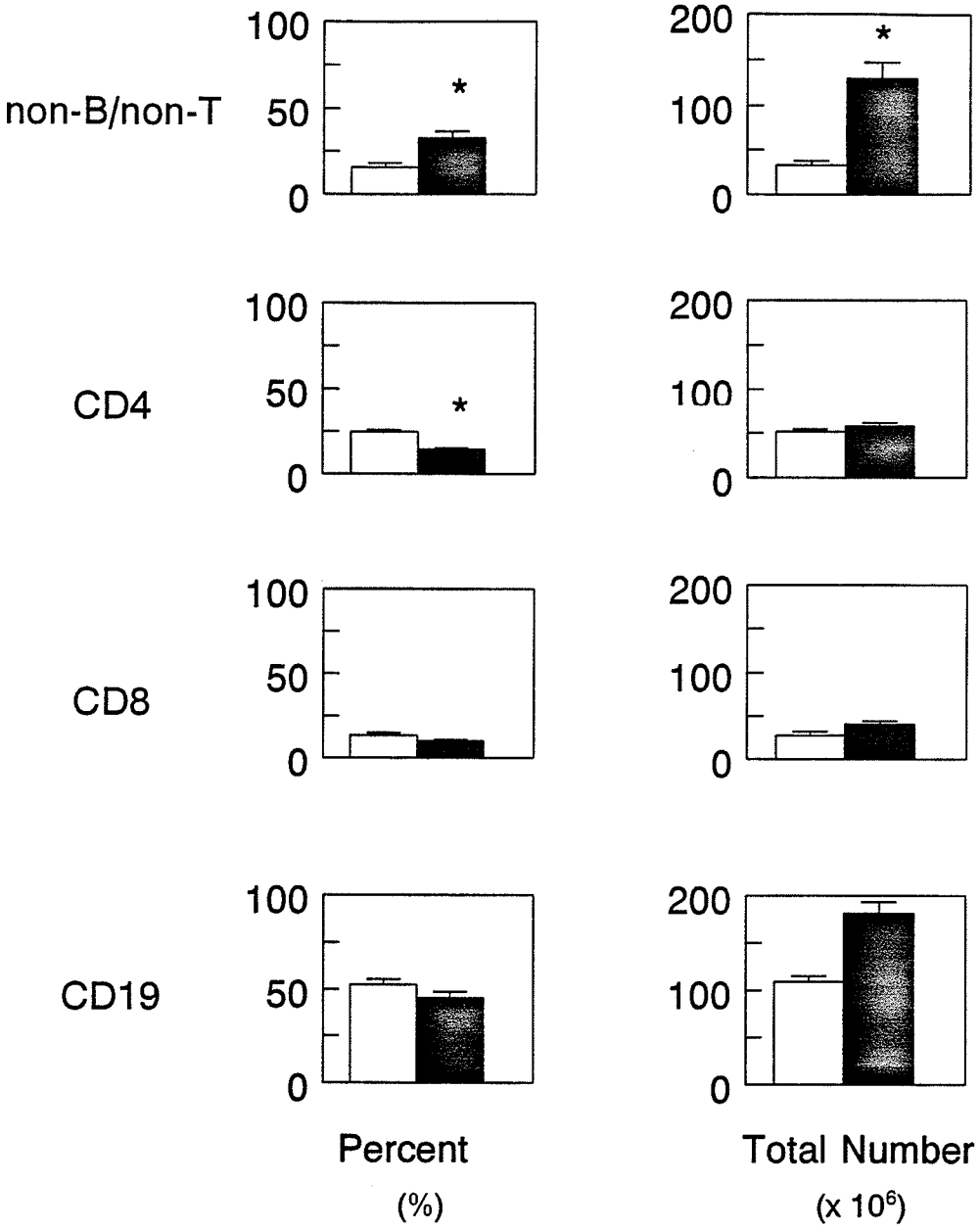

Figure 18. IL-12 increases the proportion of non-B/non-T cells in the spleen. Mice were OVA (0.2 μg, alum) immunized and treated with 3 courses of 5 days at 0 (□) or 200 ng/day (■) of IL-12, beginning the day prior the secondary boost (0.2 μg OVA, alum), two weeks later and four weeks later (the day prior to the tertiary boost). Spleen cells were harvested 5 days after final boost and the proportion of CD4, CD8 and CD19 cells was determined by flow cytometry. Means ± SEM (n = 4) are shown from two experiments. Significant differences to untreated immunized cohorts are indicated ($p<0.005$*).

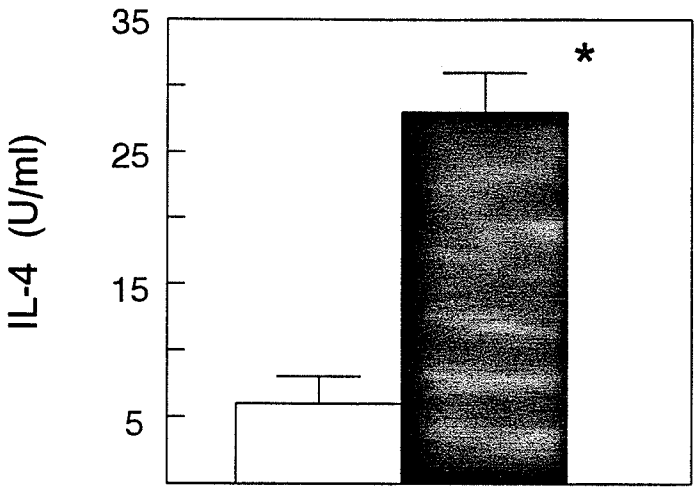

Figure 19. In vivo administration of IL-12 increases the relative production of Ag-driven IL-4 from non-B/non-T cells in the spleen. Mice were OVA (0.2 μg, alum) immunized and treated with 3 courses of 5 days at 0 (□) or 200 ng/day (■) of IL-12. Courses were given beginning the day prior the secondary boost (0.2 μg OVA, alum), two weeks later and four weeks later (the day prior to the tertiary boost). Spleen cells were harvested 5 days after final boost. Non-B/non-T cells were negatively selected (CD4-, CD8- and CD19-) from the spleens of IL-12 treated (■) and control (□) mice. Non-B/non-T cells were cultured (0.5 million cells/ 200 μl) with OVA for 24 h. Supernatants were harvested and analyzed for IL-4 production. Mean (± SEM) (n = 4) are shown. Significant difference to untreated immunized cohort is indicated ($p<0.05$*).

for IL-4 secretion. Non-B/non-T cells from mice receiving IL-12 produced 5 times more IL-4 than culture of an equal number of corresponding cells from mice that did not receive IL-12. Taken together with the increase frequency of non-B/non-T cells described above, this indicated that not only did IL-12 treatment increase the number of non-B/non-T cells within the spleen, but on a cell-to-cell basis non-B/non-T cells from boosted/treated mice produced more IL-4 than non-B/non-T cells isolated from mice boosted in the absence of IL-12.

In summary, the administration of IL-12 to mice with established type 2 dominated responses failed to inhibit recall IgE responses, but greatly increased IgG_{2a} production. Despite elevated IFNγ synthesis in IL-12 treated mice, a simultaneous increase in non-B/non-T derived IL-4 production was most likely responsible for maintained IgE levels. IL-4 production has been shown to be required for establishing and maintaining Ag-driven IgE responses (Finkelman, 1990). Thus, the therapeutic use of IL-12 in individuals with atopy could potentially result in detrimentally high IL-4 production by non-B/non-T cells.

8.0 Endogenous IL-12 regulation of antibody responses

8.1 In vivo neutralization of IL-12 moderately reversed the capacity of OA-POL to inhibit IgE production

HayGlass *et al.* (HayGlass, 1991c) had previously shown that treatment with OA-POL prior to OVA (alum) immunization inhibits the development of Ag-specific IgE production. In contrast, Ag-specific IgG_{2a} and IgG_1 were elevated in OA-POL treated OVA alum

Table 10. Pretreatment with OA-POL inhibits IgE production upon OVA (alum) immunization.

Ab Isotypes		Conditions[a]	
		OVA (alum)	OVA (alum) + OA-POL treatment
IgE	Ag-specific (PCA titer)[b]	1,620 ± 320	197 ± 53
	Total (μg/ml)[c]	9.3 ± 3.2	4.4 ± 1.1
IgG_{2a}	Ag-specific (ELISA titer)	62 ± 13	5,5100 ± 13,030
	Total (μg/ml)	257 ± 53	341 ± 80
IgG_1	Ag-specific (ELISA titer)	29,000 ± 4,000	1,244,000 ± 15,700
	Total (μg/ml)	392 ± 28	631 ± 116

[a] Mice were immunized with 2.0 μg of OVA (alum) on day 0. Mice (n = 7 mice) were either pretreated with nothing or 80 μg/day of OA-POL on days -14, -12 and -10. Mice were bled on day 10 and 14 for IgE and IgG's determination respectively.
[b] Geometric means ± SEM are shown for PCA titers.
[c] Means ± SEM are shown for remaining isotypes.

immunized mice. This capacity of OA-POL was also seen in recent experiments (Table 10). Not previously demonstrated, OA-POL treatment also inhibited total IgE levels.

Since (1) IL-12 administration, at the time of OVA (alum) immunization can largely abolish IgE production in response to OVA (alum) immunization, and (2) OA-POL can also prevent IgE production, we hypothesized that the processing of OA-POL results in enhanced localized IL-12 production which acts to inhibit IgE production.

To examine this, mice were treated with OA-POL, OA-POL and anti-IL-12, OA-POL and normal goat IgG, or nothing. Mice were OVA (alum) immunized (Fig. 20 and 21). The neutralization of endogenous IL-12 enhances IgE synthesis (6 fold), while decreasing IgG_{2a} production (3.5 fold) in OA-POL treated mice. However, these responses were only partially altered, indicating that mechanisms other than IL-12 regulation may also be involved. Conversely, anti-IL-12 may have failed to neutralize IL-12 completely within our system. Interestingly, anti-IL-12 also enhanced OA-POL induced IgG_1 production suggesting that feedback mechanisms may restrict the induction of IL-12 upon OA-POL treatment.

Endogenous IL-12 was at least partly responsible for the effect that OA-POL treatment has on humoral responses, seen most clearly by a reversal in OA-POL induced inhibition of Ag-specific IgE. Due to the limited supply of anti-IL-12, we continued these investigations with IL-12 knockout (KO) mice.

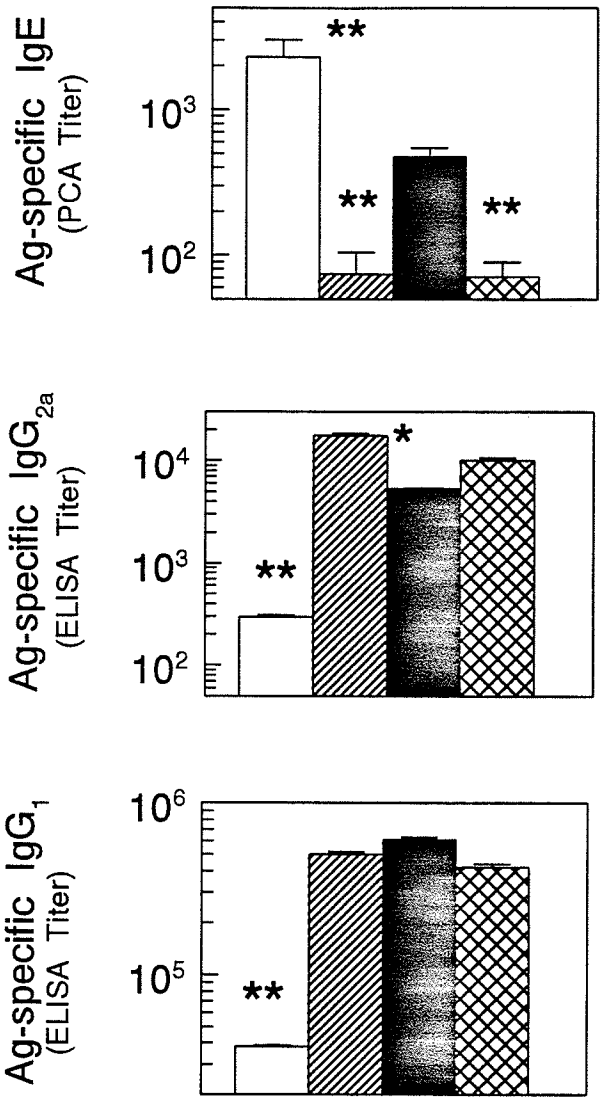

Figure 20. Neutralization of endogenous IL-12 somewhat reverses the ability of OA-POL to prevent the induction of OVA-specific IgE. Mice were immunized with 2 μg of OVA (alum) on day 0. Mice were either treated with nothing (☐), OA-POL (80 μg on days -14, -12 and -10) (☑), OA-POL and goat IgG anti-mouse-IL-12 (p70) (1 mg on days -15, -7 and -1) (■), or OA-POL and normal goat IgG (1 mg on days -15, -7 and -1) (☒). Mice were bled on days 10 and 14 for IgE and IgG's synthesis respectively. Geometric means (IgE) and means (IgG_1 and IgG_{2a}) ± SEM (n = 9 mice) are shown. Significant differences to OA-POL and anti-IL-12 treated group are shown (p<0.01*, 0.0001**).

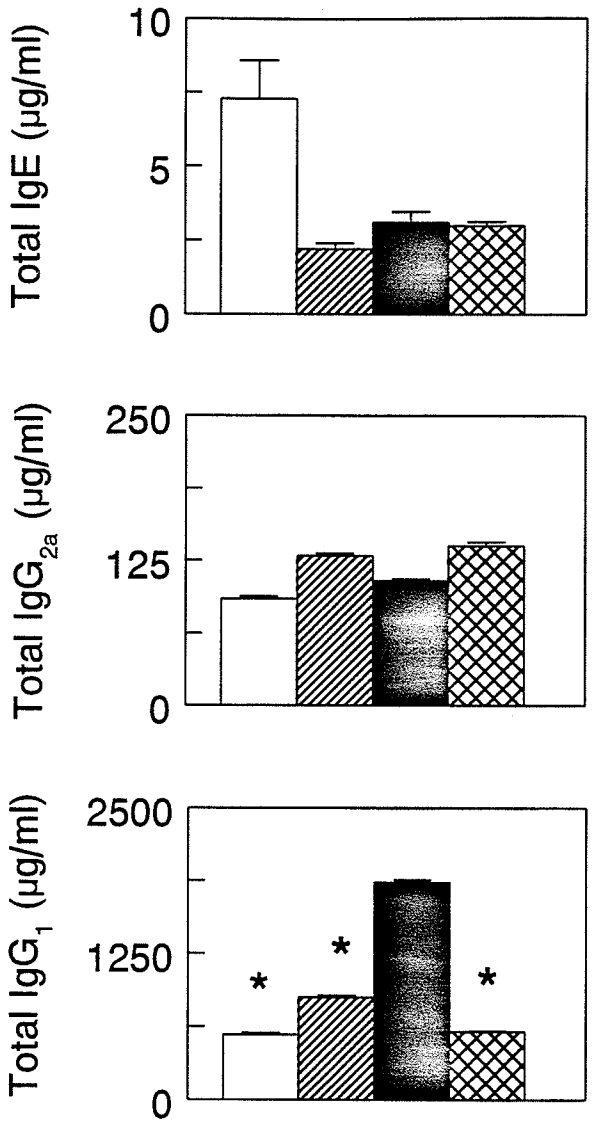

Figure 21. The impact of anti-IL-12 on OA-POL induced total Ab production. Mice were immunized with 2 µg of OVA (alum) on day 0. Mice were either treated with nothing (☐), OA-POL (80 µg on days -14, -12 and -10) (▨), OA-POL and goat IgG anti-mouse IL-12 (1 mg on days -15, -7 and -1) (■), or OA-POL and normal goat IgG (1 mg on days -15, -7 and -1) (▩). Mice were bled on days 10 and 14 for IgE and IgG's synthesis respectively. Means ± SEM (n = 9 mice) are shown. Significant differences to OA-POL and anti-IL-12 treated group are shown ($p<0.001$*).

8.2 IL-12 deficiency fails to enhance IgE synthesis upon immunization

We (Table 4) and others (Finkelman, 1994; McKnight, 1994; Morris, 1994) showed that administration of exogenous IL-12 inhibits primary IgE synthesis upon protein immunization or parasite infection. Furthermore, endogenous IL-12 appeared to play an important role in the capacity of OA-POL to inhibit OVA (alum) induced IgE production. Thus, we hypothesized that endogenous IL-12 would be important in the negative regulation of type 2 immunity, such that in the absence of endogenous IL-12, IgE and type 2 cytokine synthesis would be markedly enhanced. To test this hypothesis, we first examined the effect of IL-12 deficiency on type 2 Ab production in response to protein immunization. WT, p35 KO and p40 KO mice were immunized with OVA (alum) and bled for serum Ab analysis (Fig 22). Contrary to our hypothesis, the absence of endogenous IL-12 production did not alter Ag-specific or total IgE synthesis. However, total IgG_{2a} was consistently lower in IL-12 KO mice strains.

Parasites are known for their ability to induce non-specific (as well as specific) IgE production. Therefore, as an alternative approach to examining the importance of endogenous IL-12 on type 2 humoral responses, WT and p35 KO mice were injected with the parasite *T. spiralis* extract in alum. The impact of *T. spiralis* extract (alum) immunization on p40 KO mice was not evaluated due to lack of mice. We were unable to generate Ab responses to the parasite extract alone, thus we needed to use an adjuvant. In addition, we had to restrict our Ab analysis to total Ab production since we were unable to analyze Ag

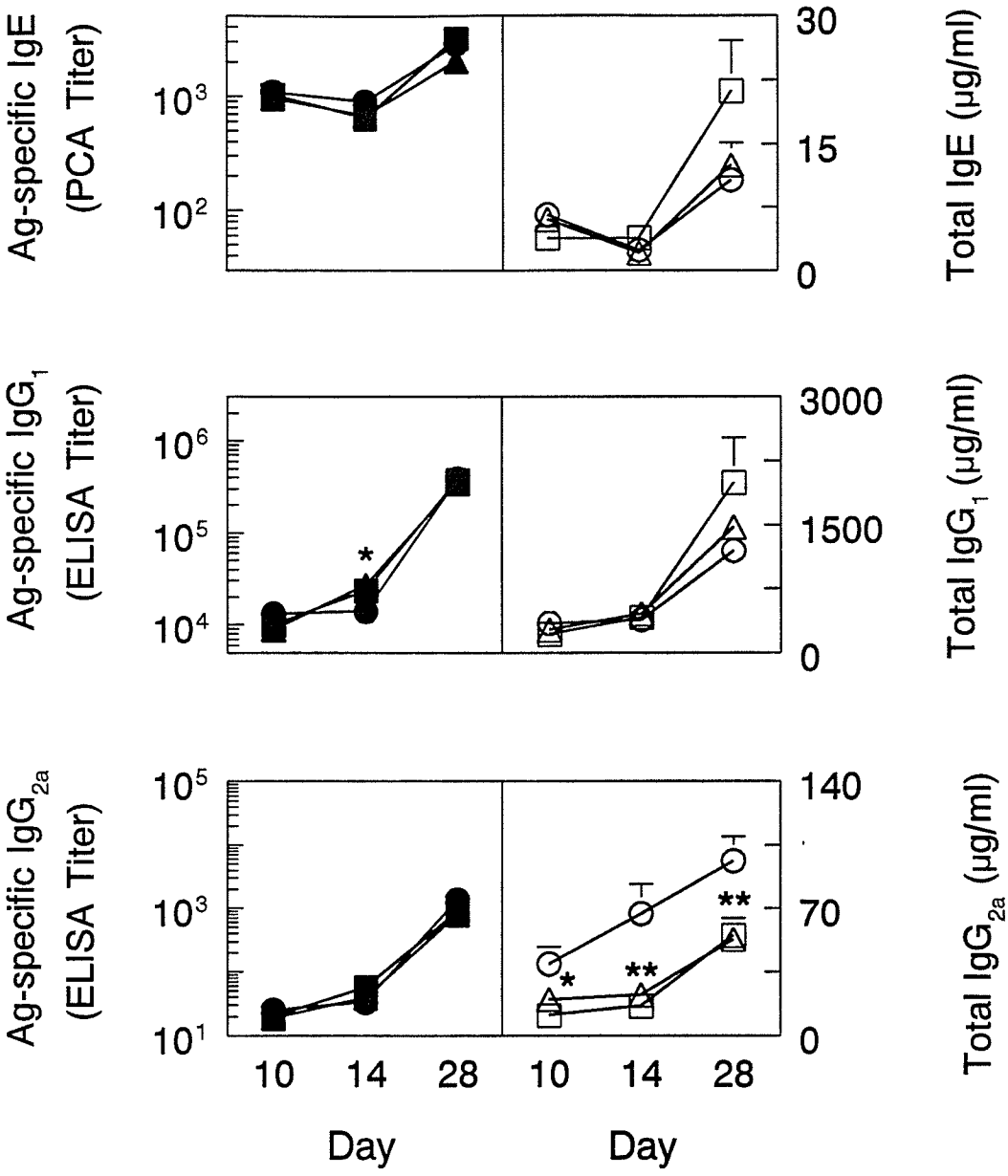

Figure 22. IL-12 deficiency fails to enhance IgE synthesis in OVA (alum) immunized mice. WT (●), p35 KO (■) and p40 KO (▲) (solid symbols indicate Ag-specific Ab synthesis; open symbols indicate total Ab synthesis) mice were immunized with 2 µg OVA (alum) on days 0 and 21. Mice were bled on days 10, 14, and 28. Means ± SEM (n = 12 mice) are shown. Significant differences to WT are shown ($p<0.05$*, 0.005**).

specific Ab synthesis. Serum IgE synthesis was generally unchanged in p35 KO mice (Fig. 23), except for a slight enhancement on day 10. Similar to the other immunization protocols, an impaired capacity to produce total IgG_{2a} in the absence of endogenous IL-12 was also seen.

Since in vivo neutralization of IL-12 enhances IgE production in OA-POL treated mice, we wanted to examine further if endogenous IL-12 was critical in the regulation of Ab responses upon type 1 humoral stimuli. CFA is an adjuvant which induces type 1 Ab profiles upon immunization with protein Ag. We immunized WT, p35 KO and p40 KO mice with OVA in the presence of CFA. The absence of endogenous IL-12 again failed to enhance IgE production in primary responses (Fig. 24). In contrast, both Ag-specific and total secondary IgE responses were suppressed in IL-12 KO mice. Decreased total IgG_{2a} was again apparent in IL-12 KO mice at all time points examined.

Taken together, this data indicates that a factor(s) (other than IL-12) acts to inhibit the production of IgE. This was demonstrated by (1) the moderate decrease in OA-POL induced IL-12 production upon anti-IL-12 treatment and (2) the similar IgE levels in IL-12 KO and WT mice following a variety of immunization protocols. Experiments examining endogenous IL-12 regulation upon OA-POL treatment are continuing in IL-12 KO mice.

8.3 IL-12 deficiency strongly suppressed IFNγ production

In addition to examining the involvement of IL-12 in Ab responses, we wanted to examine

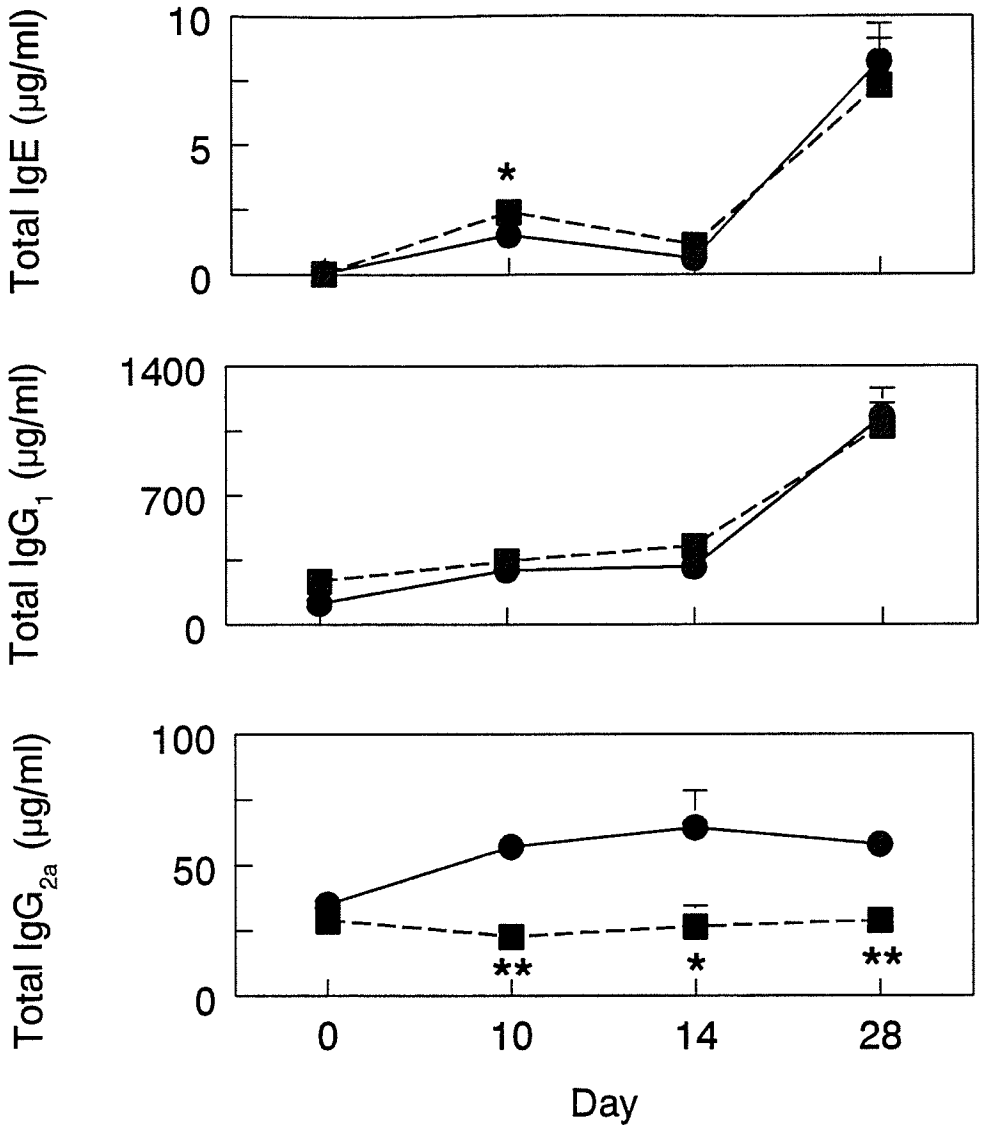

Figure 23. The absence of endogenous IL-12 does not greatly alter total IgE in mice injected with parasite extract. *T. spiralis* extract was prepared from a sterile filtered whole larvae homogenate. WT (●), and p35 KO (■) mice were injected with 50 µg of extract in the presence of alum on days 0 and 21. Mice were bled on days 10, 14, and 28. Means ± SEM (n = 12 mice) are shown. Significant differences to WT are indicated ($p<0.05$*, 0.005**).

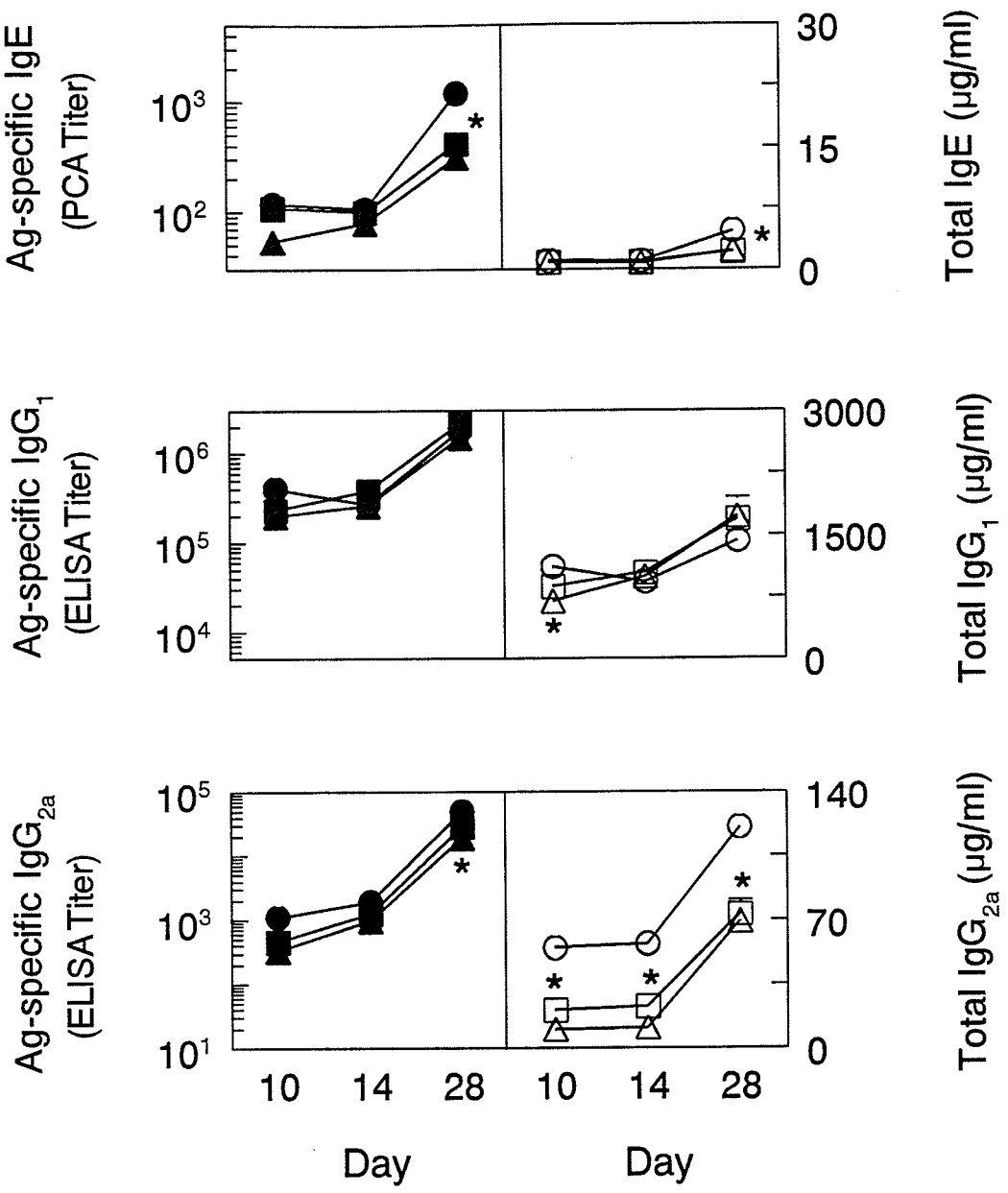

Figure 24. Secondary IgE production is decreased in IL-12 KO mice upon OVA (CFA) immunization. WT (●), p35 KO (■) and p40 KO (▲) (solid symbols indicate Ag-specific Ab synthesis; open symbols indicate total Ab synthesis) mice were immunized with 100 µg OVA (CFA) on days 0 and 21. Mice were bled on days 10, 14, and 28. Means ± SEM (n = 12 mice) are shown. Significant differences to WT are shown ($p<0.05$*).

spleen cell cytokine responses. Therefore, spleen cells harvested from WT, p35 KO and p40 KO mice which had been immunized with either OVA (alum) or OVA (CFA), were cultured in the presence of OVA. As previously reported (Magram, 1996), we observed greatly inhibited IFNγ production in IL-12 KO mice independent of the means of immunization (OVA, alum: Fig. 25; OVA, CFA: not shown). Addition of anti-CD4 mAb to culture eliminated WT IFNγ production, suggesting that IL-12 deficiency was predominately affecting CD4 T cell driven (rather than CD8 or NK cell derived) IFNγ synthesis. In contrast, type 2 associated cytokine production was not generally altered (Fig. 26). Ag driven IL-4 secretion was not significantly inhibited in OVA (alum) immunized mice. Stimulation of spleen cells with Con A or anti-CD3 similarly resulted in suppressed IFNγ synthesis, with minor modification of type 2 cytokine production (data not shown).

Spleen cell cytokine responses to *T. spiralis* extract (in the absence of adjuvant) were determined in WT and p35 KO mice. As seen with OVA immunized mice, IFNγ synthesis was inhibited (Fig. 27). Addition of anti-CD4 to culture essentially abolished IFNγ synthesis (data not shown). Unlike mice immunized with OVA, IL-10 synthesis was elevated approximately 2 fold in cultures from p35 KO animals.

The absence of endogenous IL-12 consistently inhibited IFNγ production. Reflective of the impact of IL-12 deficiency on humoral responses, type 2 cytokine production was not generally enhanced in IL-12 KO mice. This indicates that endogenous IL-12 promotes type 1 immunity by positively regulating type 1 cytokine without directly negatively regulating type

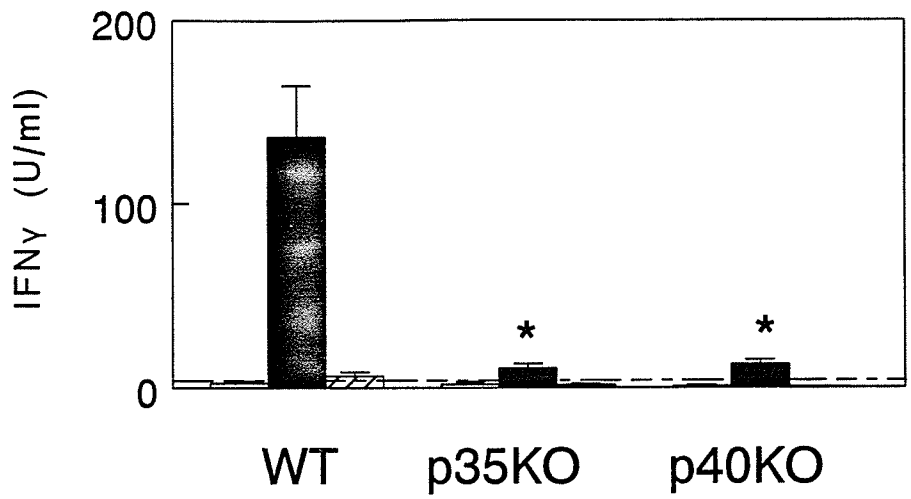

Figure 25. IFNγ production is reduced in IL-12 KO mice upon immunization. WT, p35 KO and p40 KO mice were immunized with 2 μg OVA (alum). Spleen cells were cultured in media alone (□), OVA (■) or OVA and anti-CD4 (▨). Supernatants were harvested at 48 hours and analyzed for IFNγ. Means ± SEM (n = 12 mice) are shown. Significant differences to WT are shown ($p<0.005$*).

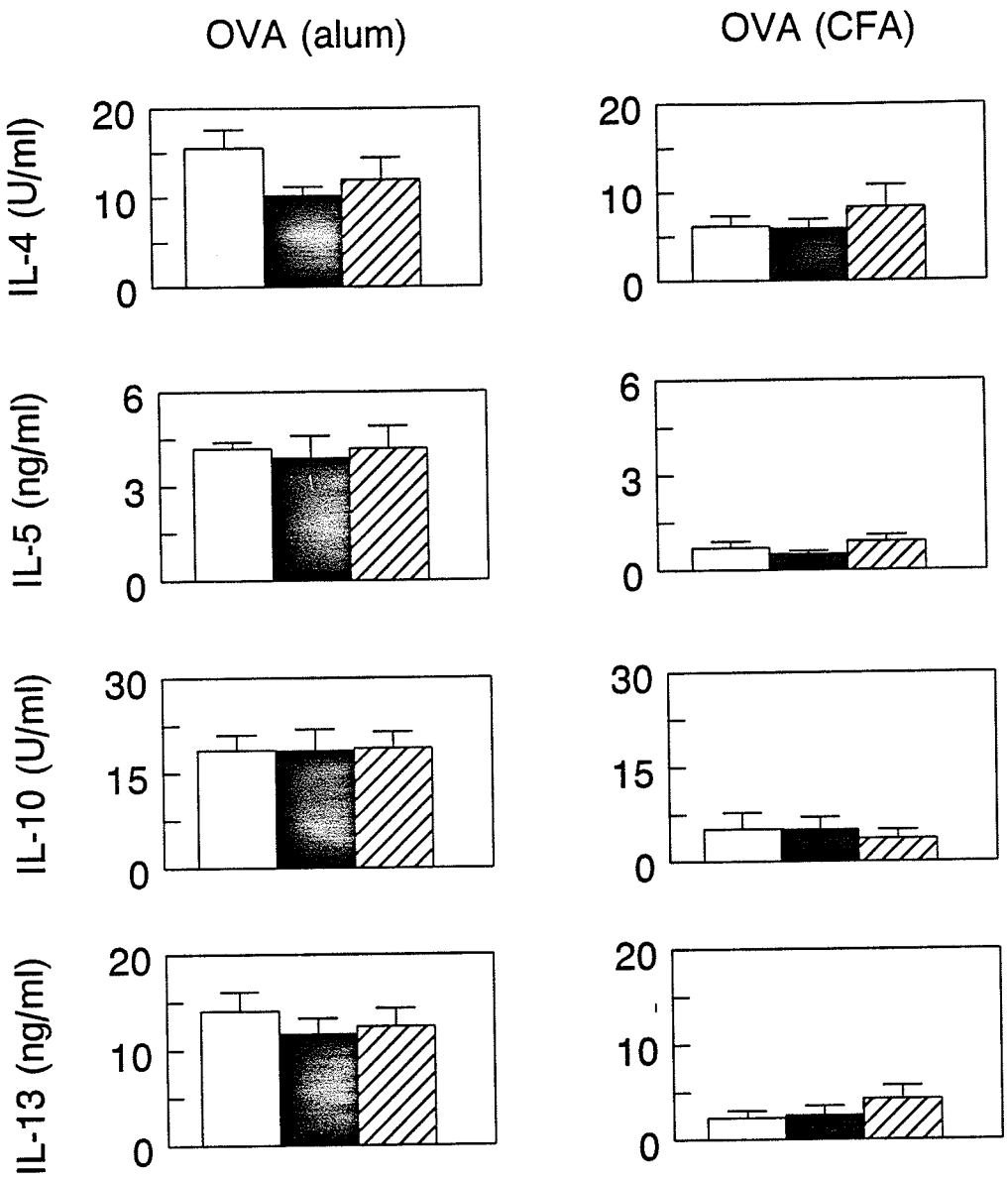

Figure 26. Type 2 associated cytokine synthesis is not greatly altered in IL-12 KO mice. WT (□), p35 KO (■) and p40 KO (▨) mice were immunized with either 2 μg OVA (alum) or 100 μg OVA (CFA) as indicated. Spleen cells were cultured in OVA. Means ± SEM (n = 12 mice) are shown.

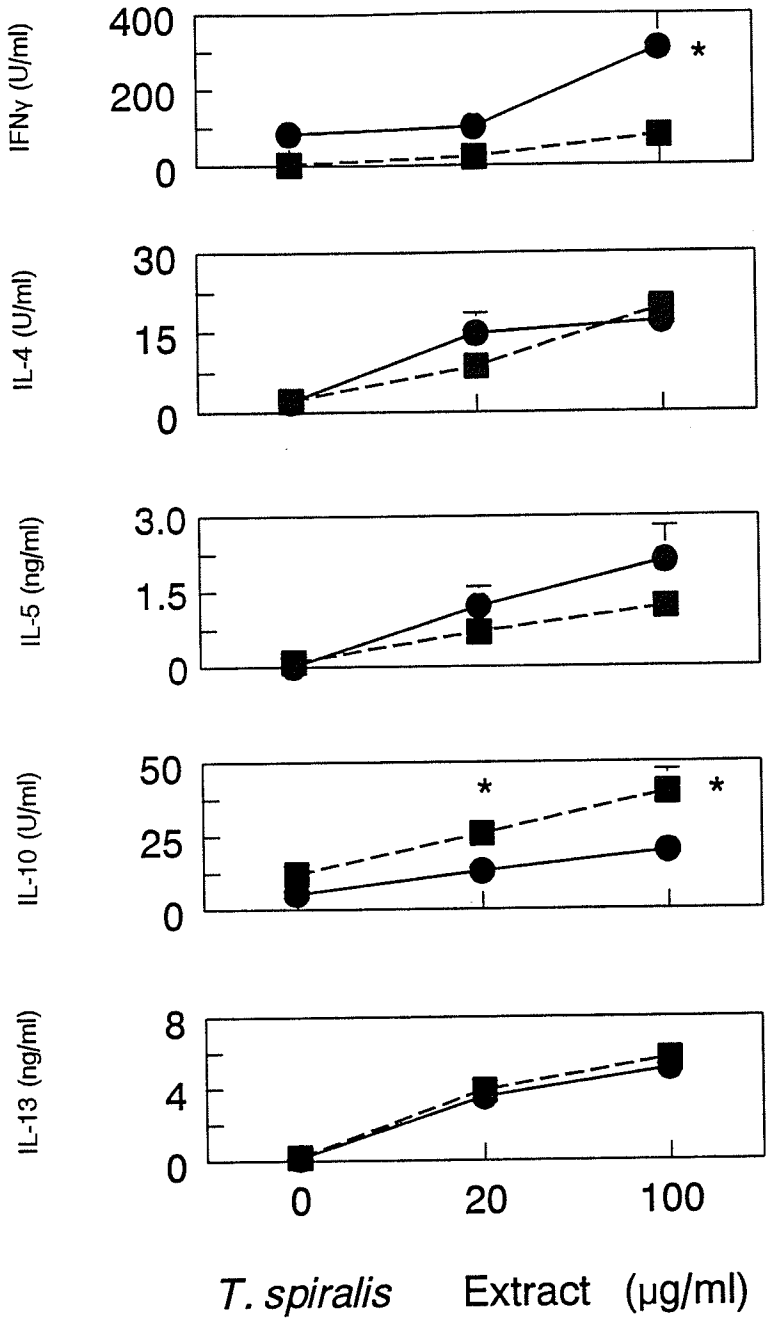

Figure 27. IFNγ production is decreased following injection of p35 KO mice with *T. spiralis* extract. WT (●) and p35 KO (■) mice were injected with 50 μg of *T. spiralis* extract in the absence of adjuvant. Five days later spleens were removed and cells were cultured in the presence of media, 20 μg/ml or 100 μg/ml of *T. spiralis* extract. Means ± SEM (n = 9 mice) are shown. Significant differences to WT are shown ($p<0.05$*).

2 cytokine production.

8.4 IL-12 deficient mice retained the capacity to respond to IL-12

IL-12 KO mice had substantially decreased IFNγ production upon stimulation of spleen cells with antigenic or polyclonal stimuli. Since IFNγ is considered to be important in maintaining IL-12R receptor expression, we wanted to determine if the absence of endogenous IL-12 (and lower IFNγ synthesis) resulted in lower spleen cell responsiveness to exogenous IL-12. The capacity of spleen cells from IL-12 KO mice to produce IFNγ upon the addition of rIL-12 to culture was analyzed. WT, p35 KO and p40 KO mice were immunized with OVA (alum). Spleen cells were harvested and stimulated with Ag in the presence and absence of IL-12. The addition of IL-12 to unstimulated and OVA stimulated cultures from p35 KO and p40 KO mice resulted in IFNγ levels similar to those seen in corresponding cultures from WT mice (Fig. 28). IL-2 was also added to cultures. While IL-2 alone did not modify IFNγ synthesis (data not shown) it did enhance the ability of IL-12 to promote IFNγ synthesis. IFNγ levels were similar in cultures receiving both cytokines from control and KO mice. This indicates that IL-12 deficiency does not in itself alter the ability of spleen cells to respond to IL-12.

Thus, although IL-12 KO mice are impaired in their ability to produce IFNγ, the absence of endogenous IL-12 and decreased IFNγ in vivo does not impact the ability of spleen cells to respond to IL-12 (added to culture). This suggests that IL-12 receptors are present to the same extent in WT and IL-12 deficient mice.

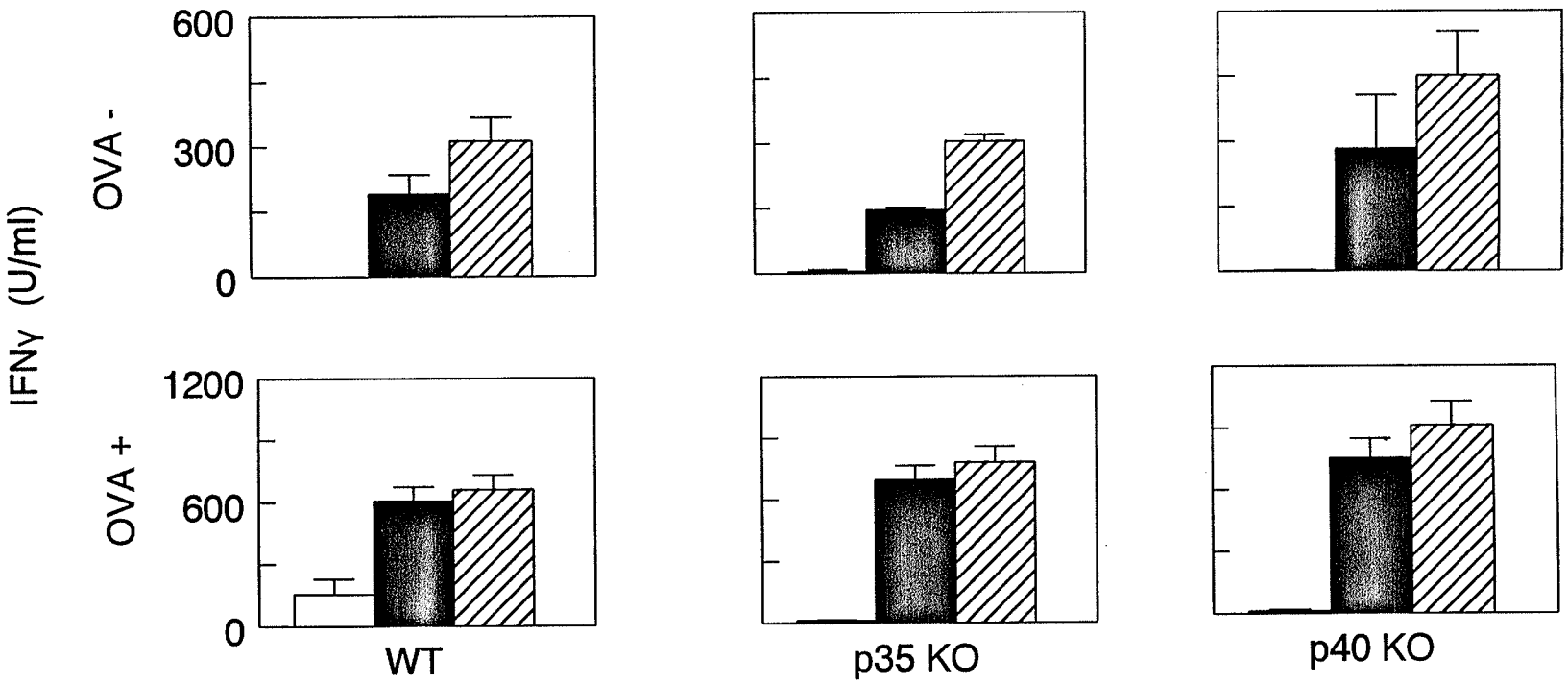

Figure 28. IL-12 responsiveness is intact in IL-12 deficient mice. WT, p35 KO and p40 KO mice were immunized with 2 μg OVA (alum). Five days later spleen cells were harvested at 48 h and cultured in the presence of media or OVA. Nothing (□), IL-12 (100 pg/ml) (■), or IL-12 and IL-2 (10 U/ml) (▨) were added to cultures. Means ± SEM (n = 6 mice cultured independently) are shown.

9.0 Impaired IgE production in IL-10 knockout mice was associated with increased type 1 cytokine production

9.1 Neutralization of IL-10 in vivo eliminated IgE production

IL-10 inhibits inflammatory cytokines including IL-12 and IFNγ (Moore, 1993; Muraille, 1998). It is speculated that a primary mechanism by which this occurs is through negative feedback loops, such that IL-12 induces the production of IL-10, which then limits IL-12 synthesis. Thus, in anti-IL-12 treated mice, the enhanced capacity of OA-POL to induce IgG_1 may reflect the absence of negative feedback mechanisms, such as the induction of IL-10, which could act on a range of cytokines. We hypothesized that the lack of IL-10 regulation would result in enhanced type 1 (IgG_{2a}) and decreased type 2 (IgE) humoral responses.

To determine the requirement for endogenous IL-10 in Ab production, WT mice were OVA immunized and treated with nothing, normal rat IgM, or rat IgM anti-mouse IL-10 (Fig.29). Administration of normal rat IgM did not significantly alter Ab production. Anti-IL-10 treatment resulted in a 54 fold suppression in Ag-specific IgE production. IgG_{2a} also appeared to be enhanced 5 fold, although the results were not statistically significant.

This indicates that endogenous IL-10 augments IgE synthesis in vivo.

9.2 IgE production was inhibited in IL-10 deficient mice

Anti-IL-10 treatment inhibited Ag-specific IgE production suggesting that endogenous IL-10

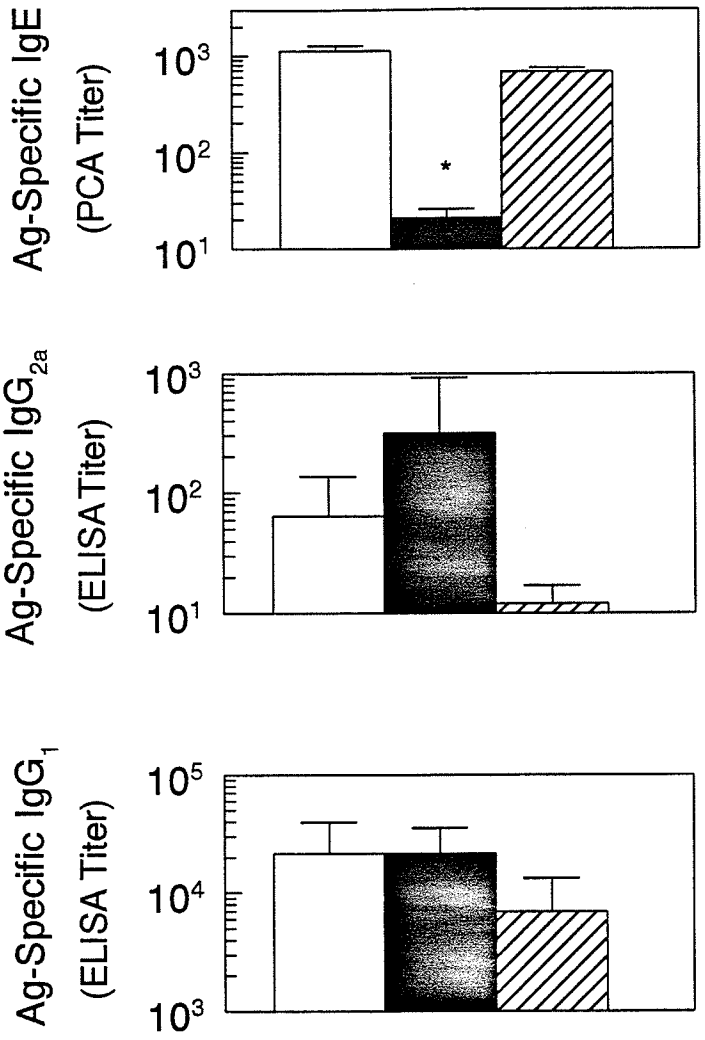

Figure 29. Anti-IL-10 treatment inhibits IgE synthesis. Mice were immunized with 2 μg OVA (alum) on d. 0. Mice were treated with nothing (□), rat IgM anti-mouse-IL-10 (■), or normal rat IgM (isolated from the sera of non-immunized rats) (▨) at 1 mg/injection on d. -2, -1, 0, 2, 3, and 5. Mice were bled on days 10 and 14 for analysis of IgE and IgG's respectively. Geometric means (IgE) and means (IgG_1 and IgG_{2a}) ± SEM (n = 11 mice) are shown. Significant differences to untreated, immunized cohorts are indicated (*p* values $<0.005^*$).

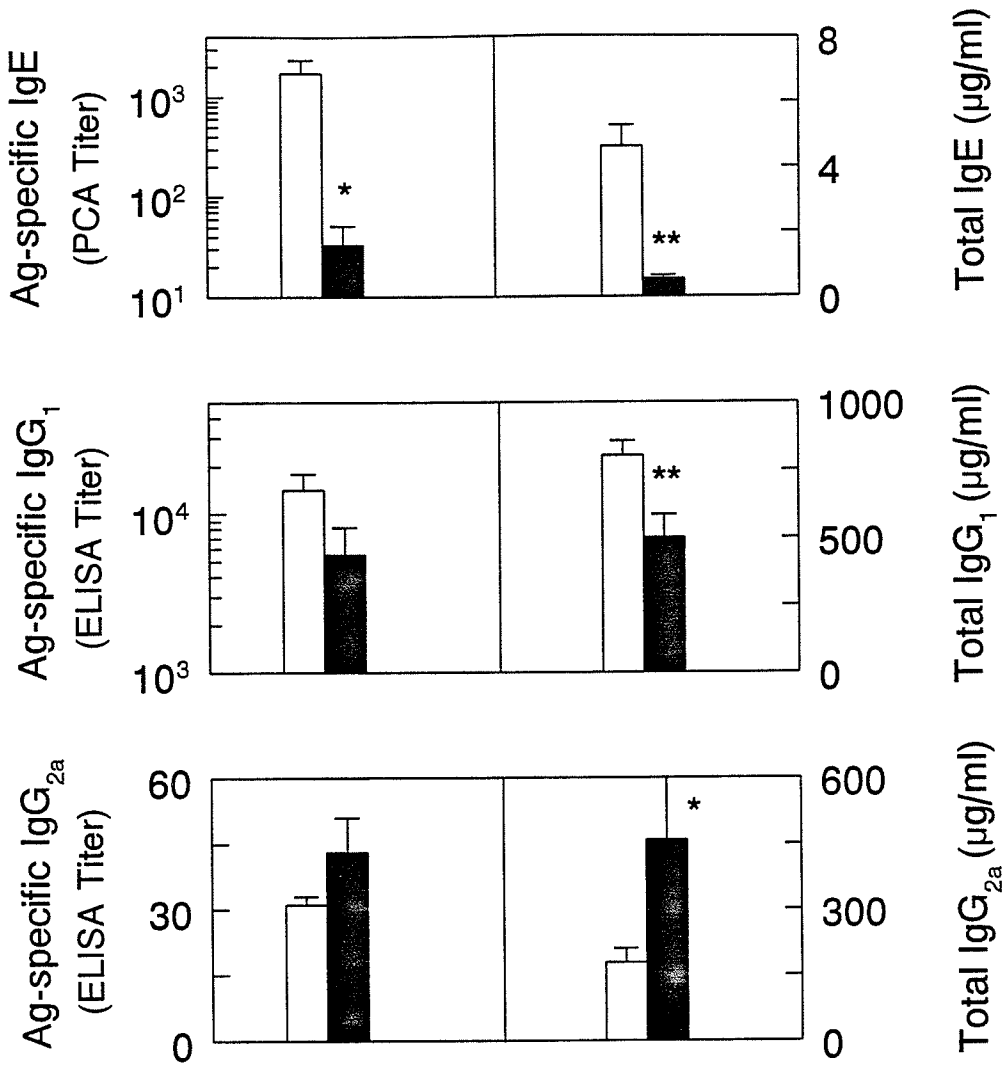

Figure 30. IgE production is inhibited in IL-10 KO mice. WT (□) and IL-10 KO (■) mice were immunized with 2 µg OVA (alum) on day 0. Mice were bled on day 10 and 14 for serum IgE and IgG's analyses, respectively. Geometric means (Ag-specific IgE) or means (all other isotypes) ± SEM (n = 9 mice) are shown. Significant differences to WT mice are indicated (p values $<0.05^{*}$ and 0.005^{**}).

is required for the production of IgE in immunized mice. We decided to continue this investigation using IL-10 KO mice as an alternative strategy.

WT and IL-10 KO mice were immunized with OVA (alum) (Fig. 30). Similar to WT mice receiving anti-IL-10 treatment, Ag-specific IgE synthesis in immunized IL-10 KO mice was suppressed approximately 60 fold.

Thus, in confirmation of data received from mice acutely treated with anti-IL-10 at the time of OVA immunization, observations from IL-10 KO mice also indicated that endogenous IL-10 is required for IgE production.

9.3 IL-10 deficiency enhanced type 1 cytokine production

To determine the importance of endogenous IL-10 on Ag-driven cytokine levels in short-term culture, spleen cells from immunized WT and IL-10 KO mice were stimulated with OVA (Fig. 31). Mean IFNγ production was increased 6 fold in the absence of IL-10. Conversely, IL-5 synthesis was decreased approximately 5 fold. However, a significant effect on IL-4 production was not observed.

IL-10 is a natural inhibitor of IL-12, preventing its uncontrolled production. To directly examine the effect of IL-10 deficiency on IL-12 production, spleen cells from immunized WT and IL-10 KO mice were cultured in different concentrations of OVA and LPS. Supernatants were harvested at various time points. In cultures established with media alone, IL-12 p40

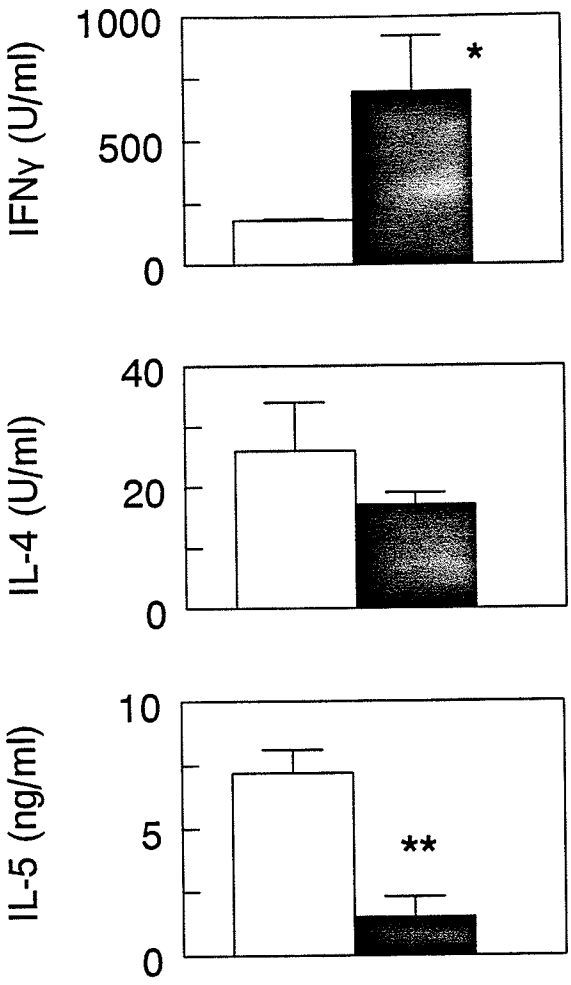

Figure 31. IL-10 KO mice have increased IFNγ and decreased IL-5 synthesis. WT (□) and IL-10 KO (■) mice were immunized with 2 μg of OVA (alum) on d. 0. On d. 5 spleen cells were harvested and cultured with or without OVA (1 mg/ml). In the absence of OVA, cytokine production was negligible (not shown). Means ± SEM (n = 7 mice) are shown. Significant differences to WT mice are indicated (p values $<0.05^{*}$ and 0.005^{**}).

production did not differ between strains. p40 production was greatly elevated in IL-10 KO mice at 0.3 and 1.0 mg/ml of OVA and 100 ng/ml of LPS (Fig. 32) and throughout the time frame of the experiment (Fig.33). Smaller increases were also seen with 10 ng/ml of LPS (Fig. 32). The extent of LPS contamination in OVA was examined to determine if p40 produced in OVA stimulated cultures was in response to antigen and not contaminating LPS. 1 mg of OVA contained 6.2 ng of LPS. As shown in Table 11, this represented approximately 1/3 of p40 production in cultures stimulated with 1 mg/ml of OVA, indicating that antigen stimulation accounts for the majority of the p40 detected.

Taken together, the data argue that, since IL-10 is a critical inhibitor of inflammatory cytokine production, the absence of endogenous IL-10 results in unrestricted type 1 cytokine synthesis. It may be that the enhancement of type 1 cytokine synthesis overwhelms the capacity of IL-4 to induce IgE with the net result being suppressed IgE synthesis.

10.0 Type 1 antibody and cytokine production were slightly enhanced in CD8 knockout mice

10.1 Total IgE and IgG_{2a} synthesis in OVA (alum) immunized mice was decreased in CD8 knockout mice

CD8 cells have been implicated in regulating IgE production. However, in rats depleted of CD8 prior to immunization, primary IgE levels remain constant or are decreased (Holmes, 1996).

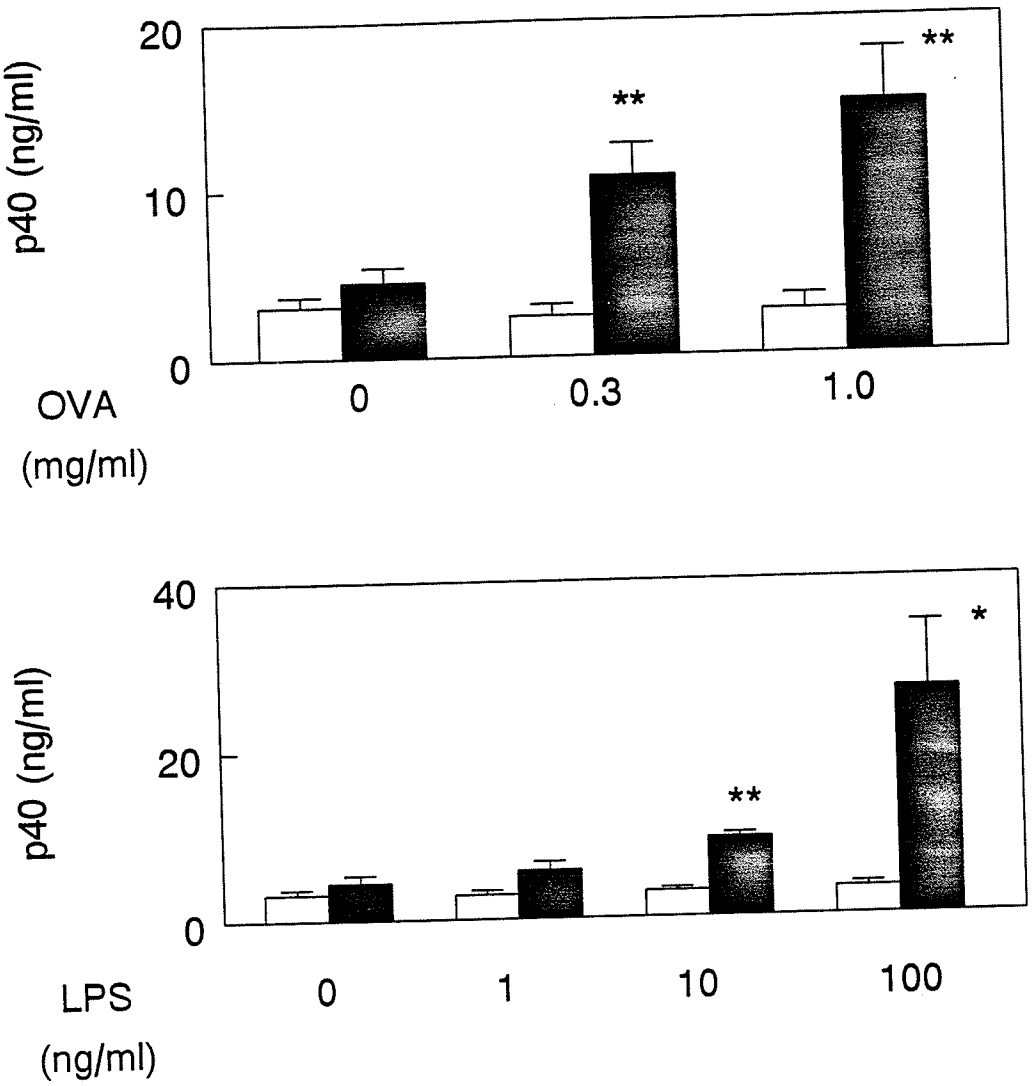

Figure 32. Endogenous IL-12 p40 production is elevated in IL-10 KO mice. WT (□) and IL-10 KO (■) mice were immunized with 2 μg OVA (alum) to activate immune system. Five days later spleen cells were removed and cultured at 1.5 million cells/200 μl. Cells were cultured in media alone, OVA or LPS at the indicated concentrations. Supernatants were harvested at 36 hours and analyzed for p40 production. Means ± SEM (n = 5) are shown (*p* values $<0.05^{*}$ and 0.01^{**}).

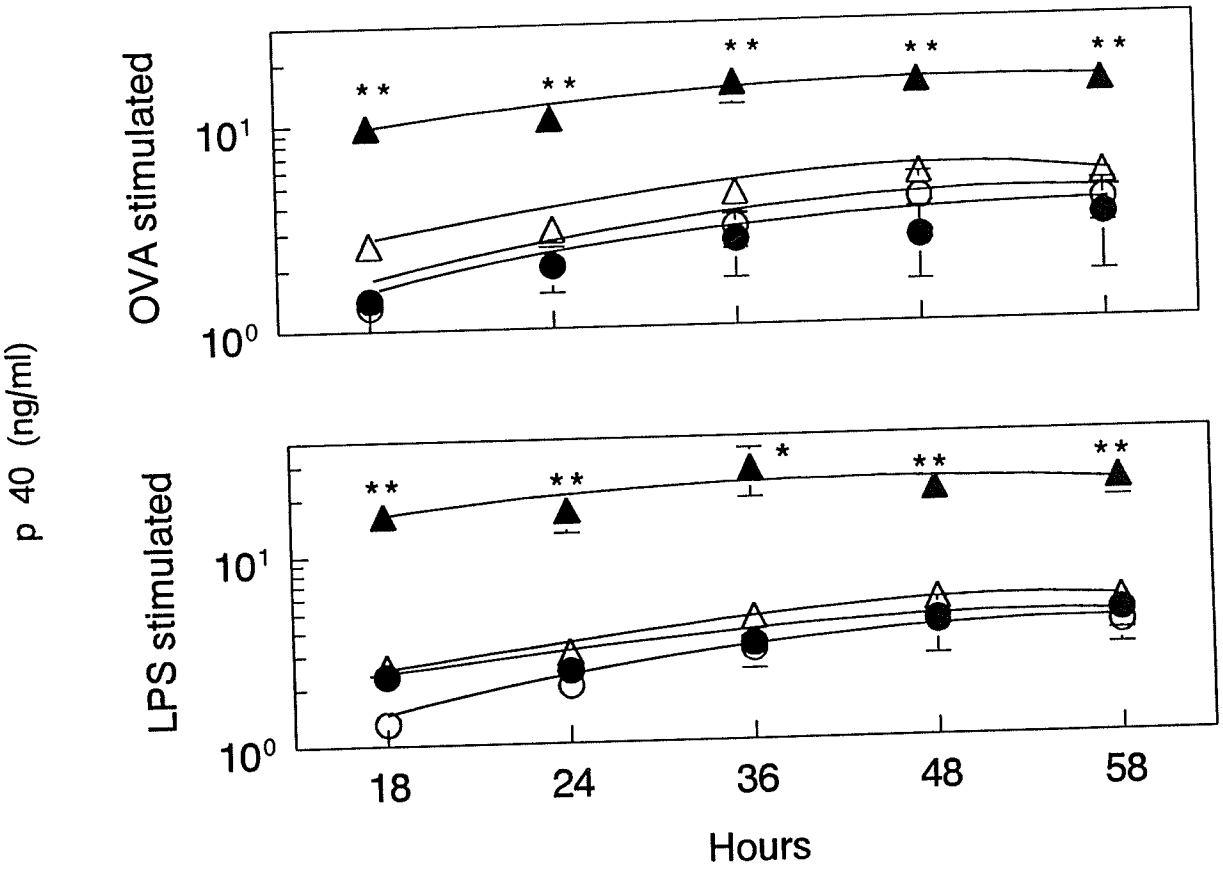

Figure 33. p40 levels are enhanced in IL-10 KO mice. WT (●) and IL-10 KO (▲) mice were immunized with 2 μg OVA (alum). Five days later, spleen cells were removed and cultured at 1.5 million cells per 200 μl. Cells were cultured in media alone (open symbols), OVA (1 mg/ml) (closed symbols) or LPS (100 ng/ml) (closed symbols). Supernatants were harvested at times indicated and analyzed for p40 production. Means ± SEM (n = 5 mice) are shown (p values $<0.05^{*}$ and 0.01^{**}).

Table 11. LPS contamination of OVA might account for one third of IL-12 p40 production in OVA stimulated cultures[a]

Spleen cell stimulation	Difference in IL-12 p40 synthesis between stimulated and media alone cultures (pg/ml)	Percentage of IL-12 p40 synthesis in OVA stimulated cultures that can be accounted for by LPS (%)	Percentage of IL-12 p40 synthesis in OVA stimulated cultures that can be accounted for by OVA (%)
6.2 ng LPS	3900	32	68
1 mg OVA	12000		

[a] 1 mg of OVA has 6.2 ng of LPS as determined by Limulus Amebocyte Lysate Pyrochrome Chromogenic Test Kit (Associates of Cape Cod, Inc.)

To further explore the impact of CD8 cells on humoral responses, WT and CD8 KO mice were immunized with OVA (alum). Total IgE titers were decreased in CD8 KO mice approximately 2.5 fold compared to WT mice (Fig. 34). While CD8 deficiency did not modify Ag-specific IgG_1 and IgG_{2a} synthesis (data not shown), total IgG_{2a} was inhibited 2 fold.

Thus, CD8 deficiency slightly, but consistently, decreases IgE production. In addition to examining the importance of CD8 cells on the induction of type 2 Ab responses, we also wanted to investigate the impact that the presence or absence of CD8 cells had on the capacity of OA-POL to switch established type 2 responses to type 1. To accomplish this we treated immunized WT and CD8 KO mice with OA-POL.

10.2 OA-POL treatment reduced established antigen-specific and total IgE levels

Previously, HayGlass and colleagues showed that OA-POL administered after OVA (alum) immunization has the capacity to abrogate OVA-specific IgE responses in WT mice (HayGlass, 1991a). Simultaneous with this decrease in IgE, OVA-specific IgG_{2a} and IgG_1 production was enhanced.

To confirm these results, C57Bl/6 mice were immunized with OVA (alum) and treated in the presence and absence of OA-POL. After three courses of OA-POL treatment mice were boosted and bled for Ab analysis. OA-POL treatment reduced Ag-specific and total IgE titres 12 and 7 fold respectively (Table 12). Ag-specific IgG_{2a} and IgG_1 titres were elevated.

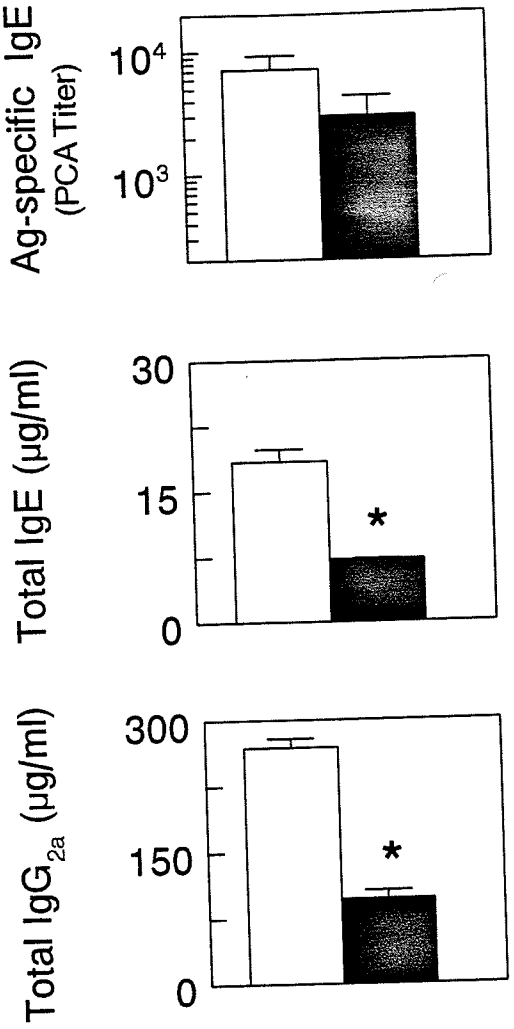

Figure 34. Total IgE synthesis is slightly inhibited in CD8 KO mice upon OVA (alum) immunization. WT (□) and CD8 KO (■) mice were immunized with 0.2 µg OVA (alum) on days 0 and 76. Mice were bled 7 days after being boosted. Geometric means (Ag-specific IgE) or means (other isotypes) ± SEM are shown from a representative experiment (n = 3 mice; $p<0.05$*).

Table 12. OA-POL abrogates established OVA specific IgE responses in vivo.

Ab Isotypes		Conditions[a]	
		OVA (alum)	OVA (alum) + OA-POL treatment
IgE	Ag-specific (PCA titer)[b]	6,939 ± 646	574 ± 133
	Total (μg/ml)[c]	12.9 ±1.7	1.8 ± 0.4
IgG_{2a}	Ag-specific (ELISA titer)	43 ± 9	2,369 ± 540
	Total (μg/ml)	218 ± 14	165 ± 31
IgG_1	Ag-specific (ELISA titer)	35,990 ± 5,675	493,000 ± 123,450
	Total (μg/ml)	849± 147	1,253 ± 230

[a] Mice were immunized with 0.2 μg of OVA (alum) on day 0. One group of mice was treated with 80 μg/day of OA-POL on 3 alternating days 2, 4 and 6 weeks after immunization. At week 8 mice were boosted. Mice were bled 7 days after boost for IgE and IgG's determinations. One representative experiment is shown (n = 3 mice).
[b] Geometric means ± SEM are shown for PCA titers.
[c] Means ± SEM are shown for remaining isotypes.

Yang *et al.*, further demonstrated that the capacity of OA-POL to alter humoral responses was associated with an increase in the IFNγ:IL-4 ratio (Yang, 1993). In a similar manner to those studies, mice were immunized and treated with and without OA-POL. After the final boost, spleen cells were stimulated with OVA. We also observed an increase in the IFNγ:IL-4 ratio (Table 13).

Thus, in agreement with earlier reports, OA-POL can abrogate established IgE responses. This capacity is associated with an increase in the Ag-driven spleen cell IFNγ:IL-4 ratio.

Using this system we sought to determine if the absence of CD8 regulation alters the ability of OA-POL to redirect a type 2 immune response into a type 1.

10.3 OA-POL induced antibody responses were slightly increased in CD8 knockout mice

Immunized WT and CD8 KO mice were treated with OA-POL, or not. After the last OA-POL treatment, mice were boosted and bled. OA-POL treatment inhibits IgE synthesis to a similar extent in WT and CD8 KO mice (Fig. 35 and 36). Although OA-POL treatment amplifies Ag-specific IgG_{2a} and IgG_1 in both groups, in KO mice Ag-specific IgG_{2a} and IgG_1 production was slightly, but significantly increased. Furthermore, total IgG_1 was elevated 3 fold in CD8 KO mice upon OA-POL treatment.

In summary, the capacity of OA-POL to skew a type 2 Ab response in a type 1 direction was

Table 13. OA-POL treatment shifts type 2 cytokine production to type 1.

IFNγ:IL-4 Ratios	
OVA (alum)[a]	OVA (alum) + OA-POL
1.5 ± 0.3	10.0 ± 2.0

[a] Mice were immunized with 0.2 μg of OVA (alum) on day 0. One group of mice was treated with 80 μg/day of OA-POL on 3 alternating days 2, 4 and 6 weeks after immunization. At week 8, mice were boosted. Mice were sacrificed 5 days after final boost. Spleen cells were cultured in the presence of OVA (1 mg/ml). Supernatants were harvested at 24 and 48 hours for analysis of IL-4 and IFNγ production respectively. Means ± SEM of IFNγ:IL-4 ratios (n = 3 mice individually cultured) are shown.

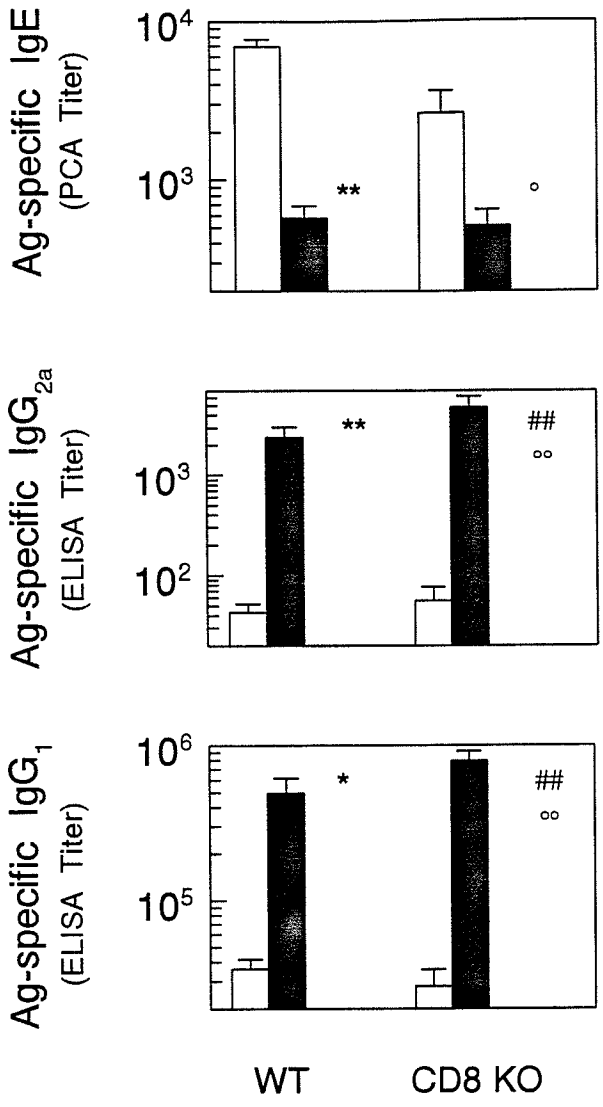

Figure 35. CD8 deficiency does not alter the ability of OA-POL to redirect pre-established type 2 humoral responses. WT and CD8 KO mice were immunized with 0.2 μg OVA (alum) on day 0 and 76. Mice were treated with 3 courses of 3 x 80 μg OA-POL (■) or not (□) beginning days 32, 46, and 60. Mice were bled 7 days after final boost. Geometric means (Ag-specific IgE) or means (other isotypes) ± SEM of 3 experiments are shown. Significant differences as compared to WT with no treatment"*", WT with OA-POL"#" and CD8 KO with no treatment"°" are indicated (p<0.05*, 0.01**).

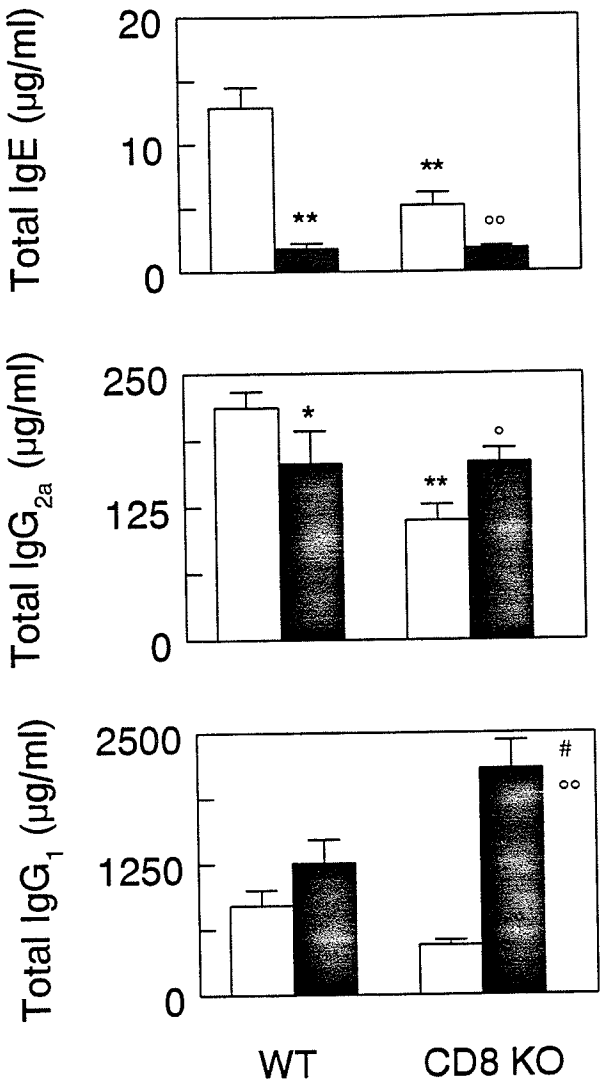

Figure 36. CD8 KO mice exhibit increased total IgG_1 following OA-POL treatment. WT and CD8 KO mice were immunized with 0.2 µg OVA (alum) on day 0 and 76. Mice were treated with 3 courses of 3 x 80 µg OA-POL (■) or not (□) beginning days 32, 46, and 60. Mice were bled 7 days after final boost. Means ± SEM of 3 experiments are indicated. Significant differences as compared to WT with no treatment"*", WT with OA-POL"#" and CD8 KO with no treatment"o" are indicated (p<0.05*, 0.01**).

somewhat enhanced in the absence of CD8 T cell regulation.

10.4 IFNγ synthesis was enhanced in CD8 deficient mice

Since CD8 deficiency appeared to favor type 1 humoral responses, we expected this to be reflected in altered cytokine production. Mice were immunized, OA-POL treated and OVA boosted. Five days after boosting, spleen cells were removed and stimulated with OVA. In the absence of OA-POL treatment, WT and CD8 KO mice had comparable levels of IL-4 and IL-5 (data not shown); IFNγ and IL-10 levels were elevated in CD8 KO mice (Fig. 37). IFNγ and IL-10 production in WT or CD8 KO mice was largely unaffected upon OA-POL treatment (data not shown).

OA-POL treatment inhibited IL-4 synthesis in both WT and KO mouse strains (data not shown). Due to the heightened IFNγ production in CD8 KO mice, CD8 KO mice had an elevated IFNγ:IL-4 ratio compared to WT mice (Fig. 38). In addition, in the absence of CD8 cells the IFNγ:IL-4 ratio observed upon OA-POL treatment was enhanced.

Finally, OA-POL treatment in the absence of CD8 cell regulation inhibited IL-5 synthesis by close to 40% (Fig. 39). IL-5 production in WT mice was unaffected by OA-POL injection.

Studies where CD8 cells had been depleted after immunization, supported the hypothesis that CD8 cells suppress type 2 responses (Holmes, 1996). However, our studies with CD8 KO expanded on observations that CD8 cells are required prior to immunization for optimal

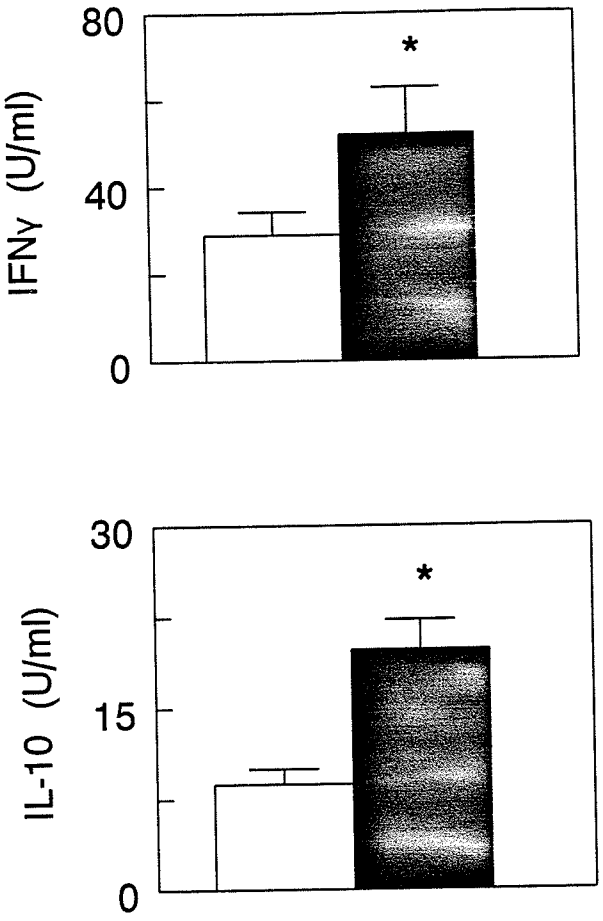

Figure 37. IFNγ and IL-10 are enhanced in CD8 deficient mice. WT (□) and CD8 KO (■) mice were immunized with 0.2 μg OVA (alum) on days 0 and 76. Mice were sacrificed 5 days after final boost. Spleen cells were cultured in the presence and absence of OVA (1 mg/ml). In the absence of OVA stimulation, spleen cells produced negligible amounts of cytokine. Means ± SEM of 3 experiments are indicated. Significant differences as compared to WT are indicated ($p<0.01$*).

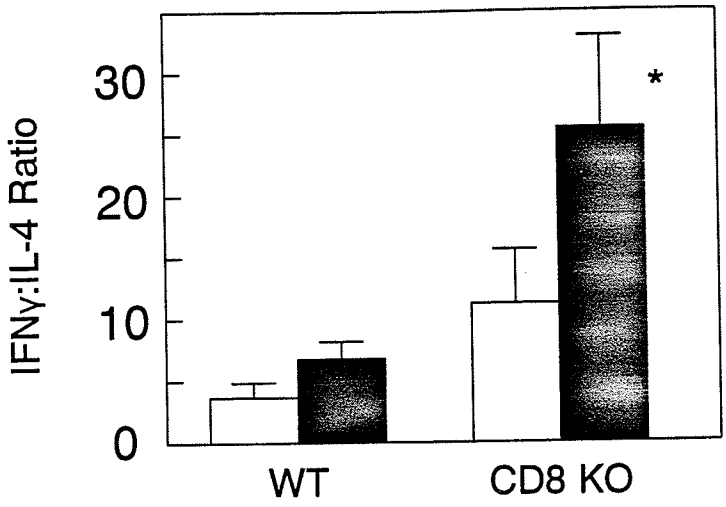

Figure 38. The IFNγ:IL-4 ratio is enhanced in CD8 deficient mice following OA-POL treatment. WT and CD8 KO mice were immunized with 0.2 μg OVA (alum) on day 0 and 76. Mice were treated with 3 courses of 3 x 80 μg OA-POL (■) or not (□) beginning days 32, 46, and 60. Mice were sacrificed 5 days after final boost. Spleen cells were cultured in the presence and absence of OVA (1 mg/ml). In the absence of OVA stimulation, spleen cells produced negligible amounts of cytokine. IFNγ and IL-4 ratios were calculated for individual wells. Means ± SEM (n = 12 mice) of ratios from cultures within a group were calculated. Significant differences as compared to WT with same treatment are indicated ($p<0.01$*).

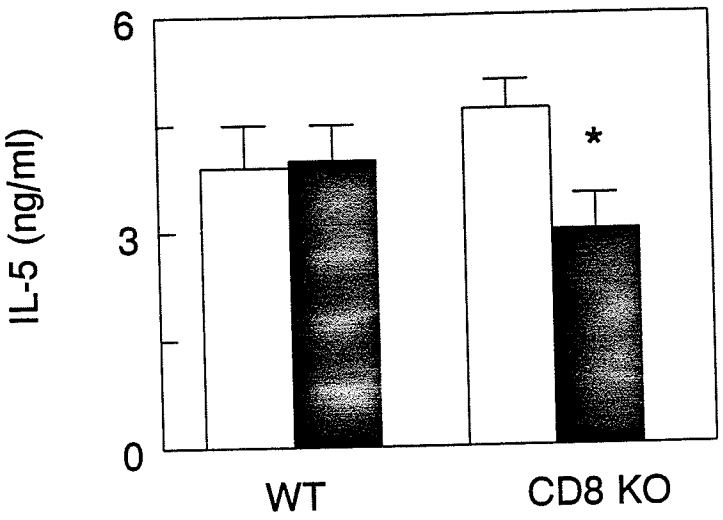

Figure 39. IL-5 production is decreased upon OA-POL treatment in CD8 KO mice. WT and CD8 KO mice were immunized with 0.2 μg OVA (alum) on day 0 and 76. Mice were treated with 3 courses of 3 x 80 μg OA-POL (■) or not (□) beginning days 32, 46, and 60. Five days after boost, mice were sacrificed and spleen cells were restimulated in the presence and absence of OVA (1 mg/ml). In the absence of OVA negligible amounts of cytokine were produced (not shown). Means ± SEM of 3 experiments are shown. A significant difference compared to CD8 KO mice without OA-POL treatment is indicated ($p<0.01$*).

production of type 2 responses. Although, changes both in Ab and cytokine production favoring a stronger type 1 response in the absence of CD8 T cell regulation were seen, the majority of these changes were less than 2.5 fold. Therefore, the biological relevance of these data may be limited.

V. Discussion

1.0 Introduction

Recognition that humoral responses are regulated by cytokines has stimulated considerable optimism for the possible pharmacologic usage of cytokines to alter detrimental Ab responses, such as IgE production in immediate hypersensitivity (Gillis, 1998). This study explored the potential adjuvant and therapeutic utility of exogenous IL-12 in the regulation of murine IgE synthesis. Furthermore, it examined the roles of endogenous IL-12 and IL-10 production in Ab regulation. Lastly, CD8 T cell regulation of Ab responses were investigated in CD8 KO mice. The primary effector response examined here was serum Ab synthesis. Cytokine secretion was analyzed in an attempt to understand the mechanisms which influence net serum Ab production.

2.0 Alum immunization results in strong Ag-specific IgE responses

This study utilized a well established animal model of immediate hypersensitivity. Mice immunized i.p. with OVA in the presence of alum, produce high levels of Ag-specific IgE, and extremely low levels of primary Ag-specific IgG_{2a}. Therefore, this approach was elected in preference to alternative methods, such as multiple courses of aerosolized Ag or epicutaneous exposure (Wang, 1996) which produce extremely weak IgE responses.

Although OVA (alum) immunization is a broadly recognized and widely used method of generating strong serum IgE responses, we wanted to verify whether or not alum alone induced large amounts of non-OVA-specific IgE. Therefore, we injected mice with OVA (alum) or alum alone. Alum injection (without Ag) resulted in Ag-specific or total IgE levels that did not differ from those seen in normal mouse sera (Fig.2). Secondly, total IgE levels were strongly correlated ($r = 0.84$) to Ag-specific levels induced upon immunization (Fig. 3). Taken together, this suggested that OVA (alum) immunization predominately induces OVA-specific IgE synthesis.

3.0 rIL-12 administration profoundly and transiently inhibited IgE production

When work on this study began, a number of reports had concluded that IL-12 possessed adjuvant qualities for the potential prevention of type 2 diseases (including allergy) based on (1) parasite and tumor studies demonstrating that IL-12 given with initial exposure to antigen was able to protect mice against ongoing exposure (Boggi, 1998; Heinzel, 1993), (2) the ability of rIL-12 to inhibit priming for IL-4 and IgE secretion in vitro (Boer, 1997; Kiniwa, 1992), and (3) the capacity of exogenous IL-12, given together with Ag, to inhibit *primary* IgE production (McKnight, 1994; Morris, 1994). Therefore, we hypothesized that IL-12 administration with primary immunization would induce and maintain long-term inhibition of IgE responses. We found that rIL-12 administered in vivo effectively abolishes primary IgE production, but upon secondary immunization (in the absence of further IL-12 treatment) IgE synthesis returned to controls levels. This could be attributed to IL-12 induction of NK cell IFNγ synthesis, in the absence of any noticeable change in the frequency of CD4 T cells

producing IFNγ or IL-4.

We began this study by verifying that we could repeat previous reports demonstrating that IL-12 induced IFNγ production (Galbiati, 1998). We found that the addition of exogenous IL-12 to culture enhanced the capacity of spleen cells from both naive and OVA (alum) immunized mice to produce IFNγ (Fig. 4, Fig. 5). When we injected rIL-12 into naive mice, a similar enhancement in serum IFNγ levels was observed (Fig. 6). This indicated that our system was responding as expected. Secondly, we examined the impact of exogenous IL-12 administration in vivo on spleen cell cytokine production in vitro (without the addition of IL-12 to culture). We found that rIL-12 administration in vivo did not alter the capacity of spleen cells from naive mice to produce IFNγ (data not shown), indicating that the changes in cytokine (observed upon Ag-driven stimulation of spleen cells from immunized/IL-12 treated mice) were dependent on Ag immunization. This was further confirmed by the negligible amounts of cytokine produced in unstimulated cultures of spleen cells from immunized/IL-12 treated mice (Fig. 10). Although it would have been possible to investigate the impact of exogenous IL-12 administration on Ab production following immunization in the absence of these controls, these controls assured us that the cytokine production seen upon OVA stimulation in vitro was Ag driven.

Having assessed culture controls, we examined the effect of rIL-12 administration in vivo on serum Ab responses. When we administered rIL-12 (200 ng x 5 days) simultaneous with OVA (alum) immunization, primary Ag-specific IgE production was inhibited by > 98 %

(Table 4, Fig. 8), a pattern similar to that observed by others. It had been reported that rIL-12 treatment inhibited IgE responses in mice injected with goat-anti-mouse IgD (Morris, 1994) or exogenous antigen (Bliss, 1996; McKnight, 1994). However, these studies only examined primary responses, making it impossible to assess the ability of IL-12 to alter long-term adaptive Ab memory responses required of a true adjuvant utility. To determine whether inhibition of primary IgE following rIL-12 administration translated into skewing of Ag-specific adaptive immunity, resulting in the continued suppression of IgE, IL-12 treated and untreated mice were OVA (alum) boosted in the absence of further rIL-12 administration. We found that initial IL-12 treatment did not result in continued inhibition of IgE upon secondary and tertiary immunization (in the absence of IL-12), such that treated and untreated mice had the same IgE levels (Fig. 8). In one report where secondary IgE responses were examined in response to Ag immunization, even continued IL-12 administration failed to inhibit secondary IgE production (Germann, 1995a). In addition, treatment of mice with high levels of IL-12 (1000 ng x 9 d) at initial parasite exposure still resulted in a 75 % increase of IgE synthesis upon secondary parasite exposure compared to primary IgE levels (Finkelman, 1994). Although 60 % inhibition of IgE levels was still observed, this may also indicate the involvement of alternate Ag processing of helminth as opposed to OVA. Together this data indicates that the presence of high levels of rIL-12 is able to greatly reduce primary IgE responses, but in the absence of IL-12 this inhibitory signal is removed and IgE production returns to normal levels upon subsequent immunization.

We also observed a 17 fold increase in primary OVA-specific IgG_{2a}. The capacity of IL-12

to increase IgG_{2a} had been previously observed (McKnight, 1994). Interestingly, IgG_{2a} synthesis remained 4 fold enhanced upon continued immunization in the absence of IL-12 (Fig. 8), indicating that IL-12 has the capacity to cause permanent switching to IgG_{2a} secretion. This is most likely mediated through IFNγ dependent mechanisms, since exogenous IL-12 was unable to enhance IgG_{2a} in IFNγ KO mice and anti-IFNγ inhibited IL-12 induction of IgG_{2a} mRNA transcription in vitro (Metzger, 1997). Furthermore, the capacity of IL-12 to increase the frequency of B cells producing IgG_{2a} was reversed by the addition of anti-IFNγ in vitro (Metzger, 1997). This supports earlier studies which demonstrated that anti-IFNγ treatment in vivo partly impaired serum IgG_{2a} production following rIL-12 administration (Germann, 1995b).

Decreased IgG_1 production (Fig. 8) following IL-12 treatment/immunization also probably reflected an IFNγ dependent mechanism. This conclusion is based on (1) the ability of IFNγ to inhibit IgG_1 and (2) in the absence of endogenous IFNγ (IFNγ KO mice), IgG_1 synthesis was enhanced following IL-12 treatment. IL-12 enhancement of IgG_1 occurred without affecting transcription of IgG_1 mRNA (Metzger, 1997). Together, this indicates that other cytokines, such as IFNγ, can influence the capacity of IL-12 to alter Ab production.

To examine whether IFNγ could be responsible for IL-12 induced changes in Ab production in our study, we examined the effect of IL-12 on IFNγ synthesis of naive and OVA (alum) immunized mice. Upon IL-12 treatment and immunization of mice, serum IFNγ levels displayed a similar early burst as in IL-12 treatment alone (Fig. 6 and 9). Ag-driven culture

IFNγ also exhibited an early increase (within 24 h) which quickly diminished. Furthermore, IL-4 synthesis was reduced, consistent with other reports (Fig. 10) (Galbiati, 1998). Thus, increased IFNγ and decreased IL-4 production by immune cells following IL-12 treatment could promote inhibition of IgE and enhancement of IgG_{2a} levels (Finkelman, 1994; Germann, 1995b).

IL-12 induces IFNγ production by T cells and NK cells. Since following rIL-12 administration (1) long-term inhibition of IgE responses did not occur (Fig. 8), and (2) increased IFNγ synthesis was apparent early after immunization and not at the peak of the CD4 T cell response (day 5, Fig. 11), this suggested that IL-12 administration did not result in a shift of the CD4 T cell population into a more type 1-like phenotype. To investigate the impact of IL-12 on CD4 T cell cytokine production directly, CD4 T cells (isolated from immunized plus/minus IL-12 treated mice) were established in short-term Ag-driven culture (to determine changes in the intensity of cytokine synthesis) and LDA (to determine changes in the frequency of CD4 T cells producing cytokine). Some inhibition of IL-4 synthesis by CD4 T cells (in short term culture, Fig. 13) from mice treated with IL-12 was observed, reflecting the decreased IL-4 production seen in bulk culture (Fig. 13) at the peak of the CD4 T cell response. Reduced IL-4 synthesis by CD4 T cells could result in impaired T cell help required for the induction of primary IgE responses. However, LDA results indicated that exogenous IL-12 treatment did not alter of the precursor frequency of CD4 T cells producing IL-4 or IFNγ (Table 5). This finding is important because it indicates that even though IL-12 treatment altered the amount of cytokine (resulting in strong type 1 cytokine profiles), in vivo

administration of IL-12 did not change the frequency of CD4 T cells able to produce these cytokines upon encounter with Ag. Thus, upon the next encounter with Ag in vivo, the OVA-specific CD4 T cells would have the same capacity to produce cytokine in response to Ag independent of IL-12 treatment.

It had been observed that IL-12 treatment increased the frequency of unseparated spleen or lymph node cells producing IFNγ and decreased the frequency of cells secreting IL-4 as assessed by immunohistochemical analysis and ELISPOT (McKnight, 1994; Morris, 1994). Unfortunately, the greatest liability of studies was that they examined whole cell populations. As a result, they could not distinguish which cell population(s) displayed altered frequency of cytokine production following IL-12 treatment. To speculate that these observations were representative of CD4 T cell activity would be premature, especially since NK1.1 depletion markedly inhibited IL-12 induced IFNγ synthesis in KLH immunized/IL-12 treated mice (McKnight, 1994).

We found that suppressed primary IgE synthesis following IL-12 treatment could be attributed to IL-12 activation of NK cells. The early burst of IFNγ levels following IL-12 treatment suggested that increases in IFNγ synthesis were the result of the capacity of IL-12 to promote NK cell IFNγ production. Heightened serum (on d. 3) and culture (on d. 1) IFNγ levels following IL-12 administration, were strongly inhibited by anti-NK 1.1 treatment, indicating that IL-12 induced early IFNγ synthesis was associated with NK cell activation (Fig.12). Moreover, anti-NK 1.1 treatment reversed IL-12 induced inhibition of IgE

responses (Fig.14), demonstrating that, in our study, IL-12 inhibition of IgE synthesis was dependent on enhanced NK cell IFNγ production.

Taken together, this component of the study indicates that IL-12 suppresses primary IgE synthesis by activation of NK cell IFNγ production. While this innate immunity was sufficient to alter primary effector responses and long-term IgG_{2a} synthesis, it did not result in a shift in the adaptive memory responses which would be required for the continued inhibition of IgE, such as the skewing of the frequency of Ag-specific CD4 T cells producing cytokine.

In contrast to the inability of exogenous IL-12 to prevent long term inhibition of IgE production, IL-12 treatment at the time of initial parasite infection in susceptible mice did promote durable protective immunity against re-infection (Heinzel, 1993). However, IL-12 induced protective immunity against parasites was associated with stronger type 1 cytokine profiles by CD4+ T cells, increases in macrophage nitric oxide cytotoxicity, in addition to heightened NK cell IFNγ production (Afonso, 1994; Scharton-Kersten, 1995; Wynn, 1996). Furthermore, IL-12 administration was shown to prevent the occurrence of tumors and cause the regression of established tumors. IL-12 mediated tumoricidal effects could be attributed to a combination of macrophage, CD8 T cell, CD4 T cell and NK cell activation involving both IFNγ dependent and independent mechanisms (Gollob, 1998).

The reason rIL-12 treatment could induce adaptive immune responses upon exposure to parasites and tumors, and not following immunization with soluble Ag remains to be

determined. However, these successes were associated with the capacity of IL-12 to enhance the type 1 CMI normally protective against parasites and tumors. This suggests that in a system were CMI is normally protective, IL-12 treatment can promote this normal CMI resulting in memory protection. In contrast, IL-12 does not appear to alter humoral immunity normally required to maintain the inhibition of IgE synthesis.

This would suggest that if IL-12 administration stimulated CMI against OVA, OVA-specific IgE responses might be inhibited. In support of this speculation, DNA vector vaccination (encoding an Ag gene) inhibited Ag-specific IgE and AHR responses upon immunization with Ag (alum), even if Ag (alum) immunization was delayed for 6 months (Raz, 1996). The importance of CD4 and CD8 T cells in this process was seen when adoptively transferred CD4 and CD8 T cells from mice immunized with DNA vectors induced lower IgE responses upon alum immunization (with corresponding protein product) (Lee, 1997).

4.0 rIL-12 administration in mice with established IgE responses

Simultaneous with our investigations on the potential of IL-12 to maintain inhibition of IgE synthesis, we began to investigate whether IL-12 could be therapeutically beneficial in abrogating IgE synthesis associated with chronic immediate hypersensitivity responses. The rationale for this study was based on (1) the capacity of IL-12 to inhibit IgE synthesis, (2) the capacity of IL-12 added to resting CD4 T cells from allergic individuals to enhance IFNγ synthesis and decrease IL-4 production (Marshall, 1995), and (3) the demonstration that a strong type 1 stimulus (OA-POL) can inhibit established IgE responses by > 90 % (HayGlass,

1991a). Therefore, we hypothesized that IL-12 administered to mice with established IgE responses would be able to abrogate type 2 immunity (IgE and IL-4 synthesis) and enhance type 1 immunity (IgG_{2a} and IFNγ production). We found that rIL-12 treatment strongly enhanced type 1 immunity, as seen by extensively increased serum IgG_{2a} (11 to 50 fold) and IFNγ levels of immunized mice. In contrast, IL-12 failed to abrogate established type 2 immunity. Furthermore, IL-4 production by non-B/non-T cells was markedly elevated in IL-12 treated mice, resulting in a net *increase* in type 2 activation.

Despite investigation with a variety of treatment protocols, IL-12 treatment did not inhibit Ag-specific IgE synthesis, and in some cases increased total IgE production (Table 6, 7, and 8). Other attempts to inhibit established IgE responses have been made without success (Finkelman, 1994). Most recently, Umetsu *et al.* observed slightly decreased Ag-specific IgE synthesis (2.7 fold compared to immunized, untreated mice) when mice immunized with OVA (alum, footpad) were treated with OVA-IL-12 fusion protein at secondary OVA challenge (Kim, 1997). Although this was interpreted as illustrating the capacity of IL-12 (directed to the appropriate APC by the attachment to the Ag) to decrease established IgE production, this decrease was minor. This is consistent with the inability of IL-12 to redirect already differentiated systems including established Th2 clones and parasitic responses (Murphy, 1996; Rogge, 1997; Szabo, 1995).

In contrast to the inability of exogenous IL-12 administration to suppress IgE production, Ag-specific IgG_{2a} was highly elevated (Table 6, 7 and 8). This was also seen with OVA-IL-12

fusion protein treatment of established responses (Kim, 1997). Increased IgG_{2a} corresponded with strong serum (Fig. 15) and culture IFNγ synthesis (Fig. 16). These increases in IFNγ were most likely associated with NK cell activation (Fig.12).

Prolonged courses of exogenous IL-12 treatment had minimal impact on the intensity (short term culture, Fig. 17) or the frequency of CD4 T cells secreting IFNγ (LDA, Table 9). Conversely, the frequency of CD4 cells producing IL-4 was consistently and strongly decreased. This contradicts what is known about the inability of IL-12 to modify cytokine profiles of conditioned Th2 clones due to their loss of IL-12R β2 chain expression (Rogge, 1997; Szabo, 1995). However, in contrast to in vitro studies which use mature homogeneous clonal populations, in vivo Ag-specific T cell responses to exogenous Ag are thought to consist of (1) differentiated T cells (producing predominately either type 1 *or* 2 associated cytokines), (2) heterogeneous memory cells capable of producing both type 1 *and* 2 cytokines, and (3) uncommitted Ag-specific T cells. Upon secondary immunization all three populations of Ag-specific T cells are likely to be present. While predominantly type 2 responses were apparent following primary OVA (alum) immunization (in the absence of IL-12 treatment), upon secondary immunization (in the absence of IL-12 treatment) increased Ag-specific IgG_{2a} and serum IFNγ levels were seen (Fig. 8, 9 and 15), suggesting that de novo commitment of naive T cells to a type 1 phenotype occurs over the course of the experiment in response to OVA (alum) immunization. Whether this reflects an attempt by the immune system to restrict type 2 immunization immunity or expansion of initial immunization responses is unknown. Thus, IL-12 treatment at secondary immunization may greatly

augment this normal development of type 1 immunity. The possibility that repeated IL-12 administration enhanced this de novo type 1 immunity by influencing uncommitted T cell activity in a Th1 direction was consistent with the clear reduction in the frequency of OVA-specific CD4 T cells producing IL-4. Similarly, altered CD4 T cell cytokine production had also been seen upon IL-12 treatment of established *B. malayi* specific responses, resulting in elevated CD4 T cell mediated IFNg production and suppressed IL-4 synthesis, despite no change in IgE levels (Pearlman, 1995). Furthermore, evidences suggests that successful immunotherapy results, not in altering established T cell responses, but in recruiting uncommitted Ag-specific T cells, which retain responsiveness to IL-12, into Th1-like cells (Jutel, 1995). Another explanation for the decrease in the frequency of IL-4 producing CD4 T cells is that extended IL-12 administration switched off IL-4 production of OVA-specific Th2-like cells. This is unlikely as IL-4 production from Th2 clones is highly stable (Murphy, 1996; Szabo, 1995).

In marked distinction to the capacity of extended IL-12 administration to inhibit the frequency of IL-4 producing CD4 T cells, OVA stimulation of unseparated spleen cells resulted in highly increased IL-4 synthesis. Enhanced IL-4 synthesis and mRNA expression following IL-12 administration in established immunization and *L. major* responses was previously reported (Bliss, 1996; Wang, 1994). But contrary to our LDA results, these reports speculated that the source of IL-4 was CD4 T cells.

IL-4 production is important for inducing primary and secondary IgE production following

immunization, since anti-IL-4 treatment eliminated the induction of primary and secondary IgE responses upon TNP-KLH (alum) immunization (Finkelman, 1990). Some secondary IgE responses to polyclonal and infectious agents also require the presence of IL-4 (Finkelman, 1990; Yamashita, 1996). However, while IL-4 driven primary IgE synthesis appeared to be T cell derived, secondary IgE responses seemed to be more dependent on IL-4 produced from non-T cell sources (Yamashita, 1996). Specifically, non-B/non-T cells have been reported to be important producers of IL-4 in systems where IgE is already present (Aoki, 1995). We observed that secondary immunization (in the absence of IL-12 treatment) resulted in 24 times more IL-4 synthesis from non-B/non-T cells than CD4 T cells (non-B/non-T cells: 6 U/ml IL-4/2.5 million cells, Fig 19; CD4 T cells: 0.5 U/ml IL-4/5 million cells, Fig. 17).

When we administered IL-12 to mice with established Ab responses the number of non-B/non-T cells (defined as CD4-, CD8- and CD19-, Fig. 18) was enhanced, without altering the total number of CD4 or CD8 positive cells in the spleen. It had been previously reported that IL-12 production enhanced the B220-, CD3- cells within the spleen (McKnight, 1994). In addition, we observed that rIL-12 treatment augmented the ability of non-B/non-T cells to produce IL-4 (Fig. 19). Thus, the 15 fold enhancement of IL-4 in spleen cell culture (Fig. 16) appears to result from the ability of IL-12 to increase the number of non-B/non-T cells in the spleen and enhance their capacity to produce IL-4.

We speculate that these non-B/non-T cells were basophilic in nature and expressed FcεRI.

It has been previously shown that non-B/non-T cells, able to produce IL-4 upon FcεRI cross-linking, possess basophilic characteristics, such as cytoplasmic granules and encapsulated histamine (Seder, 1991). In addition, human basophils have been cited as a major source of IL-4 in Ag stimulated cultures from atopic individuals (Kasaian, 1996).

The mechanism of IL-12 action on these cells is under investigation. Since up regulation of FcεRI on basophils by IgE has been reported (Lantz, 1997), we are currently attempting to determine whether exogenous IL-12 administration enhances FcεRI expression.

In summary, this component of the study demonstrated that IL-12 administration after the development of type 2 humoral responses resulted in enhancement in type 1 immunity exhibited by strongly elevated IgG_{2a} production, enhanced IFNγ synthesis and a consistent reduction in the frequency of IL-4 producing CD4 T cells. However, despite the generation of type 1 immunity, IL-12 administration markedly increased spleen cell IL-4 levels, attributed to an increase in the number of non-B/non-T cells within the spleen and their capacity to produce IL-4. These increased IL-4 levels would contribute to the inability of exogenous IL-12 treatment to decrease IgE synthesis in mice with established Ab responses. Collectively, this argues that the potential for exogenous IL-12 treatment to exert beneficial effects on ongoing type 2-dominated humoral responses, such as in the case of atopy, may be limited.

A potential trivial explanation for our inability to maintain inhibition of IgE or alter established IgE responses is that we did not administer sufficient IL-12. In an effort to inhibit IgE

responses following *N. brasiliensis* infection, Finkleman *et al.* administered 1000 ng x 9 days of IL-12 to mice (9 times our amount) (Finkelman, 1994). However, even at this dose secondary IgE production was enhanced 75 % compared to primary inhibition of IgE, indicating that the inhibition of primary responses was unstable. Furthermore, in our attempts to abrogate established responses, mice were treated with a wide range of protocols (Fig. 6). These ranged from mice receiving a single course of IL-12 injections over 6 days beginning immediately prior to secondary immunization, to repeated series of five courses (200 ng x 5) of IL-12 treatment with the final course being administered with secondary immunization, 225 days after initiation of the experiment. Although it remains possible that administration of substantially higher amounts of IL-12 might be more effective, this would need to be balanced against the severe toxicity commonly associated with such levels (Ryffel, 1997), which could have been responsible for the high mortality we saw at doses of 1000 ng/ml. Taken together, it is unlikely that higher doses of exogenous IL-12 administration would have proven beneficial (and may have actually have been detrimental) in maintaining inhibition of IgE responses upon subsequent immunization or abrogating established responses.

5.0 Endogenous IL-12 regulation of humoral mediated immunity

Increasing evidence indicates that endogenous IL-12 is important in regulating T cell and macrophage responses in autoimmune and inflammatory diseases (Davidson, 1998; Leonard, 1995), as well as in the induction of protective immunity in parasite resistant mice (Anderson, 1998; Cooper, 1995; Mattner, 1996). However, there is limited information on the role of

endogenous IL-12 in modulating Ab responses in vivo. Thus, we examined the importance of endogenous IL-12 in the induction of type 1 and type 2 Ab responses.

Our interest in endogenous IL-12 regulation of IgE synthesis was driven by our observations that OA-POL treatment (a strong inducer of OVA-specific type 1 immunity), given prior to OVA (alum) immunization, is able to induce and maintain pronounced inhibition (~ 95 %) of IgE production upon subsequent immunizations (Table 10) in a CD4 T cell dependent manner and is associated with an increase in the ratio of IFNγ:IL-4 secretion (Yang, 1993). Large chitin complexes have also been shown to induce strong type 1 responses (stronger IL-12 production) upon mannose receptor-mediated phagocytosis by macrophages, compared to smaller structures (Shibata, 1997). Since the M_r of OA-POL is 400 times that of naive OVA and it is glycosylated with many mannose residues (Sherblom, 1989), this suggests that OA-POL could be processed by APC in a similar manner to chitin particles resulting in the greater induction of endogenous IL-12, Th1-like OVA-specific CD4 T cell development and the inhibition of IgE synthesis. In addition, the presence of IL-12 (as seen in the first component of this study) did inhibit primary IgE. Therefore, we hypothesized that the capacity of OA-POL to inhibit IgE is dependent upon OA-POL induction of endogenous IL-12 secretion.

To examine this possibility, immunized mice were pretreated with OA-POL and anti-IL-12 polyclonal Ab. OA-POL treatment decreased OVA (alum) induced Ag-specific IgE by about 30 fold. Anti-IL-12 administration substantially (but not completely) reversed the capacity of OA-POL to inhibit IgE synthesis (Fig. 20). These OA-POL induced changes in Ab

production were neutralized by anti-IFNγ mAb treatment, indicating that this effect is IFNγ dependent (HayGlass, 1991b). The inability of anti-IL-12 to totally reverse OA-POL inhibition of IgE suggested that (1) OA-POL induced IFNγ directly or through cytokines, in addition to IL-12, or (2) IL-12 was incompletely neutralized. It was not possible for us to distinguish between these possibilities using this experimental approach. However, independent studies with heat killed *B. abortus* and *Listeria* indicated that other endogenous cytokines (in addition to IL-12) were involved in the inhibition of IgE synthesis (Scott, 1997; Yeung, 1998).

Immunization of Ag with heat killed *B. abortus* and *Listeria* initiated strong type 1 innate immune responses associated with enhanced IL-12 and IFNγ production and decreased Ag-specific IgE responses (Scott, 1997; Yeung, 1998). Using neutralizing anti-IL-12 Ab, it was examined whether inhibition of IgE could be attributed to enhanced endogenous IL-12 production upon injection with heat killed *B. abortus* and *Listeria*. Anti-IL-12 treatment did not alter the capacity of *B. abortus* to inhibit IgE, although a partial increase in the frequency of IL-4 producing spleen cells was seen (Scott, 1997). Treatment with anti-IL-12 mAb partially restored heat killed *Listeria* inhibition of Ag-specific IgE responses (64 %) (Yeung, 1998). In addition, neutralization of endogenous IL-12 returned the altered cytokine production (i.e. increased IFNγ and decreased IL-4) seen following heat killed *Listeria* treatment to control levels. Since anti-IFNγ mAb reversed the inhibited IgE synthesis to the same extent as anti-IL-12 mAb, it appears that heat killed *Listeria* induction of IL-12 results in IFNγ dependent inhibition of IgE production.

Together, this suggests that the inhibition of IgE and IL-4 induced by OA-POL, heat killed *B. abortus* and *Listeria* treatments are mediated by a number of factors, including enhanced IL-12 production. The importance of endogenous IL-12 in OA-POL treatment is being further investigated by I. Lewkowich and K. HayGlass in IL-12 KO mice. That approach, while interesting, may not solve the puzzle due to redundancy within the immune system apparent in congenitally defective mice.

Taking an independent experimental strategy, we continued to examine the importance of endogenous IL-12 in the regulation on Ab responses using p40 and p35 KO mice. There are indications that p40 homodimers inhibit the induction of type 1 immunity, possibly by acting as a direct antagonist of IL-12 receptor binding (Gillessen, 1995). In vitro and in vivo models have shown differences in the generation of type 1 immunity either in the excess or absence of p40 (Heinzel, 1997; Yoshimoto, 1998). Therefore, to account for a potential role $(p40)_2$ may have in humoral immunity, we felt that it was important to use both p35 and p40 KO mice. However, we did not see differences in Ab or cytokine responses between p35 and p40 KO mice. Thus, the term IL-12 KO mice will be used to denote both p35 and p40 KO mice. One limitation of working with p35 and p40 KO mice is that p40 homodimer inhibition of p70 activity can not be detected, since p70 does not exist in either model. Thus, differences between the two strains would have reflected the capacity of p40 to act on immune responses independent of regulating IL-12 activity.

To specifically investigate the role of endogenous IL-12 in the regulation of Ab responses

generated by a type 1 stimulus, IL-12 KO mice were immunized using type 1 (CFA) and type 2 (alum) promoting conditions. Contrary to the hypothesis that we and others (Magram, 1996) proposed that endogenous IL-12 acts as a constitutive inhibitor of type 2 cytokines (IL-4 and IL-5) and Ab (IgE) responses in vivo, IL-12 KO mice did not exhibit increased IgE production (Fig. 24). Likewise, IgE responses to type 2 stimuli (OVA (alum), Fig. 22 and *T. spiralis* (alum), Fig. 23) were not enhanced in p35 and p40 KO mice. The conclusion that endogenous IL-12 is not a central inhibitor of IgE responses in vivo is further supported by reports that (1) primary IgE levels were somewhat enhanced in IL-12 KO mice in response to KLH (100 μg; alum) immunization (2.4 fold) (Schijns, 1998), upon anti-IL-12 treatment of mice exposed to nebulized Ag (2 fold) (Keane-Myers, 1998) or injection with irradiated *S. mansoni* (Anderson, 1998), (2) no differences in IgE levels between p40 KO and WT mice were seen following infection with *S. mansoni*, and (3) the capacity of anti-IL-12 to inhibit IgE in mice exposed to nebulized Ag was dependent on mouse strain (Keane-Myers, 1998). In addition, we observed that upon secondary immunization, IgE levels were slightly suppressed in IL-12 KO mice, suggesting that IL-12 may contribute to secondary IgE levels.

In contrast to the partial or null impact of endogenous IL-12 on IgE production, IL-12 appears to be critical in the regulation of total IgG_{2a}, as it was consistently suppressed in IL-12 KO mice (Fig. 22, 23 and 24). Likewise, we observed that anti-IL-12 treatment was able to partially reverse OA-POL induced IgG_{2a} synthesis (3.5 fold, Fig. 20). Enhanced IgG_{2a} synthesis following heat killed *B. abortus* and *Listeria* treatment showed a stronger

dependence on endogenous IL-12, since neutralization of endogenous IL-12 greatly inhibited increased serum IgG_{2a} synthesis (Scott, 1997; Yeung, 1998). Decreased IgG_{2a} production was also seen following anti-IL-12 treatment in *Borrelia burgdorferi* infected mice (Anguita, 1996) and after KLH (alum) immunization in IL-12 KO mice. However, this was not universal. IL-12 deficiency did not alter IgG_{2a} or IgG_1 production upon mouse hepatitis virus (Schijns, 1998) or *S. mansoni* infection (Anderson, 1998), indicating that these immune responses have a lesser dependence on IL-12. Endogenous IL-12 appeared to have little impact on IgG_1 production (Fig. 22, 23 and 24) (Anguita, 1996; Schijns, 1998).

Furthermore, the ability of endogenous IL-12 to increase IgG_{2a} showed a strong dependence on IFNγ since (1) anti-IL-12 treatment reversed the frequency of IFNγ producing spleen cells following heat killed *B. abortus* treatment (Scott, 1997), (2) anti-IL-12 treatment reversed the amount of IFNγ produced by lymph node cells upon heat killed *Listeria* treatment (Yeung, 1998), and (3) anti-IFNγ mAb inhibited the potential of heat killed *Listeria* to enhance IgG_{2a} to the same extent as anti-IL-12 mAb (Yeung, 1998).

We observed consistently lower IFNγ synthesis by spleen cells from IL-12 KO mice under all conditions examined (Fig. 25 and 27). Decreased IFNγ production has generally been found in p35 and p40 KO mice in response to different stimuli in vivo and in vitro. In lyme disease, anti-IL-12 treatment resulted in decreased IFNγ synthesis (Anguita, 1996). Naive, KLH (CFA) immunized or LPS treated IL-12 KO mice all exhibited impaired serum and culture IFNγ responses (Magram, 1996; Schijns, 1998). Aside from increases in IL-10

synthesis in p35 KO mice immunized with *T. spiralis* extract (Fig. 27), there was no indication that endogenous IL-12 regulated type 2 affiliated cytokine profiles (Fig. 26). Unaltered IL-4 synthesis was seen in infectious and nebulized Ag IL-12 KO models (Anguita, 1996; Keane-Myers, 1998). Slightly increased (approximately 2.4 fold) IL-4 production was also seen by lymph nodes cells upon KLH (CFA) immunization (Magram, 1996). Collectively, these data indicate that endogenous IL-12 is an important promoter of IFNγ responses, but not a critical inhibitor of type 2 cytokine production upon immunization.

In parasite models, increased IL-4 production was observed upon *L. major* infection of IL-12 deficient mice (either p35 or p40) on a resistant background (Mattner, 1996). This result was consistent with the loss of the healing response in resistant mice treated with anti-IL-12 (Heinzel, 1995). In addition, BAL cells from p40 KO mice infected with *S. mansoni* after vaccination with irradiated *S. mansoni* exhibited elevated IL-4 production compared to WT mice (Anderson, 1998). IL-12 is considered important in the healing responses to parasites it because induces CMI, including IFNγ production, required for macrophage microbicidal activity (Belosevic, 1989). Furthermore, IL-12 production from infected macrophages plays a role in directly or indirectly suppressing detrimental IL-4 synthesis, strongly associated with susceptibility to *L. major*. Thus, the elimination of endogenous IL-12 in resistant mice allows for the unrestricted IL-4 synthesis normally countered by the generation of endogenous IL-12 in response to infection.

Taken together, our work and the studies cited above indicate that IL-12 has a consistent role

in the promotion of type 1 Ab synthesis. However, it appears to have minimal impact on the inhibition of type 2 Ab and cytokine responses. This implies that negative regulation of IgE synthesis (seen upon OA-POL and heat killed *B. abortus* and *Listeria* treatment, or CFA immunization) is induced by a type 1 factor(s) in addition to endogenous IL-12. Other evidence for a factor capable of inducing type 1 immunity in the absence of IL-12 (and IFNγ) was observed following attenuated schistosome vaccination in p40 KO mice by the partial elimination of parasites (Anderson, 1998). In addition, the maintenance of IL-12 responsiveness in IL-12 KO mice (Fig. 28) suggests the presence of a factor(s) which prevents the suppression of IL-12R β2 associated with Th2 clonal responses.

IL-18 (IFNγ-inducing factor) is another recently discovered stimulator of IFNγ. While in vitro studies showed that IL-18 enhanced type 1 responses in the presence of IL-12 (including the inhibition of IgE and induction of IFNγ synthesis), in the absence of IL-12, IL-18 did not enhance T cell IFNγ production or cellular proliferation (Okamura, 1995; Yoshimoto, 1997). Thus, IL-18 may be an unlikely candidate for the induction of type 1 immunity in the absence of endogenous IL-12, since it appears to act predominately in concert with other cytokines (i.e. IL-12).

6.0 Endogenous IL-10 regulation of IgE synthesis

Immune responses can be differentially regulated by the balance of endogenous type 1 and type 2 cytokines, particularly IL-12 and IL-10. IL-10 is a known inhibitor of pro-

inflammatory cytokines. In an apparent autoregulatory mechanism, IL-12 (and other type 1 cytokines) have been shown to induce IL-10 production, which in turn decreases their synthesis and limits their capacity to drive immune responses (Shibata, 1998; Takenaka, 1997). Therefore, in addition to examining the role of endogenous IL-12 in Ab responses, we investigated the importance of endogenous IL-10 in IgE production and in down regulating type 1 cytokine responses induced in response to immunization.

We found that elimination of endogenous IL-10 (either by treatment with anti-IL-10 mAb or by genetic KO of the IL-10 gene) inhibited IgE production in OVA (alum) immunized mice by > 95 % (Fig. 29 and 30). This contradicts previous reports on in vivo studies by three laboratories which indicated that IgE production was unchanged in the absence of endogenous IL-10. Treatment of neonatal mice with rat anti-mouse IL-10 resulted in little alteration of basal (pre-immunization) IgE synthesis (Ishida, 1993). The difficulty with that treatment regimen (3 injections x 8 weeks) was that would likely have generated mouse immunity against the rat Ab (anti-IL-10), contributing to ineffective IL-10 neutralization (Ishida, 1993). However, IL-10 KO mice also displayed basal serum IgE levels equivalent to their WT counterparts (Kuhn, 1993), supporting the conclusion that endogenous IL-10 is not involved in the regulation of basal IgE levels. Normal levels of IgE were also seen when IL-10 KO mice were immunized with 100 μg of haptenated chicken γ-globulin (alum) (HP-CG) (Kuhn, 1993), and when anti-IL-10 treated mice were injected with aqueous influenza antigens (Dobber, 1995). However, in both cases, the immunization protocols used (the high dose of Ag or the lack of adjuvant) themselves result in weak IgE production making it

difficult to assess the importance of endogenous IL-10 on IgE synthesis in the absence of a substantive positive control. What these observations may indicate is that in naive mice or in the absence of a strong IgE inducer, endogenous IL-10 is of little consequence in the production of IgE.

Equivalent IgE production in IL-10 KO and WT mice was also seen upon *A. fumigatus* infection. However, this was accompanied by increased IL-4 and IL-5 synthesis in addition to IFNγ production (Grunig, 1997). In our study, while IL-4 production was unchanged, and IL-5 synthesis was decreased (Fig. 31). This may be relevant since IL-5 was demonstrated to augment IL-4 induced IgE synthesis (Pene, 1988b).

In anti-IL-10 treated mice, we observed a 5 fold increase in Ag-specific IgG_{2a} production (this failed to reach statistical significance, perhaps due to an insufficient number of mice; this is currently being examined). In contrast, little change was seen in IgG_{2a} (or IgG_1) production in IL-10 KO mice, although total IgG_{2a} and IgG_1 were marginally increased (Fig. 29 and 30). Generally little impact on IgG_{2a} and IgG_1 (total and Ag-specific) had been reported in IL-10 KO mice (Grunig, 1997). In contrast, anti-IL-10 treatment substantially increased IgG_{2a} levels in naive or immunized mice (Ishida, 1993; Kerdine, 1996). Furthermore, excessive IL-10 production inhibited IgG_{2a} synthesis (Bai, 1997; Kerdine, 1996). Thus, anti-IL-10 and IL-10 treatment studies indicate that IL-10 negatively regulates IgG_{2a} synthesis, perhaps secondary to enhanced IFNγ production. This is generally unconfirmed in IL-10 KO mice. Unaltered IgG_{2a} synthesis in IL-10 KO mice may signify the induction of a factor which limits

IgG_{2a} production in IL-10 KO mice, despite the increased IFNγ synthesis.

Decreased IgE seen in our study was associated with enhanced type 1 cytokine synthesis and decreased IL-5 production. Enhanced IFNγ was consistently found in the absence of endogenous IL-10 by ourselves (Fig. 31) and others (Dai, 1997; Grunig, 1997; Shibata, 1998). Since decreased IFNγ production, but not IFNγ mRNA expression, was observed when IL-10 levels were artificially elevated in vivo, this suggests that IL-10 acts by inhibiting translation, but not transcription, of IFNγ (Kerdine, 1996).

Increased levels of serum and culture IL-12 in IL-10 KO mice have been reported in response to infectious stimuli (Grunig, 1997; Shibata, 1998). IL-10 was observed to inhibit both T cell (Ag and CD40:CD40L) dependent and independent induction of IL-12 synthesis. Therefore, we evaluated whether antigenic (T cell dependent) IL-12 p40 synthesis, in addition to LPS stimulation was affected in immunized IL-10 KO mice. We found that IL-12 p40 was enhanced in IL-10 KO mice relative to WT controls in response to both antigenic (OVA) and polyclonal (LPS) stimulation (Fig. 32). It is common knowledge that protein preparations contain LPS. Therefore, we assayed the amount of LPS in OVA and determined that 1/3 of enhanced IL-12 p40 production upon OVA stimulation might be attributable to the low level of LPS seen in the 5 times recrystalized OVA used in this study (Table 11). This means OVA stimulation accounted for 2/3 of IL-12 p40 production, indicating that in IL-10 KO mice there was an enhancement of both T cell dependent and independent mechanisms of IL-12 p40 induction. LPS stimulation of spleen cells is non-specific, but the presence of Ag induced IL-

12 p40 production indicates that p40 (and by extrapolation IL-12) synthesis was elevated in the absence of IL-10 regulation in response to immunization.

IL-10 KO mice displayed decreased IL-5 production upon exogenous Ag immunization (Fig. 31), or following helminth infection (Kuhn, 1993). In AHR models, where decreased IL-5 synthesis was seen following IL-10 treatment, it was argued that IL-10 activity altered macrophage signalling to T cells, impairing IL-5 production (Zuany-Amorim, 1996). In IL-10 KO mice, IL-5 inhibition also may be a secondary event, the result of elevated type 1 cytokine production (particularly IL-12) which can suppress IL-5 synthesis (Warren, 1995). The difference between these results probably reflects the pleiotropic nature of IL-10, as treatment with pharmacologic doses of IL-10 and removal of endogenous IL-10 could effect the cytokine network at a variety of intersecting points.

Upon primary immunization with OVA (alum) both type 1 and type 2 cytokine production is triggered, as seen by IL-4 and IFNγ synthesis in Ag-driven culture of WT (i.e. C57Bl/6) mice (Fig. 10). Since the peak of primary spleen cell culture IL-10 production (96 h harvest) is after that of IFNγ (48 h harvest), IL-10 may serve to suppress primary type 1 cytokine responses, and subsequently promote IgE production. As IL-4 synthesis was unaltered in IL-10 KO mice, it appears that the removal of inhibitors (such as IFNγ, IL-12 or other cytokines not evaluated here) were required for IgE responses. We did not observe spontaneous cytokine production ex vivo in IL-10 KO mice (Fig. 31 and 32) indicating that changes in cytokine production were initiated in response to immunizing, and not environmental, Ag.

The finding that elimination of IL-10 results in enhanced IFNγ and IL-12 p40 production and inhibition of normally substantial IgE responses in mice, does not necessarily translate into a possible therapeutic benefit for the use of anti-IL-10 in allergy. IL-10 was reported to enhance IgE secretion from differentiated B cells, yet in AHR models, IL-10 treatment reduced symptoms (Zuany-Amorim, 1996). Furthermore, both increased and decreased IL-10 were reported following successful allergen immunotherapy (Akdis, 1998b; Bellinghausen, 1997). Increased IL-10 was argued to (1) contribute to immunotherapy by causing anergy of allergen specific T cells and decreasing IgE production (Akdis, 1998b) or (2) restrict potential damage associated with enhanced type 1 cytokine production (i.e. the potential for autoimmune disease) (Bellinghausen, 1997). Thus, despite the finding that murine IgE synthesis can be inhibited by extreme increases in type 1 cytokines in the absence of endogenous IL-10, it is difficult to extrapolate this as indicating that reduced endogenous IL-10 would be therapeutically beneficial in allergy.

7.0 CD8 T cell regulation of type 1 and type 2 humoral responses

In the fifth component of this research, we investigated the effect of CD8 deficiency on the induction of IgE synthesis. It had been previously observed in our laboratory that OVA primed CD4 T cell IFNγ production could be enhanced by the addition of OVA primed CD8 T cells (X. Yang and K. HayGlass, unpublished observation), suggesting that CD8 T cells assisted CD4 T cell IFNγ production. This observation on our part initiated our interest in CD8 T cell regulation in immune responses pertaining to IgE regulation and was consistent with reports by Holt and Kemeny's groups showing that activated CD8 T cells suppressed IgE

production (Holmes, 1996; Holmes, 1997; McMenamin, 1995). However, one of these studies also suggested that CD8 T cells were required for the initiation of IgE production (Holmes, 1996). Therefore, we hypothesized that in CD8 KO mice we would see an inhibition of type 2 Ab (decreased IgE synthesis, increased IgG_{2a} synthesis) and cytokine profiles (increased IFNγ:IL-4 ratio) in response to OVA (alum) immunization. In addition, we hypothesized that strong type 1 Ab patterns normally seen after OA-POL treatment would be enhanced.

Since we were interested in later evaluating the ability of OA-POL to abrogate IgE responses in WT and CD8 KO mice, mice were immunized with 0.2 μg of OVA (alum), a dose at which OA-POL treatment can consistently inhibit established IgE responses (~ 90 %). No difference was seen in basal, primary or secondary Ag-specific IgE responses between WT and CD8 KO mice (Fig. 34). Total IgE levels were also similar except that CD8 KO mice display slightly decreased total IgE upon secondary immunization with 0.2 μg OVA (alum) (Fig. 34), further arguing against a role for CD8 T cells in the induction of IgE production. This complements our finding that Ag-specific IgE synthesis (basal, primary and secondary) was not altered in CD8 KO verses WT mice immunized with 2.0 μg of OVA (W. Stefura, J. Rempel, and K. HayGlass, unpublished observation).

A similar observation had been made in an AHR model examining the role of naive CD8 T cells in the induction of airway responses. Mice rendered CD8 T cell deficient prior to sensitization had decreased AHR, IL-5 and eosinophil responses upon inhalation nebulized

antigen (Hamelmann, 1996), but total and Ag-specific IgE levels remained consistent between sensitized CD8 depleted mice and sensitized undepleted animals. When rats were depleted of CD8 cells prior to primary PLA/ricin immunization i.p., a 5 fold decrease in total IgE, but not Ag-specific IgE, was seen (Holmes, 1996). However, under different housing conditions the ricin experiments could not be duplicated (B. Holmes, discussion), indicating that the results were highly dependent on other factors. Thus, while under well defined conditions CD8 T cells may have a role in the induction of IgE responses, generally it appears that they do not.

In addition to the impact of CD8 T cells on OVA (alum) immunization responses, we examined the role of CD8 T cell regulation in the capacity of the type 1 polarizing Ag OA-POL to switch established type 2 Ab and cytokine responses into type 1 responses. To accomplish this, 0.2 μg OVA (alum) immunized WT and CD8 KO mice were treated with OA-POL and boosted. No difference in the capacity of OA-POL to abrogate Ag-specific or total IgE were observed between strains in the presence or absence of CD8 T cells. Furthermore, although both OA-POL induced Ag-specific IgG_{2a} and IgG_1 were enhanced in CD8 KO mice compared to OA-POL treated WT mice, these increases were minimal. Previous reports have shown increased IgG_1 synthesis upon anti-CD8 treatment of naive mice, or upon viral infection of CD8 KO mice (Coutelier, 1991; Yee, 1996). The effects of CD8 cells on IgG_{2a} levels were less consistent. Taken together with our results, this indicates that CD8 T cells does not greatly impact the induction of type 1 (IgG_{2a}) or type 2 (IgE) Ab responses.

Our analysis of cytokine production in OVA immunized CD8 deficient mice indicated that CD8 KO mice had a slightly type 1 skewed cytokine profile. Upon secondary OVA (alum) immunization, CD8 KO mice displayed a minor *increase* in Ag-driven IFNγ levels compared to WT mice. Following OA-POL treatment, there was almost a 4 fold increase in the IFNγ:IL-4 ratio in OA-POL treated CD8 deficient mice compared to OA-POL treated WT mice. Neither OA-POL nor CD8 cell deficiency affected IL-5 synthesis; but a slight decrease in IL-5 production was observed upon OA-POL treatment of CD8 KO mice. Although this decrease was minor, lower IL-5 production (2.5 fold) following the depletion of CD8+ cells prior to airway sensitization had been reported (Hamelmann, 1996), suggesting CD8 T cells may contribute to, or participate in the regulation of, IL-5 secretion.

It has been argued that activated CD8 T cells are important in the regulation of established responses (Holmes, 1997); whereas, CD8 T cells are required for optimal immunity in the induction of Ab responses (Holmes, 1996). We found slightly decreased total IgE and enhanced IFNγ in CD8 KO mice compared to WT mice. In addition, OA-POL treatment resulted in enhanced IgG_{2a} and IFNγ:IL-4 ratios, along with lower IL-5 production, in CD8 deficient mice. The almost 4 fold increase in IFNγ:IL-4 ratios was suggestive of skewed T cell responses. However, the differences seen in the remaining responses (approximately 1.5 - 2.5 fold), although statistically significant, indicate that Ab responses were largely unaffected by the chronic absence of CD8 T cells.

8.0 Summary

The primary goal of this research was to investigate mechanisms of IL-12 regulation of Ab responses.

We began this study by confirming other reports that the exogenous IL-12 administration has the ability to profoundly inhibit the development of primary IgE responses. We went on to demonstrate that this initial inhibition was attributed to a burst of NK cell dependent IFNγ production following IL-12 treatment and was not associated with long-term skewing of CD4 T cell profiles or inhibition of IgE production. The observation that the presence of excessive IL-12 and IFNγ in vivo was able to suppress IgE immunization responses, was further supported by our demonstration that the inhibition of primary IgE responses, in the absence of endogenous IL-10 production, was associated enhanced type 1 cytokine production.

The most important conclusion of this thesis is that endogenous IL-12 production plays a key role in the generation of type 1 cytokine responses and IgG_{2a} synthesis, but has little impact on type 2 cytokine and IgE production. This is supported by our findings that (1) despite the inability of IL-12 to maintain restricted IgE production (when administered with primary immunization), IgG_{2a} remained approximately 4 fold enhanced, (2) IL-12 given during established responses resulted in an 11-50 fold increase in IgG_{2a} synthesis, without an alteration in IgE production, and (3) immunization responses in IL-12 KO mice revealed decreased IgG_{2a} levels, but IgE production was generally unaffected.

This contradicts what is known about the importance of IL-12 in generating protective CMI

immunity against parasites, where IL-12 treatment has the capacity to induce protective adaptive memory responses. It may be important to note that in models where IL-12 treatment resulted in long-term protection of parasites in susceptible mice, endogenous IL-12 was also reported to be critical in protective immunity in resistant mice, as observed upon the neutralization of endogenous IL-12. While protective responses showed some dependence on IL-12 induced NK activity, the presence of IL-12 (exogenous or endogenous) did translate into long term adaptive immune responses, including CD4 T cell activation and altered cytokine production (Afonso, 1994; Heinzel, 1993; Scharton-Kersten, 1995; Wynn, 1996). Thus, IL-12 treatment in susceptible mice mimics endogenous IL-12 activity in resistant mice, triggering protective responses naturally induced by infection. Similarly, exogenous IL-12 administration prevented the occurrence of tumors and endogenous IL-12 activity appears to be an important component of normal anti-tumor immunity (Bianchi, 1996; Nakajima, 1998; Rao, 1997). Since endogenous IL-12 had little effect on IgE synthesis, perhaps it should not (in hindsight) be surprising that exogenous IL-12 was not therapeutically beneficial in the long-term reduction of IgE levels. Thus, it may be more profitable to first determine what endogenous factor naturally restricts IgE production in response to type 1 stimuli (such as with OA-POL or heat killed *B. abortus* and *Listeria* treatment or CFA immunization) and subsequently attempt to manage IgE levels by manipulating that factor.

Another implication of this relationship between the endogenous regulation of IL-12 and successful exogenous IL-12 treatment is our findings indicating that endogenous IL-12 may have role in promoting secondary IgE responses by modulating non-B/non-T cell activity.

This is supported by the observations that (1) exogenous IL-12 administration in mice with established IgE responses markedly enhanced non-B/non-T cell numbers and IL-4 production within the spleen, in addition to displaying increases in total IgE production, and (2) in IL-12 KO mice secondary IgE responses were lower, while primary IgE production was unaffected.

There are at least two difficulties with exogenous cytokine administration. The first is trying to determine which cytokine to administer. For example, our study indicates that IL-12 is a strong promoter of type 1 Ab responses, but it seems that another factor(s) is required for the negative regulation of IgE synthesis. This factor will be harder to identify since a negative impact on IgE synthesis is not as obvious as one that strongly induces type 1 immunity. The second difficulty with cytokine therapy is that cytokines generally exhibit multiple effects. In our study this was seen by the discovery that administration of exogenous IL-12 to mice with established immediate hypersensitivity responses resulted in the *increased* IL-4 production from non-B/non-T cells, which could augment the IgE synthesis we were trying to inhibit by IL-12 treatment. We also observed enlarged spleens and fluid in the peritoneal cavity following IL-12 administration (at all doses of IL-12 examined) and high mortality at doses of 1000 ng/ml. Complications resulting from large increases in IgG_{2a} (a complement binding Ab) and IFNγ synthesis, seen when we administered IL-12, could also be harmful. Large doses of IFNγ are known to be toxic and are shown to be responsible for many of the toxicities associated with IL-12 treatment in mice and other animals (reviewed in Ryffel, 1997). Thus, even if exogenous IL-12 had excellent adjuvant qualities for the prevention of

IgE, or therapeutic promise in being able to inhibit established IgE responses, caution would have been warranted in endorsing it for human trials (Cohen, 1995). Exogenous IL-12 therapy does appear to have promise in treating cancer. Here, the benefits probably out weight the risks.

Lastly, some studies indicated that CD8 T cells were required for optimal induction of type 2 responses when mice were treated with anti-CD8 mAb prior to immunization (Hamelmann, 1996; Holmes, 1996). Although our study with CD8 KO mice was consistent with these findings, it is difficult to attribute any biological significance to it since the differences we obtained between CD8 KO and WT mice were generally small. The caveat in this argument is that redundancy in the immune system, strikingly apparent in many genetic knockouts, may have resulted in the replacement of CD8 T cell production of cytokines important in the initiation of responses by other lymphocytes.

It is becoming more apparent that the immune system has many compensating pathways, critical in the ability of a host to maintain immunologically responsiveness in the event that one pathway is compromised. As such, a negative result in a KO mouse strain may only indicate that other cytokines are compensating for functions normally associated with the missing cytokine. Perhaps the most renowned case of such redundancy is the capacity of IL-2 KO mice to develop mature T cell responses (Kundig, 1993). In our laboratory, OA-POL induced inhibition of IgE was shown to be dependent on IFNγ when anti-IFNγ was administered (HayGlass, 1991b). However, OA-POL inhibition of IgE was unaltered in IFNγ

KO mice (I. Lewkowich and K. HayGlass, unpublished observation). In contrast, observed differences in a KO mouse strain would strongly argue for a critical role for that cytokine in a certain response (such as decreased IFNγ and IgG_{2a} production in IL-12 KO mice).

VI. References

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