Endogenous control of allergic disease

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BY

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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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Dedicated to my parents,

whose unwavering support made this work possible

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Abbreviations

α-GalCer - alpha galactose ceramide

^{3}H - tritiated

Ag - antigen

APC - Antigen presenting cell

BAL - Bronchoalveolar lavage

Ci - Curie

ConA - Concanavilin A

Da - Daltons

DTH - delayed type hypersensitivity

EBI3 - Epstein-barr virus induced gene 3

ELISA - Enzyme linked immunosorptive assay

FcεR1 - High affinity IgE receptor

HKL - heat killed *Listeria monocytogenes*

i.p. - intra-peritoneal

ICOS - Inducible costimulator

ICS - inhaled corticosteroids

ICSBP - IFNγ consensus binding protein

IFN - Interferon

Ig - Immunoglobulin

IL - Interleukin

IL-12R - IL-12 receptor

IL-18BP - IL-18 binding protein

IP-10 - Interferon γ inducible protein - 10 kDa

i.v. - intra-venous

LPS - lipopolysaccharide

MHC - Major histocompatibility complex

NK cell - Natural Killer cell

NKT cell - NK 1.1^+ TCR^+ natural killer cell

$O.D._{405\text{-}690}$ - optical density at 405 nm - optical density at 690 nm

OA-POL - chemically polymerized ovalbumin, $\sim M_r$ 3.5×10^7 Da

OVA - Ovalbumin

$p(40)_2$ - IL-12 p40 homodimer

PBMC - Peripheral blood mononuclear cell

PMA - Phorbol 12-myristate, 13-acetate

RANTES - regulated upon activation normal T cell expressed and secreted chemokine

STAT - signal transducer and activator of transciption

TCR - T cell receptor

Th cell - $CD4^+$ helper T cell

TLR - Toll-like receptor

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I - Abstract

Allergic disease is a result of an unusually excessive and inappropriate immune response towards normally innocuous environmental antigens (allergens). This response is biased towards a type 2 dominated immune response characterized by increased serum IgE levels, enhanced production of type 2 cytokines and chemokines, airway hyperresponsiveness, and eosinophilic inflammation. While the individual contributions of these components to the overall immunopathology of disease is increasingly well understood, the identity of the endogenous controls responsible for limiting the initiation and severity of the allergic response - if they exist at all - remain unclear. This study seeks to identify the endogenous controls required for preventing the induction, and allowing the redirection of existing type 2 dominated immune responses. We hypothesize that owing to their type 1 promoting activities, the cytokines IL-12, IFNγ and IL-18 act as endogenous negative regulators of type 2 immunity.

IL-12 is the most potent inducer of type 1 immunity and the key cytokine involved in promoting Th1 cell differentiation. IL-12 is also cited as an important negative regulator of type 2 immune responses through its ability to induce NK and Th1 cell IFNγ production, and direct effects on Th2 cell cytokine production. However, the conclusion that *endogenous* IL-12 is a negative regulator of type 2 immunity is largely based on studies in anti-IL-12 treated or IL-12 -/- mice which report increases in IL-4 *alone*, a notoriously difficult to detect cytokine. We first undertook a thorough analysis of the role of endogenous IL-12 in limiting the induction of type 2 immunity by comparing *in vivo* Ab production and *in vitro* Ag-restimulated cytokine production in OVA immunized IL-12

deficient mice and C57Bl/6 controls. We found that, regardless of immunization conditions, there was no observable elevation of serum IgE levels or Ag-driven spleen cell type 2 cytokine synthesis (IL-4, IL-5, IL-10 or IL-13) in IL-12 -/- mice. In contrast, Ag-driven IFNγ production by spleen cells from IL-12 -/- mice was only 10% of that seen in wild type mice. Thus, the absence of endogenous IL-12 results in substantial reductions in the intensity of type 1 responses yet we detect no elevation of type 2 cytokine of Ab production in its chronic absence. This suggests that endogenous IL-12 plays a redundant role in limiting the intensity of developing type 2 immune responses.

In vitro, rIFNγ inhibits Th2 cell proliferation and cytokine production and prevents B cell class switch to IgE. Surprisingly, the effect of endogenous IFNγ production on Ag-driven type 2 cytokine production in murine models of allergic disease has not been examined. Given the demonstrated anti allergic activities of IFNγ, we hypothesized that endogenous IFNγ synthesis was critical for preventing hyperexpression of type 2 cytokine and antibody production *in vivo*. Specifically, we examined the importance of endogenous IFNγ in limiting the intensity of *de novo* type 2 immune responses using IFNγ -/- mice. These mice had substantially decreased IP-10 production following *in vitro* Ag restimulation suggesting a weakened type 1 immune response, as seen in IL-12 -/- mice . However, in contrast to IL-12 -/- mice, IFNγ deficient mice developed approximately three fold more intense Ag-driven type 2 cytokine and chemokine responses. Collectively, these data suggest that endogenous IFNγ and IL-12 are *both* essential for optimal induction of a type 1 immune response. In contrast, endogenous IFNγ production, but *not* IL-12, is required for optimal regulation of Ag-driven, type 2 cytokine production.

IL-18 is an inducer of IFNγ production, and shares many biological activities with IL-12. Due to its IFNγ promoting activities, we hypothesized that endogenous IL-18 was responsible for regulating the residual IFNγ response in IL-12 deficient mice, and was therefore an important endogenous control on type 2 cytokine and antibody production. We observed that IL-12 -/- mice have a significantly increased capacity to produce IL-18 in response to LPS stimulation. Since recent reports also attribute IL-18 with type 2 cytokine inducing activities, we subsequently examined the effect of increased IL-18 levels on Ag-driven type 2 cytokine production. Addition of rIL-18 to spleen cell cultures from OVA (alum) immunized mice resulted in a marked decrease in the amount of IL-4 released upon Ag restimulation. The inhibitory effect of IL-18 was specific for IL-4, as the production of other type 2 cytokines and chemokines examined was unaltered. Surprisingly, IL-18 mediated inhibition of IL-4 production was also seen in cultures derived from IFNγ -/- spleen cells. These findings demonstrate, for the first time, that IL-18 has the capacity to inhibit the production of the type 2 cytokine, IL-4, and that this inhibition is not dependant on the capacity of IL-18 to induce IFNγ production. This suggests that IL-18 and IFNγ represent independent, non-redundant, negative regulators of type 2 cytokine production.

The principal focus of this thesis thus far has been to identify endogenous controls which are important in limiting the *development* of type 2 cytokine and antibody production *in vivo*. A more clinically relevant question, however, is what endogenous factors are important in redirecting an *existing* type 2 immune response to a type 1 dominated one. This represents the ideal therapy for allergic disease. Specific immunotherapy seeks to do just this, yet unfortunately, does not provide a universal "cure". It does not work in all patients, or for all allergens. To explore the mechanism

involved in successful *immune redirection*, we utilized a murine model which uses chemically polymerized allergen to redirect a type 2 dominated immune response, to a type 1 dominated one. Treatment of mice with glutaraldehyde polymerized OVA (OA-POL) either prior to, or following OVA (alum) immunization, results in a dramatic shift from a type 2 dominated to a type 1 dominated Ab production profile. This shift is characterized by >90 % decreases in total and OVA-specific IgE, and simultaneous increases in IgG_{2c} levels (up to ~ 500-fold). This redirection is reminiscent of the shift seen following successful immunotherapy.

Previous studies have demonstrated that OVA-specific antibodies have markedly reduced affinity for OA-POL. We hypothesized that this results in an inability of B cells to capture OA-POL, suggesting that these cells are not critical antigen presenting cells involved in OA-POL mediated immune redirection. Using B cell deficient (μMT) mice, analysis of *in vitro* cytokine production in treated and untreated C57Bl/6 and μMT mice revealed that OA-POL was equally effective in both strains of animals. This suggests that B cells are not required as APCs in OA-POL mediated immune redirection.

Owing to their potent type 1 promoting activities, we hypothesized that both endogenous IL-12 and IFNγ were required for optimal induction of type 1 immunity and inhibition of IgE production following OA-POL treatment. Thus, as an alternative method of assessing the importance of IL-12 and IFNγ in inhibiting type 2 immunity we treated IL-12 -/- or IFNγ -/- mice with OA-POL. Compared to OA-POL treated C57Bl/6 controls, treatment of IL-12 -/- animals with OA-POL resulted in a similar or more complete inhibition of IgE production. This argues that endogenous IL-

12 production is not required for inhibition of existing IgE responses. In contrast, in IFNγ -/- mice the inhibition of IgE production following OA-POL treatment was reduced, arguing that IFNγ plays an important role in limiting IgE production *in vivo*. The capacity of OA-POL to upregulate IgG_{2c} levels was decreased in both IL-12 and IFNγ -/- mice compared to C57Bl/6 controls. These two cytokines are clearly required for optimal induction of type 1 immune responses. Collectively, these data support the previous findings that endogenous IFNγ, but not IL-12 production, is needed for limiting the intensity of type 2 immune responses, but that both are required for optimal upregulation of type 1 immunity.

OA-POL treatment did not result in increased production of IL-12 and IL-18 (both inducers of IFNγ production), yet we demonstrate that IFNγ is critical for OA-POL triggered immune redirection. In an attempt to resolve this apparent contradiction, we hypothesized that OA-POL treatment resulted in increased *responsiveness* to endogenously produced IL-12 and IL-18, thereby allowing increased IFNγ production in the absence of enhanced IL-12 or IL-18 production. In support of this hypothesis, we observed that spleen cells taken from OA-POL (saline) immunized mice did produce approximately three fold more IFNγ in response to *in vitro* IL-12 stimulation than spleen cells from OVA (saline) immunized mice. Similar increases in IFNγ production were seen in NK cell depleted spleen cell cultures from OA-POL treated mice following stimulation with IL-12 or IL-18. This suggests that T cells were the source of increased IFNγ production. In turn, increased T cell IL-12/IL-18 responsiveness was accompanied by increased T cell mRNA levels of IL-18Rα, IL-18Rβ and IL-12Rβ2. Taken together, these mechanistic experiments argue that OA-POL treatment decreases the intensity and prevents the initiation of type 2 cytokine and antibody production by

increasing T cell responsiveness to endogenous IL-12 and IL-18, *not* by increasing the expression of type 1 inducing monokines. This suggests that treatments which increase biological responsiveness to IL-12 and/or IL-18 may have potential therapeutic value.

In summary, the work presented here argues that endogenous IL-12, while important for optimal induction and promotion of type 1 immune responses, does not play a critical role in inhibiting the induction or the overall intensity of the type 2 immune response. While IFNγ is similarly important for induction and maintenance of type 1 immunity, unlike IL-12, endogenous IFNγ plays a non-redundant role in inhibiting the initiation and severity of type 2 immunity. Finally, we also demonstrate that IL-18 has the capacity to directly and selectively inhibit the production of allergen-driven IL-4. Importantly, the inhibition is not dependent on IFNγ, a finding which suggests that IL-18 may represent a critical inhibitor of type 2 cytokine production, even in IFNγ deficient environments. Furthermore, we also demonstrate that IL-12 and IL-18 responsiveness, perhaps more so than overall production of these monokines, is involved in negative regulation of developing and existing type 2 immune responses.

II - Introduction

1.0 - Epidemiology of allergic disease

The term "allergic disease" comprises a range of different immunological disorders, including atopic dermatitis (skin), allergic rhinitis (nasal mucosa), allergic asthma (the lung) or anaphylaxis (systemic). Allergies have been increasing in both incidence and severity over the past decades, and are considered a disease of affluence. It is estimated that upwards of 20% of the population in developed countries suffer from allergic disease, constituting the most prevalent immune disorder in these nations. In contrast, the prevalence remains much lower in poorer, developing nations (Gergen, Mullally et al. 1988; Weitzman, Gortmaker et al. 1992; 1994; 1998; Mannino, Homa et al. 1998; Mannino, Homa et al. 2002). Allergy is primarily a disease of childhood: the most affected nations report that >30% of children aged 13-14 demonstrate some symptoms of an allergic disease (1998). Allergic disease, particularly allergic rhinitis, can result in a significantly reduced quality of life (Meltzer 1997) and constitutes a major healthcare burden. The direct and indirect costs to the health care system of asthma alone was estimated to be nearly 700 million dollars in Canada in 1990 (Krahn, Berka et al. 1996), and greater than ten times that in the United States in 1998 (Sullivan and Weiss 2001; Weiss and Sullivan 2001). Despite this alarming increase in the prevalence of allergic disorders, there is at present no convincing explanation as to the cause. While the growing problem demonstrates the increasing need for an effective, universal therapy to "cure" or prevent the diseases, no such therapy exists.

1.1 - Pathogenesis of the allergic response

Allergic disease is characterized by an excessive immune response to normally innocuous environmental antigens, termed allergens. Common allergens include pollens, molds, and proteins in animal and insect dander/excretions. In allergic individuals, the immune response to these allergens is characterized by the production of IgE, a critical mediator of allergic disease. In contrast, there is limited allergen specific IgE production in non-allergic individuals. It is presently unclear why an IgE response is elicited only in some individuals and to only some allergens. It is, however, clear that IgE production is driven by type 2 cytokines like IL-4. These cytokines are ultimately responsible for controlling the type 2 dominated immune response characteristic of allergies. The initial source of IL-4 has not been identified, but a variety of cell types, including mast cells, basophils, γδ T cells, αβ T cells, and NKT cells have been implicated.

Production of allergen specific IgE following initial allergen exposure results in allergic sensitization. IgE is present in the serum at very low levels (representing <0.01 % of all immunoglobulins in the serum of a normal individual), has a very short half-life when in circulation (~ 2 days), but is bound with unusually high affinity by membrane FcεR1. Through binding FcεR1, IgE is concentrated and stabilized on the cell surface, increasing its half-life. FcεR1 is expressed primarily on mast cells, but has also been reported on neutrophils, basophils, and eosinophils (reviewed in Gould, Sutton et al. 2003). IgE priming of mast cells is critical in systemic reactions which occur following subsequent allergen exposure.

Upon re-exposure to the allergen, FcεR1-bound IgE is cross-linked, resulting in activation and degranulation of the mast cell. Contained within mast cell granules are potent mediators of inflammation including histamine, heparin, platelet-activating factor, proteolytic enzymes, and a variety of chemotactic and regulatory cytokines. Furthermore, activation of mast cells also results in increased lipid metabolism and release of arachidonic acid metabolites such as thromboxanes, leukotrienes, and prostaglandins. The release of these inflammatory mediators results in vasodilation, increased vascular permeability and smooth muscle contraction which are responsible for the immediate effects of allergen exposure seen in immediate hypersensitivity diseases, particularly strikingly in acute asthma and anaphylaxis.

In addition to these acute effects, the release of chemical mediators results in cellular infiltration into the site of allergen exposure. The appearance of neutrophils, basophils, eosinophils and lymphocytes, initially attracted by potent chemotactic chemokines such as RANTES, IL-8 and eotaxin, facilitates further cellular recruitment and effector cell development which contribute to the so called late phase response. These cells produce cytokines known to be potent mediators of allergic disease. IL-4 is required for class switch to IgE (Finkelman, Katona et al. 1988), increases the production of eotaxin (Rothenberg, Luster et al. 1995), upregulates adhesion molecule expression (Schleimer, Sterbinsky et al. 1992), and facilitates the development of additional allergen specific Th2-like effector T cells (O'Garra 1998). Locally produced IL-5 facilitates eosinophil maturation, but is also a chemotactic agent and survival factor for eosinophils within the tissue (Cameron, Christodoulopoulos et al. 2000). IL-13 is a critical effector molecule as it further potentiates IgE production and enhances mucus production, which is particularly important in the pathogenesis of

asthma (Wills-Karp, Luyimbazi et al. 1998). This cascade of mediator production and release by newly recruited cells results in persistent tissue inflammation which leads to dramatic structural changes in the inflamed tissue. In particular, chronic inflammation of the nasal mucosa results in allergic rhinitis, but in the airways results in severe remodeling which causes difficultly in breathing and enhanced sensitivity to environmental provocations which are the hallmarks of allergic asthma.

Moreover, while the initial inflammatory insult is mediated by a type 2 dominated immune response to an environmental allergen, any subsequent inflammatory response in the airways, regardless of etiology, can result in exacerbations of the disease. This is particularly true in the case of allergic asthma where insults to the airways, whether caused by inhalation of environmental pollutants (tobacco smoke, diesel exhaust), development of a lung specific type 1 immune response (viral or bacterial airway infection), or inhalation of additional environmental allergen (triggering additional rounds of type 2 mediated airway inflammation), result in acute asthma symptoms.

1.2 - The hygiene hypothesis

The basis for the "hygiene hypothesis" stems from studies examining the relationship between family size, socioeconomic status and the prevalence of allergic disease. These studies indicate that children in smaller households generally have increased incidence of hay fever, skin prick positivity to common allergens and allergen specific-IgE levels (von Mutius, Martinez et al. 1994; Strachan, Taylor et al. 1996; Forastiere, Agabiti et al. 1997; Jarvis, Chinn et al. 1997; Strachan, Harkins et al. 1997; Matricardi, Franzinelli et al. 1998; Svanes, Jarvis et al. 1999). In contrast, a decreased risk of

developing allergic disease (hay fever, eczema and skin prick test reactivity) is found in children growing up in households of low socioeconomic status within the UK, Italy, GDR and Ghana (Broder, Higgins et al. 1974; Gergen, Turkeltaub et al. 1987; Addo Yobo, Custovic et al. 1997; Butland, Strachan et al. 1997; Forastiere, Agabiti et al. 1997; Heinrich, Popescu et al. 1998). The hypothesis developed to explain these findings argues that the reduced susceptibility to allergic disease is a result of a greater exposure to childhood infectious disease (as would be expected for those growing up with many siblings or in a household with low socioeconomic standing). Furthermore, the development of improved health care practices in the 20th century (resulting in a reduction in the overall frequency of severe childhood infections and rapid antibiotic treatment of those that did occur) correlate well with increases seen in allergic disease since the industrial revolution. This is taken as further evidence of a protective effect of frequent childhood infection. Basically, as a result of society becoming "cleaner", the incidence of allergic disease is increasing. The search for a single infection or organism that confers protection from the future development of allergies have yet to bear fruit, although studies focussing on infectious diseases commonly transmitted via the oral-fecal route (eg Hepatitis A, *Toxoplasma gondii*) have yielded potentially interesting results (Matricardi, Rosmini et al. 1999; Bodner, Anderson et al. 2000; Matricardi, Rosmini et al. 2000).

The relatively short period during which allergies have been increasing (since ~1900 for allergic rhinitis, or hay fever, and since the 1960s for allergic asthma), makes it unlikely that genetic differences are the sole determinant for the increased prevalence of allergic disease. Decreasing family size and improved health care practices represent attractive *environmental* factors which are

associated with the observed increase. It is important to stress, however, that reports demonstrating an association between increased allergic disease and increased hygiene and improved health care practices are still largely observational. The volume of reports which demonstrate support for such an association suggest that it is likely "real", but *causality* has not yet been adequately demonstrated, and the immunological mechanisms involved in the protective effect of "poor hygiene" is not yet fully understood. There are, however, two prominent theories as to the mechanism behind the observed associations. These theories will be discussed below.

1.3 - Bacterial infections as promoters of type 1 immunity

To explain the protective effect of infectious disease on subsequent development of allergies, proponents of the hygiene hypothesis argue that the repeated childhood infections are necessary for "re-educating" the immune response away from the type 2 dominance required for a successful pregnancy (and commonly seen in neonates) towards a more balanced profile normally found in adults. Thus, in the absence of these infections, the "re-educating" of the immune system is delayed resulting in the development of type 2 dominated immune responses to environmental antigens rather than a more balanced response associated with clinical tolerance. Recently, it was reported that children raised on a farm (but not those raised in urban, or rural, non-farming environments) are protected from the development of asthma, hay fever and allergic sensitization (Riedler, Braun-Fahrlander et al. 2001). It was later determined that these farm children are exposed to greater levels of Lipopolysaccharide (LPS) (Gereda, Leung et al. 2000; Braun-Fahrlander, Riedler et al. 2002), and have increased expression of CD14 and Toll-like receptor 2 (TLR 2) (required for

responsiveness to LPS) in peripheral blood mononuclear cells (PBMC) (Lauener, Birchler et al. 2002). Indeed, natural exposure to increasing doses of household LPS, a known inducer of IL-12, IFNγ production, correlates, in a dose dependent manner, with decreased skin prick test reactivity, hay fever and asthma (Braun-Fahrlander, Riedler et al. 2002). It is suggested that early life exposure to high LPS environments on these farms is the cause of the protection from allergic disease noted in these populations. These data are taken to support the idea that exposure to bacterial products, or bacterial infections, represent a strong type 1 promoting influence that is responsible for protecting the host from subsequent development of allergic disease.

However, the hypothesis that a lack of type 1 promoting signals in early childhood results in a population skewed towards type 2 dominance of the immune system is *not* supported by studies examining the incidence of organ-specific autoimmune diseases. In parallel with the rise in allergic diseases seen over the past decades is a rise in autoimmune diseases, which show a similarly increased prevalence in children from small families and those from low socioeconomic status (2000; Bingley, Douek et al. 2000; McKinney, Okasha et al. 2000; Stene and Nafstad 2001). Interesting preliminary evidence also indicates that, contrary to expectations, there is actually an *increased* prevalance of allergic disease in individuals suffering from rheumatoid arthritis, celiac disease or type 1 diabetes, diseases normally associated with type 1 dominated immune responses (Kero, Gissler et al. 2001; Stene and Nafstad 2001; Sheikh, Smeeth et al. 2003). In other words, in individuals with an immune response *supposedly* skewed towards a type 2 dominated immune response (demonstrated by an increased incidence of allergic disease), there is a simultaneous *increase* in the incidence of type 1 dominated immune pathologies. Moreover, this increase in both

type 1 and type 2 dominated disease states can co-exist within the same individual. This caveat is strongly suggestive that the observed association between increased cleanliness and increased incidence of allergic disease is *not* a result of a generalized shift towards a type 2 dominated immune profile.

1.4 - The hygiene hypothesis, an alternative view

A potential explanation for these observations comes from the equally counter-intuitive observation that individuals chronically infected with helminths demonstrate lower skin prick test reactivity to common allergens, yet exhibit high allergen specific IgE levels (Lynch, Lopez et al. 1987; Faniran, Peat et al. 1999; Araujo, Lopes et al. 2000; van den Biggelaar, van Ree et al. 2000; Nyan, Walraven et al. 2001; Scrivener, Yemaneberhan et al. 2001). Furthermore, treatment of patients with anti-helminth drugs to reduce parasite load actually *increases* both skin test reactivity and serum specific IgE levels (Lynch, Hagel et al. 1993), This suggests that the immune response to the parasite is preventing the manifestation of allergic disease (skin prick test reactivity and overt clinical symptoms), but not necessarily preventing allergic sensitization (as infected individuals possess specific-IgE levels).

IL-10 is an anti-inflammatory cytokine which globally downregulates immune responses. Indeed IL-10 -/- mice produce significantly more IFNγ and succumb to fatal intestinal inflammation within weeks of birth (Kuhn, Lohler et al. 1993). Helminth infection results in increased PBMC IL-10 production and decreased T cell proliferation to both worm antigens and non-related antigens, a

deficiency which is reversed by the addition of antibodies to IL-10 or TGFβ (King, Mahanty et al. 1993; Mahanty and Nutman 1995; King, Medhat et al. 1996; Doetze, Satoguina et al. 2000). IL-10 is also produced in lower quantities by bronchoalveolar lavage (BAL) cells from asthmatics compared to those from non-asthmatic controls (Borish, Aarons et al. 1996). Further, the size of the wheal in a positive skin test reaction is inversely proportional to the magnitude of allergen driven PBMC IL-10 production (Macaubas, Sly et al. 1999). Thus, IL-10 is produced in excess in individuals with parasitic infections, and is associated with control of allergic disease suggesting that perhaps the anti-inflammatory properties of IL-10 are lacking in allergic individuals.

Applying the data on helminth infection and IL-10 to the hygiene hypothesis has resulted in an alternate view of the effects of childhood infection. Instead of tipping the balance from type 2 dominance to a more balanced immune response, it is now hypothesized that recurrent childhood infections result in the formation of a healthy "regulatory network" of IL-10 producing, regulatory T cells (discussed in more detail in section 2.4), which act to efficiently control chronic inflammatory responses (Levings, Bacchetta et al. 2002). In the absence of these infectious "pressures", this regulatory network does not develop, and allowing inflammatory responses to become chronic and damaging. This chronicity is seen whether the inflammation is in the lung and type 2 dominated, or in the joint, pancreas or gut and type 1 dominated. This mechanism to explain the protective effects associated with childhood infection is attractive in that it potentially explains the observed pattern of *both* increased allergic and autoimmune diseases described earlier.

Thus, while the collected observational data strongly suggest that there is a link between the increased incidence of *both* type 1 and type 2 dominated pathologies, and increased cleanliness in developed nations, the mechanisms to account for this association are not yet clear. While the model of weakened regulatory networks due to fewer early childhood infectious challenge can nicely address the increases in both Th1 and Th2 dominated disease, further experimentation is needed to confirm or refute this hypothesis.

2.0 - T cell subsets and control of the immune response

Historically it was observed that there is often a reciprocal regulation of cellular and humoral immunity. That is, the immune response to foreign pathogens is predominantly either inflammatory (cell mediated) or humoral (antibody mediated), with little overlap between the two. Thus, protective immunity to viruses and intracellular bacteria or pathogens is a result of primarily cell-mediated immune responses and characterized by the production of pro-inflammatory mediators such as IFNγ and TNFα. In contrast, immunity to extracellular bacteria or parasites is a result of a humoral response characterized by high levels of parasite-specific immunoglobulins designed to bind, and neutralize, the invading organism. The reciprocal nature of these immune responses is thought to be of great importance in successful control of infection because induction of an inappropriate immune response frequently results in uncontrolled infection, severe pathology and in some circumstances, death of the host.

Almost 20 years ago, it was observed that T cell clones can be placed in one of two distinct subsets on the basis of the profile of cytokines they produce. Th1 clones produce IL-2 and IFNγ upon stimulation while Th2 cells produce IL-4 and IL-5 to the exclusion of IFNγ (Mosmann, Cherwinski et al. 1986). Furthermore, not only do these subsets of T cell produce different profiles of cytokines *in vitro*, when administered to mice, they induce very different types of immune responses *in vivo*: Th1 cells administered to the footpad of mice with the appropriate antigen cause a delayed type hypersensitivity (DTH) reaction (Cher and Mosmann 1987). In contrast, Th2 cells are able to provide potent B cell help and allow vigorous IgG_1 and IgE production (Killar, MacDonald et al. 1987; Coffman, Seymour et al. 1988). That there is little overlap in profiles of cytokine production and the biological immune responses which the two subsets of T cells mediate suggest that there is a strong correlation between these T cell subsets and ultimate control of the immune response.

2.1 - Th1 and Th2 cells: controlling T cell differentiation

Following the initial classification of Th1 and Th2 subsets on the basis of the cytokines produced following antigenic stimulation and the observation that these cytokine profiles correlated well with the control of cellular and humoral immune responses respectively, there has been a great deal of interest in identifying those factors which cause the preferential development of Th1 or Th2 subsets. As controllers of the fate of T cell differentiation, these factors would allow unprecedented control of the immune response.

The first of these factors are cytokines. To gauge the ability of various cytokines to influence T cell differentiation, T cells were stimulated with various cytokines in the presence of phorbol myristic acetate (PMA), Concanavalin A (ConA), anti-CD3 or antigen and re-stimulated in the absence of exogenous cytokines. The cytokine profile produced by these cells was determined following this restimulation. From such studies, IL-4 is identified as the chief cytokine responsible for promoting development of T cells producing Th2 patterns of cytokine production (Swain, Weinberg et al. 1990; Hsieh, Heimberger et al. 1992; Seder, Paul et al. 1992). IL-6, IL-25 and IL-1β are also implicated in the development of IL-4 producing Th2 cells, although the influence of these cytokines is consistently less powerful than that of IL-4 (Brigelius-Flohe, McCarthy et al. 1993; Fort, Cheung et al. 2001; Diehl, Chow et al. 2002). IL-12 clearly acts on developing T cells to promote their differentiation into cells which produce a Th1-like pattern of cytokines (Hsieh, Macatonia et al. 1993; Manetti, Parronchi et al. 1993; Seder, Gazzinelli et al. 1993; Manetti, Gerosa et al. 1994). In contrast, the role of IFNγ in inducing the differentiation of naive T cells into Th1-like cells, is more controversial. While some reports claim that IFNγ alone is sufficient to induce differentiation into Th1 cells (Bradley, Dalton et al. 1996; Lu, Ebensperger et al. 1998), others have shown that IFNγ alone is insufficient to support such differentiation (Macatonia, Hsieh et al. 1993; Wenner, Guler et al. 1996; Smeltz, Chen et al. 2002). It is important to note, however, that studies supporting a role for IFNγ in directly inducing Th1 cells differentiation make use of C57Bl/6 or B10.BR mice, strains typically considered to be biased towards a Th1 immune response. In contrast, studies in which IFNγ was insufficient to induce Th1 cell differentiation rely on BALB/c mice, arguing that this difference is a function of genotypic variations. The recently discovered cytokines IL-18, IL-23 and IL-27 also

exert influence over the development of a Th1 cells. The actions of these cytokines in particular will be discussed in greater detail later.

Although cytokines are perhaps the most potent regulators of T cell differentiation, other factors also influence T cell differentiation. It is hypothesized that weak engagement or engagement of only a few TCR molecules on the T cell surface results in a qualitatively different signal than high affinity or numerous ligations. In a model of collagen induced arthritis, administration of a collagen peptide with adjuvant results in the development of arthritic inflammation while the use of an altered peptide ligand, modified at a single amino acid and displaying 10,000 fold weaker affinity for the MHC (resulting in a lower MHC:peptide complex density on the surface of the APC and thus the engagement of fewer TCR molecules) results in a primarily humoral immune response (Murray, Pfeiffer et al. 1992; Pfeiffer, Stein et al. 1995). Similarly, increasing the affinity of an EAE inducing peptide for the MHC complex (and thus increasing the number of possible MHC:peptide:TCR complexes) increases IFNγ production, induction of Th1 differentiation and worsens disease status (Kumar, Bhardwaj et al. 1995). *In vitro* stimulation of naive T cells with a very low dose of peptide (<0.05 μM) stimulates the development of Th2-like effectors, whereas stimulation with intermediate doses (1 μM - 50 μM) results in the differentiation of Th1-like effectors (Constant, Pfeiffer et al. 1995; Hosken, Shibuya et al. 1995). Thus, the intensity of the signal delivered through the TCR can have dramatic effects on T cell differentiation: a collectively weak signal (generated by a low affinity interaction or triggering of only a few TCR molecules) triggers differentiation of Th2 cells, while a collectively strong signal (high affinity interactions, with many TCR ligations) results in the development of a Th1-like effector.

Finally, the nature of the co-stimulatory molecules engaged during T cell differentiation is also hypothesized to influence T cell differentiation. Both CD80 and CD86 on the surface of the APC interact with CD28 expressed on recently activated T cells, but the nature of the signal delivered to the T cell may be different. CD80 promotes the development of Th1 cells while CD86 ligation of CD28 results in the differentiation of Th2 cells (Freeman, Boussiotis et al. 1995; Kuchroo, Das et al. 1995). However, there is controversy regarding this characterization as other studies report the reverse (Lenschow, Ho et al. 1995), or no preferential induction (Chen, Ashe et al. 1992; Lanier, O'Fallon et al. 1995). The ligation of ICOS (inducible costimulator) on the surface of T cells induces Th2 cells (Dong, Juedes et al. 2001; Sharpe and Freeman 2002) while the ligation of LFA-1 on T cells promotes Th1 development (Bleijs, de Waal-Malefyt et al. 1999). Thus, cytokines provide perhaps the most important signals, but other molecules may also influence this differentiation.

2.2 - T cell differentiation at the molecular level

A great deal of attention has focussed on trying to identify the intracellular signalling molecules and events involved in differential induction and stabilization of Th1 and Th2 phenotypes. It is increasingly evident that the development of Th1 cells is largely dependent upon the intracellular induction of the transcription factor T-bet, upregulated in the process of Th1, but not Th2 differentiation (Szabo, Kim et al. 2000). T-bet expression increases in response to TCR ligation and activation of the IFNγ/STAT1 pathway, but surprisingly, not following activation of the IL-12/STAT4 pathway despite IL-12's role as a central regulator of T cell differentiation (Lighvani, Frucht et al. 2001; Mullen, High et al. 2001; Afkarian, Sedy et al. 2002). Overexpression of T-bet in

polarized Th2 cells (through transfection) increases IFNγ production but only variably reduces type 2 cytokine synthesis, arguing that T-bet expression alone is not sufficient to downregulate an existing Th2 phenotype (Szabo, Kim et al. 2000; Afkarian, Sedy et al. 2002). T-bet also upregulates IL-12Rβ2 expression (Mullen, High et al. 2001; Afkarian, Sedy et al. 2002) and is very important in controlling *initial* IFNγ production as T-bet -/- T cells produce little IFNγ even if cultured in Th1 inducing conditions (Szabo, Sullivan et al. 2002). However, *sustained* IFNγ production is controlled by the transcription factors Hlx and STAT4 (Park, Hondowicz et al. 2000; Stobie, Gurunathan et al. 2000; Mullen, Hutchins et al. 2002). Importantly, T-bet also directly induces chromatin rearrangement and the appearance of DNAase I hypersensitivity sites in the IFNγ locus, a hallmark of stable differentiated Th1 cells (Mullen, High et al. 2001; Mullen, Hutchins et al. 2001).

In contrast to Th1 cells, promoting development of Th2 cells results in upregulation of the transcription factor GATA-3 (Mullen, High et al. 2001; Mullen, Hutchins et al. 2001). Overexpression of GATA-3 in transfected T cells increases expression of IL-4, IL-5 and IL-13, with concomitant downregulation of IFNγ and IL-12Rβ2 expression, even if grown in Th1 promoting conditions (Zheng and Flavell 1997; Ouyang, Ranganath et al. 1998; Ferber, Lee et al. 1999; Lee, Takemoto et al. 2000). Although GATA-3 is the master controller of Th2 cytokine production, increasing expression of all type 2 cytokines simultaneously, inducing chromatin remodelling and appearance of DNAase I hypersensitivity sites in the type 2 cytokine loci (Takemoto, Koyano-Nakagawa et al. 1998; Lee, Takemoto et al. 2000; Ouyang, Lohning et al. 2000), the transcription factor c-Maf appears to be the major regulator of IL-4 transcription (Agarwal, Avni et al. 2000).

The major controls on Th1 and Th2 development are increasingly well understood, but two models have been proposed to explain how T cell differentiation actually proceeds. The instructional model of T cell differentiation states that each T cell has the potential to become either a Th1 or a Th2 cell, and immediately upon activation the genes for both subsets of cytokines are readily accessible. This is due to a small, but sufficient induction of both T-bet and GATA-3. Upon receiving additional signals from the environment (cytokines, ligation of different co-stimulatory molecules) the ability to produce one profile is abrogated according to the signal received. Thus, IL-12/STAT4 activation abrogates the ability to produce and respond to IL-4 by downregulating GATA-3 expression, while IL-4/GATA-3 signalling causes the extinction of IL-12 signalling and T-bet activation. This model is supported by the observation that within minutes after TCR stimulation, the heterochromatic nucleus of the naive T cell decondenses facilitating the rapid appearance (<1 hr) of message for both IFNγ and IL-4 (Lederer, Perez et al. 1996; Grogan, Mohrs et al. 2001).

In contrast, the stochastic model argues that an individual naive T cell will randomly produce either Th1 or Th2 cytokines upon stimulation of the TCR regardless of the activating conditions. These conditions, however, allow the outgrowth of only one type of cell. In the presence of IL-12, the growth of IFNγ producing, IL-12 responsive cells will be supported and IL-4 producing, IL-12 unresponsive cells will die from "neglect". Numerous studies demonstrate that, at a single cell level, most T cells do not produce the entire panel of Th1 or Th2 cytokines, but rather one or maybe two cytokines (Bucy, Karr et al. 1995; Kelso, Groves et al. 1995; Openshaw, Murphy et al. 1995; Saparov, Elson et al. 1997; Kelso, Groves et al. 1999). These data are taken to demonstrate that the expression of an individual cytokine gene is a random, rare event and that expression of each

cytokine gene is independently regulated, a finding consistent with the stochastic model of T cell differentiation.

Despite many years of striving to define the mechanisms controlling T cell differentiation, the mechanisms are still largely unclear. It is interesting to note that while the two opposing theories designed to explain the processes which facilitate the generation of Th1 or Th2 effector cells seem to describe two distinctly different mechanisms (and indeed were originally derived as such, one based on extracellular, the other on intracellular events), in an attempt to incorporate new observations, the two theories have invariably become less distinct. Both theories now explain T cell differentiation as an internal event (induction of both Th1 and Th2 transcription factors (instructional) *versus* random production a single transcription factor (stochastic)) followed by selection by external pressures (extinction of one pathway (instructional) *versus* selection of responding cells (stochastic)). As new observations are made on the kinetics and order of the activation of transcription factors/cytokines genes following TCR engagement, it is likely that the lines between the two theories will continue to blur, allowing a single unified theory to emerge.

2.3 - In vivo induction of Th1 and Th2 immune responses

The development of Th1 and Th2 subsets, and ultimately cell mediated or humoral immunity also occurs *in vivo* and is of great consequence in determining disease outcome. The generation of an immune response improperly equipped to deal with the pathogen can result in severe pathology or even death of the host. This is particularly well studied in murine models of Leishmaniasis.

A great deal of evidence indicates that the most important types of cells in determining the outcome of infection with *Leishmania* are CD4+ T cells. C57Bl/6 mice are resistant to infection with *Leishmania* major parasite and suffer from small lesions, eventually clearing the disease (Handman, Ceredig et al. 1979). In contrast, BALB/c mice suffer from large lesions and eventually succumb to the disease (Howard, Hale et al. 1980). While BALB/c mice are susceptible to infection with *Leishmania major*, a number of techniques can be used to "rescue" these mice, many of which focus on manipulating the phenotype of CD4+ T cells normally induced by infection in these mice. Administration of anti-CD4 antibodies (Titus, Ceredig et al. 1985) or sublethal, whole body γ-irradiation (Howard, Hale et al. 1981) prior to infection renders BALB/c mice phenotypically resistant to infection. The protective effects of these therapies can be reversed with injection of CD4+ T cells from syngeneic donors, or mice with progressive disease (Howard, Hale et al. 1981). Analysis of cytokine mRNA in the draining lymph nodes of BALB/c mice demonstrate these mice produce high levels of IL-4 which persists over the course of infection, but demonstrate little induction of IFNγ (Locksley, Heinzel et al. 1987). In contrast, resistant C57Bl/6 mice demonstrate elevated transcripts for IFNγ, and a transient increase in IL-4 transcripts which returns to baseline after the initial period of infection (Heinzel, Sadick et al. 1989). Furthermore, BALB/c mice cured by treatment with αCD4 Ab demonstrate a similar pattern of cytokine induction normally seen in resistant mice (Titus, Ceredig et al. 1985). In general, exacerbation of disease through a variety of methods is usually associated with an upregulation of type 2 cytokines (Hoerauf, Solbach et al. 1994; Saha, Chattopadhyay et al. 1998) and protection is associated with increased expression of type 1 cytokines (Brown, Titus et al. 1996; Murphy, Engwerda et al. 1997; Flohe, Bauer et al. 1998; Saha,

Chattopadhyay et al. 1998). Similarly, human patients who cure the disease display elevated IFNγ synthesis and depressed IL-4 production, while non-healing patients display the reverse (Ajdary, Alimohammadian et al. 2000).

There is clearly a dichotomy between healing and non-healing responses in mice. A Th1 response leads to protective immunity, and a Th2 response leads to progressive disease. It is ultimately the cytokines, produced *early* in infection with *Leishmania major* that are the key factors in determining the outcome of infection. This is demonstrated in studies where BALB/c mice are made resistant to infection by inhibiting type 2 inducing cytokine activity (αIL-4 mAb) (Sadick, Heinzel et al. 1990), or promoting type 1 immunity (with IL-12) (Heinzel, Schoenhaut et al. 1993; Sypek, Chung et al. 1993). In these cases, the treatment must be administered within the first week of infection for protection. Conversely, resistant mice are made susceptible by inhibiting type 1 immunity (αIFNγ mAbs), within the first week of infections (Belosevic, Finbloom et al. 1989).

The differential induction of Th1 and Th2 immune responses in resistant and susceptible mice is compelling evidence that Th1 and Th2 responses are important in dictating the severity and outcome of a disease state. Furthermore, the successful manipulation of the normal immune response to *Leishmania* has lead to use of cytokine administration in an attempt to achieve clinically relevant improvements in numerous animal models and even in human disease, including allergic disease.

2.4 - Regulatory T cells

Regulatory T cells (T_r) represent a subset of T cells distinct from Th1 or Th2 cells which are characterized chiefly by their overall inhibitory effects on T cell responses. Their cytokine production profile consists exclusively of IL-10 and/or TGF-β (Roncarolo, Bacchetta et al. 2001). It is chiefly through these cytokines that T_r cells mediate their immunosuppresive effect. IL-10 acts on APCs to downregulate the expression of CD80, CD86 and MHC class II, which results in decreased antigen presenting capacity (Moore, de Waal Malefyt et al. 2001). Additionally, IL-10 has direct effects on the T cell as stimulation of T cells with anti-CD3 plus IL-10 results in the development of anergic T cells (Groux, Bigler et al. 1996). TGF-β also has antiproliferative effects due to its capacity to inhibit IL-2 production, and prevent the activation of both T-bet and GATA-3 (Heath, Murphy et al. 2000; Gorelik, Constant et al. 2002; Gorelik and Flavell 2002). As such, it is thought that TGF-β and IL-10 are both important for the observed inhibitory effects of T_r on immune responses.

The factors controlling differentiation of naive T cells into T_r remains controversial, but it appears that the differentiation is a two step process. In the presence of cognate Ag plus IL-10 (with or without TGF-β) a naive T cell will become anergic, refractory to further antigenic stimulation. This anergic T cell has the capacity to inhibit T cell responses in a cell-contact dependent manner (Steinbrink, Graulich et al. 2002). The development of a true T_r cell with full IL-10 and TGF-β producing capabilities only occurs after a round of cell division, typically achieved *in vitro* through the use of high concentrations of anti-CD3 (Groux, O'Garra et al. 1997). It is presently unknown to what extent this second round of forced proliferation is possible *in vivo*.

While the search for a definitive marker of regulatory T cells has not been successful, there are two recognized populations of regulatory T cells: T_{r1} cells and $CD4^+CD25^+$ regulatory T cells. T_{r1} cells constitutively express IL-2Rβ and common γ chains, but lack expression IL-2Rα (CD25) (Bacchetta, Sartirana et al. 2002). T_{r1} cells produce IL-10 and TGF-β in similar quantities as Th2 cells (lacking the capacity to produce other type 2 cytokines) and thus supernatants of cultures of T_{r1} cells are sufficient to mediate immune suppression (Cavani, Nasorri et al. 2000; Kitani, Chua et al. 2000; Lecart, Boulay et al. 2001). In contrast, $CD4^+CD25^+$ regulatory T cells constitutively express CD25, express *more* IL-10 than Th2 cells typically do (Papiernik, do Carmo Leite-de-Moraes et al. 1997; Dieckmann, Plottner et al. 2001; Stephens, Mottet et al. 2001) and inhibit IL-2 production through as yet undefined, cell-contact dependent mechanisms. This may be mediated through the actions of CTLA-4 or the glucocorticoid-induced TNF receptor (GITR) (Takahashi, Tagami et al. 2000; Stephens, Mottet et al. 2001; McHugh, Whitters et al. 2002; Shimizu, Yamazaki et al. 2002; Zelenika, Adams et al. 2002). It has recently been proposed that cell bound TGF-β may also play an important role in the immunosuppresive activities of $CD4^+CD25^+$ T_r (Nakamura, Kitani et al. 2001).

The majority of work on T_r has focussed on autoimmunity, but the importance of $CD4^+CD25^+$ regulatory T cells has been examined in allergic disease. In murine models, IL-10 and TGF-β producing $CD4^+CD25^+$ cells have been shown to play a critical role in mediating the induction of immune tolerance associated with mucosal administration of allergens (Tsitoura, Blumenthal et al. 2000; Akbari, DeKruyff et al. 2001; Wiedermann, Herz et al. 2001; Zhang, Izikson et al. 2001; Hall, Houghton et al. 2003; Zemann, Schwaerzler et al. 2003). Furthermore, in human studies, the

development of IL-10 producing $CD4^+CD25^+$ T cells was associated with successful immunotherapy for allergies to grass pollen (Francis, Till et al. 2003), house dust mite allergen (Der p1) (Jutel, Akdis et al. 2003) and bee venom (phospholipase A_2) (Blaser, Akdis et al. 1999). These studies provide interesting preliminary evidence arguing for the importance of regulatory T cells (and the development of a robust "regulatory network") in the control of chronic allergic inflammation.

While regulatory T cells are being intensely studied at present, there are currently many fundamental questions which remain unanswered. While the functions of IL-10 and TGF-β are indisputably immunosuppressive, the mechanisms involved in controlling the development of regulatory T cells *in vivo* is largely still a mystery. The processes which are required to generate these cells in *in vitro* culture (forced proliferation through the use of high levels of anti-CD3) possess no readily identifiable homologues *in vivo*. As such, it is unclear the extent to which regulatory T cells influence immune responses naturally *in vivo*. Furthermore, the two classes of regulatory T cells, T_{r1} and $CD4^+CD25^+$ T cells, are very similar: both are characterized by their capacity to produce IL-10 and TGF-β, yet mediate their suppressive effects through apparently unique mechanisms (secreted factors versus cell contact mediated respectively). Thus it is presently unclear whether these two subsets of regulatory T cells represent distinct cellular sub-populations, or different stages in the differentiation of a true regulatory T cell. These fundamental questions about regulatory T cells must be addressed before a deeper understanding of the role that T_r play in regulating allergic disease can be achieved.

2.5 - Limitations of the Th1/Th2 paradigm

The Th1/Th2 paradigm has proven invaluable for explaining the apparent dichotomy observed between cellular and humoral immune responses. It has also been instrumental in exploring the pathologies associated with various disease states and understanding the underlying processes of these diseases. However, it is important to acknowledge the limitations of the paradigm. It is becoming increasingly evident that the "Th1" and "Th2" cells are the abstract ideal. If a discrete Th1 cell expressing *all* of the effector functions associated with Th1 cells exists, it is likely only as an artifact of overly vigorous *in vitro* stimulation. At a population level a "Th2 clone" may produce all the cytokines normally associated with a Th2 cell, but it has been observed that the majority of individual T cells taken directly *ex vivo* and from well developed T cell clones do not express the entire panel of cytokines associated with either Th1 or Th2 phenotypes (Bucy, Karr et al. 1995; Kelso, Groves et al. 1995; Openshaw, Murphy et al. 1995; Saparov, Elson et al. 1997; Kelso, Groves et al. 1999). Similarly, the classification of type 1 immune responses as cell mediated while type 2 immune responses as humoral is a gross oversimplification. Type 1 immune responses induce significant Ab production (particularly IgG_{2a}/IgG_{2c}) and significant cellular infiltration can occur in type 2 dominated immune responses (particularly eosinophils). Furthermore, although diseases like allergy and autoimmunity are strongly associated with Th2 and Th1 responses *in vivo*, there still exist IFNγ producing, allergen specific T cells and IL-4 producing autoantigen specific T cells in these diseases. An immune response is never exclusively Th1 or Th2, there is always a mixture of both. Thus it is important to characterize allergy not, for example, as a Th2 disease, but rather more accurately as a result of a type 2 *dominated* immune response.

3.0 - IL-12

3.1 - Structure and Regulation

IL-12 is a heterodimeric cytokine, consisting of disulfide bonded subunits of 35 and 40 kDa (p35 and p40 respectively) resulting in a biologically active protein of ~70 kDa (p70) (see Table 1 for summary)(Kobayashi, Fitz et al. 1989). For production of biologically active IL-12 p70, both subunits must be expressed and disulfude bonded within the cell, as mixing the subunits outside the cell does not result in bioloically active IL-12 p70 (Gubler, Chua et al. 1991; Wolf, Temple et al. 1991; Schoenhaut, Chua et al. 1992). p40 can be secreted alone or in the form of p40 homodimers ($(p40)_2$) (D'Andrea, Rengaraju et al. 1992). In contrast, p35 cannot be secreted in the absence of p40 (D'Andrea, Rengaraju et al. 1992). The amount of $(p40)_2$ produced in IL-12 producing cells is frequently 3 or 4 orders of magnitude higher than that of p70 (Salkowski, Detore et al. 1997; Aste-Amezaga, Ma et al. 1998). mRNA for p35 is ubiquitously expressed, but p40 mRNA is more restricted in expression, being produced only in cells capable of producing biologically active IL-12 (Gubler, Chua et al. 1991; Bette, Jin et al. 1994). IL-12 production was originally detected in EBV-transformed B cells (Kobayashi, Fitz et al. 1989), but its expression is now largely thought to be restricted to professional APCs to the exclusion of normal B cells (Guery, Ria et al. 1997; Gately, Renzetti et al. 1998).

Due to the more restricted production profile of p40, the majority of studies examining the regulation of IL-12 production have focussed specifically on regulation of the p40 subunit. LPS enhances transcription of p40 mRNA and increases the activity of the p40 promoter, an effect further enhanced by exposure to IFNγ (Ma, Chow et al. 1996; Aste-Amezaga, Ma et al. 1998; Cowdery, Boerth et al.

Table 1: Properties of Th1 promoting cytokines

Cytokine	Composed of	Natural Antagonist	Principal biological activities	Receptor Subunits	
				Signalling	Binding
IL-12	p35 - p40 heterodimer	$(p40)_2$	Induces IFNγ from NK and T cells, Promotes T cell proliferation, Increases NK cell cytotoxicity	IL-12Rβ2	IL-12Rβ1
IL-18	Caspase 1 processed pro-IL-18	IL-18 binding protein	Synergizes with IL-12 to promote Th1 immunity, Promotes Th2 immunity in certain circumstances	IL-18Rβ	IL-18Rα
IL-23	p19 - p40 heterodimer	$(p40)_2$	Induces IFNγ from *activated* T cells, Promotes *activated* T cell proliferation	IL-23R	IL-12Rβ1
IL-27	p28 - EBI3 heterodimer	Unknown, possibly p35 - EBI3 heterodimer	Induces IFNγ from *naive* T cells, Promotes *naive* T cell proliferation	WSX-1	Unknown

1999; Wang, Contursi et al. 2000). More recent work however, provides compelling evidence that there is also significant regulation of p35 production suggesting that the regulation of IL-12 production at the molecular level is far more complex than originally envisioned (Babik, Adams et al. 1999; Grumont, Hochrein et al. 2001; Vaidyanathan, Gentry et al. 2001). The signals which are known to trigger IL-12 p70 production fall within two categories: those derived from bacterial or parasitic products, and those derived from T cells. Bacterial or parasitic products such as LPS, oligonucleotides containing CpG motifs, and *Staphylococcus aureas* Cowan strain (SAC) act predominantly on macrophages and monocytes (Sieling and Modlin 2001), recognized chiefly by the recently identified TLRs. In contrast, dendritic cells are less responsive to bacterial products, particularly LPS due to lower expression CD14 (Kato, Yamane et al. 1997).

Alternatively, T cell stimulated, APC IL-12 production is mediated by the interaction of CD40 on the surface of the APC, with CD40L (CD154) on the surface of activated T cells. Culture of T cell clones with APCs in the presence of antigen triggers IL-12 production (DeKruyff, Gieni et al. 1997; Guery, Ria et al. 1997; Kato, Yamane et al. 1997; Ria, Penna et al. 1998; Skok, Poudrier et al. 1999; Yamane, Kato et al. 1999). The production of IL-12 in this system is both T cell and antigen dependent (DeKruyff, Gieni et al. 1997; Guery, Ria et al. 1997). Neutralizing the CD40:CD40L signalling pathway through the use of a blocking anti-CD40 or CD154 antibody (Kato, Hakamada et al. 1996; DeKruyff, Gieni et al. 1997; Yamane, Kato et al. 1999) or APCs derived from CD40 -/- mice (Skok, Poudrier et al. 1999) completely prevents IL-12 production. Similarly, disrupting the TCR:MHC Class II interactions (DeKruyff, Gieni et al. 1997; Guery, Ria et al. 1997; Yamane, Kato et al. 1999) also abrogates IL-12 production, likely a result of blocking T cell activation and

subsequent CD154 expression (Yamane, Kato et al. 1999). It is also interesting to note that *both* Th1 and Th2 clones can induce IL-12 production from APC populations, although IL-12 production by Th2 clones is significantly increased by the inclusion of neutralizing anti-IL-10 or recombinant IFNγ (DeKruyff, Gieni et al. 1997; Ria, Penna et al. 1998).

While APC are the principal source of IL-12 production, both B cells and NKT cells influence IL-12 production. Depletion of B cells from spleen cell cultures dramatically increases the production of IL-12 p70 (up to 100 fold) following stimulation with bacterial products, or polyclonal T cell activators (Maruo, Oh-hora et al. 1997; Skok, Poudrier et al. 1999; Yang, Tomura et al. 2000). The inhibitory effects of B cells have been ascribed to the production of soluble CD40 (Maruo, Oh-hora et al. 1997) or to their production of IL-6 and IL-10, which in turn inhibit IL-12 production (Skok, Poudrier et al. 1999). Stimulation of NKT cells with α-galactosyl ceramide (α-GalCer), the only known ligand for the invariant TCR found on NKT cells, elicits production of IL-12 *in vitro* and *in vivo* (Tomura, Yu et al. 1999; Yang, Tomura et al. 2000). Production of IL-12 is completely abrogated in the presence of a blocking anti-CD40L, and CD40L expression is found on $CD4^+$ (but not $CD4^-$) NKT cells suggesting that NKT cell activation facilitates APC production of IL-12 through a CD40:CD40L pathway (Tomura, Yu et al. 1999). However, NKT cells from IFNγ -/- mice cannot elicit IL-12 production following α-GalCer stimulation despite equivalent expression of CD40L on NKT cells from WT and IFNγ -/- animals (Yang, Tomura et al. 2000) suggesting that IFNγ plays an important role in enabling CD40L induced IL-12 production which is mediated by NKT cells. Thus IL-12 is produced in response to signals from both the innate immune system (LPS,

NKT cells) and adaptive immunity (Ag-stimulated T cells) suggesting an important role in general immune responsiveness.

3.2 - IL-12 Receptor

The IL-12 receptor complex comprises two distinct subunits, termed IL-12Rβ1 and IL-12Rβ2 which share significant homology with the β subunit of the IL-6 receptor (Gubler and Presky 1996). As the cytoplasmic tail of the IL12Rβ1 chain does not contain any tyrosine residues, it appears that signalling IL-12 binding is primarily the function of the IL-12Rβ2, which contains multiple tyrosine motifs in the cytoplasmic tail (Presky, Yang et al. 1996; Zou, Presky et al. 1997). It is interesting that high affinity binding of human IL-12 requires the presence of both subunits on the surface of the cell (Presky, Yang et al. 1996), but high affinity binding of mouse IL-12 requires only the presence of the IL-12Rβ1 subunit, although this is not sufficient to mediate IL-12 signalling (Wu, Wang et al. 2000). Functional IL-12 receptor (both IL-12Rβ1 and IL-12Rβ2) is found on activated αβ T cells (Szabo, Jacobson et al. 1995; Szabo, Dighe et al. 1997; Chang, Shevach et al. 1999), αGalCer activated NKT cells (Kitamura, Iwakabe et al. 1999), IL-2 activated NK cells (Chakir, Camilucci et al. 2000), activated γδ T cells (Yin, Zhang et al. 2000) and macrophages (Fantuzzi, Puddu et al. 2000). Signalling after ligation of the IL-12 receptor involves the JAK2 pathway (Yamamoto, Shibata et al. 1999), and the phosphorylation of STAT1, STAT3, STAT4 and STAT5. IL-12 (and IL-23 - see below) are the only known cytokines which trigger the activation of STAT4 (Yao, Niu et al. 1999), an important transcription factor involved in Th1 effector differentiation.

Expression of IL-12Rβ1 is constitutive on T cells, and can be found on both resting and activated Th1 and Th2 cells (Szabo, Dighe et al. 1997). In contrast, expression of IL-12Rβ2 is upregulated upon ligation of the TCR (Szabo, Jacobson et al. 1995; Szabo, Dighe et al. 1997). In the presence of IL-12 the increase in IL-12Rβ2 expression is much greater and the presence of IL-4 causes the rapid disappearance of IL-12Rβ2 (Szabo, Jacobson et al. 1995; Szabo, Dighe et al. 1997). Indeed, the loss of IL-12 responsiveness observed in well developed Th2 clones is likely due to the disappearance of IL-12Rβ2. TCR triggered expression of IL-12Rβ2 is enhanced via CD80/CD86 mediated signalling through CD28 (Igarashi, Yamane et al. 1998; Chang, Segal et al. 2000; Yamane, Igarashi et al. 2000; Elloso and Scott 2001), and enhanced in the presence of IL-18 (Chang, Segal et al. 2000) and TNFα (Ahlers, Belyakov et al. 2001), but inhibited by CTLA4 mediated signalling through CD28 (Yamane, Igarashi et al. 2000).

Studies examining the importance of IFNγ exposure on the subsequent expression of IL-12Rβ2 have yielded somewhat conflicting results. Studies examining the consequence of the presence of both IL-4 and IL-12 during initial T cell priming reveal the dominance of IL-4 (i.e. - differentiation of IL-4 producting Th2-like effectors), but these cells maintain IL-12Rβ2 expression can respond to IL-12 by proliferating and producing IFNγ (Szabo, Dighe et al. 1997; Nishikomori, Ehrhardt et al. 2000). IL-12Rβ2 expression can be abrogated by adding anti-IFNγ Ab to the initial cultures and can be enhanced by inclusion of IFNγ suggesting that IFNγ is required to maintain IL-12Rβ2 expression (Szabo, Dighe et al. 1997; Nishikomori, Ehrhardt et al. 2000). However, subsequent experiments also suggest that IFNγ is not required for maintenance of IL-12Rβ2 expression, as IL-12Rβ2^{+} T cells are found in IFNγ -/- mice (Chang, Shevach et al. 1999) and IL-12Rβ2 expression is unaltered on

TCR transgenic T cells (C57Bl/6 background) cultured with Ag + IL-12 + anti-IFNγ in the initial priming conditions (Smeltz, Chen et al. 2002). The discrepancy was resolved with the observation that in the presence of IL-4 (as in cell cultures from BALB/c mice), IFNγ must be present in order to maintain functional levels of IL-12Rβ2 expression on the surface of the newly differentiated T cell. In contrast, in the absence of IL-4 (as in cultures from other strains of mice), IL-12 alone is sufficient to promote the differentiation of IL-12Rβ2 expressing T cells.

3.3 - IL-12 p40 Homodimers

As stated above, the secretion of bioactive IL-12 is associated with the simultaneous production and release of excess IL-12 $(p40)_2$ (Aste-Amezaga, D'Andrea et al. 1994; Salkowski, Detore et al. 1997). $(p40)_2$ binds with high affinity to the IL-12Rβ1 without signalling, and prevents binding of bioactive IL-12 p70 to the IL-12 receptor complex (Mattner, Fischer et al. 1993; Gillessen, Carvajal et al. 1995; Gately, Carvajal et al. 1996). IL-12 $(p40)_2$ inhibits IL-12 driven T cell proliferation, increases in NK cell cytolytic activity, IFNγ production and Th1 effector cell differentiation (Gillessen, Carvajal et al. 1995; Gately, Carvajal et al. 1996; Heinzel, Hujer et al. 1997). As IL-12 $(p40)_2$ also inhibits *in vivo* induction of type 1 dominated immune responses following LPS administration (Gillessen, Carvajal et al. 1995; Heinzel, Rerko et al. 1995; Gately, Carvajal et al. 1996; Mattner, Ozmen et al. 1997), in a murine model of diabetes (Rothe, O'Hara et al. 1997) and in a cancer model (Schmidt, Brijs et al. 1998), $(p40)_2$ is typically regarded as a natural antagonist of IL-12 function.

However, reports of IL-12 $(p40)_2$ promoting type 1 immunity (i.e - acting as an agonist for the IL-12R complex) do exist (Piccotti, Chan et al. 1997; Decken, Kohler et al. 1998; Yoshimoto, Wang et al. 1998; Brombacher, Dorfmuller et al. 1999; Kopp, Kieffer et al. 2001). It is important to note that these studies, especially those utilizing p40 transgenic mice (Yoshimoto, Wang et al. 1998; Kopp, Kieffer et al. 2001), are confounded by the recent identification of IL-23, another member of the IL-12 "family" which consists of a heterodimer of IL-12 p40 and a novel subunit labelled p19. As IL-23 has similar activites to IL-12 (see section 5), it may be that overexpression of IL-12 p40 may allow for increased IL-23 secretion and potentially explain some conflicting results in regards to the role of endogenous IL-12 $(p40)_2$.

3.4 - Biological Activities of IL-12

IL-12 provides a proliferative signal to activated T cell and NK cells (Bertagnolli, Lin et al. 1992; Perussia, Chan et al. 1992), and is a potent inducer of IFNγ from NK and αβ and γδ TCR expressing T cells (Kobayashi, Fitz et al. 1989; Chan, Perussia et al. 1991; Otani, Nakamura et al. 1999). The presence of IL-12 and other mitogenic stimuli (anti-CD3, anti-CD28 for T cells, or anti-CD16 on NK cells) results in synergistic production of IFNγ (Chan, Perussia et al. 1991; Aste-Amezaga, D'Andrea et al. 1994; Kubin, Kamoun et al. 1994). IL-12 also synergizes with TNFα and IL-1β to increase production of IFNγ (D'Andrea, Aste-Amezaga et al. 1993; Tripp, Wolf et al. 1993) and increases the cytotoxic activity of both CTLs and NK cells (Chehimi, Valiante et al. 1993; Gately, Warrier et al. 1994). IL-12 induces the production of IL-10 (Morris, Madden et al. 1994), which is interesting in light of the fact that IL-10 inhibits IL-12 synthesis. It is believed that this represents an important

negative regulatory mechanism to prevent overexpression of IFNγ and limit type 1 dominated inflammatory responses. This is supported by the observation that IL-10 -/- mice produce elevated levels of IFNγ and are prone to chronic entrocolitis when kept in non-sterile environments (Kuhn, Lohler et al. 1993).

Perhaps the most important activity of IL-12 is its ability to prime naive T cells to become IFNγ producing effector cells both *in vitro* and *in vivo* (Hsieh, Macatonia et al. 1993; Seder, Gazzinelli et al. 1993; Afonso, Scharton et al. 1994; McKnight, Zimmer et al. 1994). The recognized ability of IL-12 to specifically promote the production of IFNγ and support differentiation has led to speculation that it would provide a valuable therapeutic for the treatment of type 2 dominated diseases like allergy.

3.5.0 - IL-12 as a negative regulator of type 2 immunity

Owing to its potent type 1 inducing activities, the effects of IL-12 exposure on type 2 immunity has frequently been examined. *In vitro* studies demonstrate that IL-12 can inhibit the production of type 2 cytokines (Manetti, Parronchi et al. 1993; Seder, Gazzinelli et al. 1993; Marshall, Secrist et al. 1995), type 2 associated chemokines (Ye, Huang et al. 2002) and *in vitro* IgE production (Kiniwa, Gately et al. 1992) which would argue that the use of IL-12 as an adjuvant might have the capacity to redirect immune responses associated with allergic responses to resemble those seen in clinically tolerant individuals. In studies exploring the *in vivo* administration of IL-12, IL-12 inhibits the production of IgE in response to a polyclonal B cell activator (anti-IgD) (Morris, Madden et al.

1994). In a model exploring the role of IL-12 in the clearance of *Schistosoma mansoni* eggs, administration of IL-12 ameliorates the granulomatous response associated with clearance of the eggs, while simultaneously changing the character of the remaining inflammation by reducing the number of eosinophils normally found in the lesions (Wynn, Jankovic et al. 1995; Wynn, Jankovic et al. 1995). Furthermore, administration of IL-12 also dramatically reduces serum IgE levels (Wynn, Jankovic et al. 1995; Wynn, Jankovic et al. 1995) thus providing evidence for a role for IL-12 in the inhibition of type 2 immunity.

3.5.1 - Administration of IL-12 at time of allergic sensitization

In murine models of allergic disease, IL-12 has demonstrated a similar capacity to negatively regulate type 2 immunity. Systemic administration of IL-12 to animals at the time of allergic sensitization significantly reduces Ag-specific and total IgE levels, while simultaneously increasing IgG_{2a} production (Germann, Guckes et al. 1995; Kips, Brusselle et al. 1996; Sur, Lam et al. 1996; Bruselle, Kips et al. 1997; Rempel, Wang et al. 1997; Lee, Fu et al. 1999). This reduction in IgE levels is accompanied by a decrease in the production of type 2 cytokines (IL-4, IL-5 and IL-13) in Ag-stimulated *in vitro* cultures of either spleen or draining lymph node cells and a simultaneous increase in Ag-stimulated IFNγ production (Sur, Lam et al. 1996; Lee, Fu et al. 1999). In studies where airway function was also examined, administration of IL-12 as an adjuvant has protective effects, abrogating the increases in airway hyperresponsiveness (Kips, Brusselle et al. 1996; Sur, Lam et al. 1996) and eosinophilia in the lung and BAL fluid (Kips, Brusselle et al. 1996; Sur, Lam et al. 1996; Bruselle, Kips et al. 1997; Lee, Fu et al. 1999) usually seen after OVA sensitization and airway

challenge. These data support the hypothesis that IL-12 can act as a potent inhibitor of the development of type 2 immunity.

While IL-12 administered as an adjuvant consistently results in the induction of a type 1 dominated immune response, in order to be of potential value as a prophylactic agent, the immune response must be stable in the face of repeated exposure to the allergen. Studies of the stability of the type 1 immune response induced in the presence of IL-12 are important, yet infrequent. In studies which do examine long-term effects of IL-12 administration at time of sensitization, IL-12 consistently fails to induce a stable shift away from type 2 immune responses following immunization with protein antigen (Germann, Guckes et al. 1995; Rempel, Wang et al. 1997; Rempel, Wang et al. 2000). Thus, while primary IgE responses are dramatically reduced, upon subsequent exposure to allergen, IgE responses are identical to, or even *higher* than those seen in mice which had not received IL-12 at the time of sensitization (Germann, Guckes et al. 1995; Rempel, Wang et al. 1997; Rempel, Wang et al. 2000). Thus, the effects of IL-12 in preventing the development of type 2 immune responses appear to be transient in nature.

3.5.2 - IL-12 as an inhibitor of *established* type 2 immune allergic responses

While consistent inhibition of type 2 immune responses is seen following administration of IL-12 as an adjuvant, the more interesting and clinically relevant question is whether IL-12 has the capacity to abrogate existing type 2 immune responses. Unfortunately, this question is much less studied. When examined, administration of IL-12 after sensitization (usually just prior to airway allergen challenge)

is consistently *unable* to decrease allergen specific or total IgE levels (Kips, Brusselle et al. 1996; Sur, Lam et al. 1996; Kim, DeKruyff et al. 1997; Rempel, Wang et al. 2000). Furthermore, examination of airway responsiveness in mice treated with IL-12 after sensitization reveals only a weak protective effect, associated with a decreased lung eosinophilia (Kips, Brusselle et al. 1996; Sur, Lam et al. 1996). In examination of antigen specific, splenic *T cell* responses to the allergen, decreases in IL-4 production and increases in IFNγ synthesis are noted, but culture of *whole spleen cells* reveals a dramatic, 15-20 fold increase in allergen induced IL-4 production from the non-B/non-T cell population (hypothesized to be NKT cells) (Rempel, Wang et al. 2000). In contrast, there is no decrease in Ag-stimulated IL-4 production from draining lymph node cells taken from mice treated with IL-12 after allergen sensitization (Kim, DeKruyff et al. 1997). Thus while the protective effects of IL-12 when administered at time of allergen sensitization are consistently evident, IL-12 does not redirect, and may in fact exacerbate, established type 2 immune responses.

3.5.3 - IL-12 inhibition of type 2 immune responses: alternative routes of administration.

In addition to the above studies where IL-12 was administered systemically, other studies examine alternative routes of delivery. Immunization of mice with an OVA-IL-12 fusion protein has a greater protective effect (greater inhibition of type 2 cytokine and IgE synthesis and increases in IFNγ production) and fewer non-specific complications than mice treated with OVA + IL-12 (Kim, DeKruyff et al. 1997). Similarly, intramuscular administration of plasmid encoded IL-12 (containing both p40 and p35 subunits separately) at the same time as immunization decreases airway responsiveness, Ag-specific IgE levels, and *in vitro* Ag driven IL-4 production (Kumar, Behera et al.

2001) suggesting that alternative methods of delivering IL-12 at the time of immunization are equally effective in preventing the initial development of a type 2 immune response. Long-term stability is not assessed in these studies.

Other routes of administering IL-12 in established type 2 immune responses, however, suffered from the same shortcomings as systemic administration of IL-12. Administration of an IL-12 encoding vaccinia virus (Hogan, Foster et al. 1998) or an IL-12 producing, replication deficient adenovirus (Stampfli, Scott Neigh et al. 1999) to the airways of mice undergoing a type 2 immune response significantly decreases airway responsiveness, decreases BAL fluid and Ag-stimulated spleen and lung mononuclear cell type 2 cytokine levels (IL-4, IL-5) while increasing type 1 cytokine (IFNγ) levels. However, such treatment is not able to consistently abrogate established IgE production (Hogan, Foster et al. 1998; Stampfli, Scott Neigh et al. 1999). Administration of nebulized IL-12 directly to the airways or intra-nasal treatment with IL-12 to sensitized mice immediately prior to challenge decreases airway responsiveness and BAL fluid eosinophilia (Schwarze, Hamelmann et al. 1998; Sur, Bouchard et al. 2000; Rais, Wild et al. 2002) but is unable to inhibit IgE synthesis (Schwarze, Hamelmann et al. 1998). Intratracheal administration of plasmid DNA encoding a single-chain IL-12 construct prior to allergen challenge decreases airway responsiveness, BALF eosinophilia and IL-5 levels but IgE production was not examined (Lee, Ye et al. 2001). Taken together, while administration of IL-12 via other routes (locally via plasmid immunization or IL-12 expressing viral construct infection) may reduce the toxicity associated with IFNγ production, they are consistently unable to downregulate existing IgE production.

3.5.4. - IFNγ dependance of IL-12 effects

As IL-12 is known to be an inducer of IFNγ production, it is also important to determine whether the effects seen following IL-12 administration were a result of increased IL-12 induced IFNγ production or a direct effect of IL-12. In mice treated with IL-12 (via i.p. administration, infection with IL-12 expressing viral constructs or local administration of IL-12) in the absence of IFNγ (through the use of IFNγ/IFNγR -/- mice or anti-IFNγ treatment), IL-12 no longer prevents pathology associated with exposure to *Schistosoma mansoni* eggs (Wynn, Jankovic et al. 1995) or decreases type 2 immune responses following protein antigen sensitization (Bruselle, Kips et al. 1997; Hogan, Foster et al. 1998; Stampfli, Scott Neigh et al. 1999; Rais, Wild et al. 2002). In contrast, only a single report, examining Ig production following administration of anti-IgD ± IL-12 argues that the effects of IL-12 (decreased IgE levels) are IFNγ independent (Morris, Madden et al. 1994). The authors of the latter study confirmed, however, that neutralization of IFNγ (through administration of anti-IFNγ Ab) was not complete (Morris, Madden et al. 1994). These studies argue very strongly that the chief type 2 inhibiting activity of IL-12 comes not from direct effects, but rather as a result of its ability to induce IFNγ production.

3.5.5 - Endogenous IL-12 as a negative regulator of type 2 immunity

Finally, while the administration of exogenous IL-12 results in only transient abrogation of *de novo* type 2 immune responses, and limited abrogation of *ongoing* type 2 immunity (with no indication of the stability of the observed effects), examination of the role of endogenous IL-12 in controlling the

immune response has largely focussed on the ability to support type 1 immunity (Heinzel, Rerko et al. 1995; Hofstra, Van Ark et al. 1998; Piccotti, Li et al. 1998). Data which lend support to the hypothesis that endogenous IL-12 production limits type 2 immunity are based on readouts of IL-4 (± IL-10) as the sole representative of type 2 cytokine synthesis, if type 2 cytokine production is examined at all (Gazzinelli, Wysocka et al. 1994; Neurath, Fuss et al. 1995; Heinzel, Hujer et al. 1997; Piccotti, Chan et al. 1997; Rao, Varalakshmi et al. 1997; Wu, Ferrante et al. 1997; Altare, Durandy et al. 1998; Brewer, Tetley et al. 1998; de Jong, Altare et al. 1998). Thus, previous studies which examine the role of endogenous IL-12 in inhibition of type 2 immunity do not contain a thorough exploration of the consequence of IL-12 deficiency, especially following immunization with exogenous protein antigen.

3.6 - Summary

IL-12 is the quintessential type 1 promoting cytokine, priming naive T cells to become IFNγ producing, IL-12 responsive, Th1-like effector cells. Indeed, such a role is largely supported by studies of rIL-12 administration and IL-12 -/- mice which report significant increases and decreases, respectively, in the intensity of Th1 associated responses. As a negative regulator of type 2 immunity, the role of IL-12 is unclear. Studies examining the administration of rIL-12 demonstrate powerful, but transient inhibition of type 2 immune response, likely mediated by IFNγ. However, the role of endogenous IL-12 as a negative regulator of type 2 immunity, and the role that the endogenous production of this cytokine may play in predisposing individuals to the development of allergic disease in is largely unexplored.

4.0 - Interleukin 18

4.1 - Structure and Regulation

IL-18 was initially purified from the livers of *Propionibacterium acnes* infected, LPS primed mice. Based on its potent IFNγ inducing activities, it was initially called interferon gamma inducing factor (IGIF) (see Table 1 for summary) (Nakamura, Okamura et al. 1993). Cloning of this gene revealed that it has little sequence homology with any known proteins (Okamura, Tsutsi et al. 1995), but structural analysis predicted that it has a 3-dimensional structure very similar to that of IL-1β (Bazan, Timans et al. 1996). IL-18 mRNA has a wide tissue distribution: in the adrenal cortex, particularly in glucocorticoid producing cells (Conti, Jahng et al. 1997), lung, thymus and spleen and resting microglia, B lymphocytes, T lymphocytes, macrophages and dendritic cells (Tone, Thompson et al. 1997; Stoll, Jonuleit et al. 1998). Interestingly, IL-18 message in both human and mouse contains no or few mRNA destabilisation sequences suggesting that IL-18 mRNA may have a long half-life (Tone, Thompson et al. 1997).

Regulation of IL-18 gene expression occurs from two promoters, one of which is constitutively active and principally controlled by the transcription factors PU.1 and AP-1 (Tone, Thompson et al. 1997; Kim, Kang et al. 1999; Kim, Im et al. 2000). The other is highly inducible and controlled by IFNγ consensus sequence binding protein (ICSBP) (Tone, Thompson et al. 1997; Kim, Kang et al. 1999; Kim, Im et al. 2000). Only one negative regulator of IL-18 gene expression has been identified to date; Bcl6 normally binds to the LPS-inducible promoter in resting conditions (Takeda,

Arima et al. 2003). Upon LPS stimulation however, Bcl6 is modified, removing the repression and allowing for the increased expression of IL-18 seen after LPS stimulation (Takeda, Arima et al. 2003).

IL-18 mRNA encodes a 24 kDa form of the protein (pro-IL-18) which displays very little biological activity (Ghayur, Banerjee et al. 1997; Kikkawa, Matsumoto et al. 2001). Generation of the biologically active, 18 kDa form of IL-18 requires the processing of the pro-peptide after Asp^{35} (Ghayur, Banerjee et al. 1997; Gu, Kuida et al. 1997). This cleavage is mediated chiefly by interleukin 1β converting enzyme (ICE or caspase-1), but can also be performed by caspase-4 and caspase-5 (Ghayur, Banerjee et al. 1997; Gu, Kuida et al. 1997). Caspase-3 also cleaves pro-IL-18, but this cleavage results in very small peptide fragments with no biologic activity and thus likely represents a negative regulatory mechanism to limit the production of IL-18 (Ghayur, Banerjee et al. 1997; Gu, Kuida et al. 1997). Extracellular cleavage of IL-1β has been extensively reported (Hazuda, Strickler et al. 1990; Nylander-Lundqvist, Back et al. 1996; Fantuzzi, Ku et al. 1997; Nylander-Lundqvist and Egelrud 1997), and there are reports detailing similar activation of IL-18 (Sugawara, Uehara et al. 2001). Thus, secretion of biologically active IL-18 requires the co-expression of pro-IL-18 and the expression and activation of ICE.

Other regulatory mechanisms on IL-18 activity exist, such as directly affecting the activity of ICE. IRF-1, through its upregulation of caspase-1 expression, can also increase the secretion of active IL-18 (Fantuzzi, Reed et al. 2001). MyD88, is a signalling molecule involved in transducing IL-18 signalling and thus regulation of MyD88 activity can also influence IL-18 activity. In MyD88 -/-

cells or cells treated with a protein synthesis inhibitor, LPS stimulated IL-1β production is dramatically lowered but IL-18 activity is only mildly decreased despite very similar requirements for the regulation of IL-18 and IL-1β (Puren, Fantuzzi et al. 1999; Seki, Tsutsui et al. 2001). This may be a result of a two-pronged effect of LPS stimulation. It is presently thought that LPS induces increased expression of IL-18, IL-1β and caspase-1 mRNA expression through signals delivered via MyD88. In contrast, LPS activates existing caspase-1 independently of MyD88 signalling resulting in the cleavage of constitutively present pro-IL-18, explaining the increases in IL-18, but not IL-1β secretion, as IL-1β is not constitutively expressed (Puren, Fantuzzi et al. 1999; Seki, Tsutsui et al. 2001). Finally, IL-18 secretion is enhanced by the addition of IFNγ both directly (increased IL-18 expression) and indirectly (through increased ICE activity) (Kim, Talanian et al. 1998; Suk, Yeou Kim et al. 2001; Vankayalapati, Wizel et al. 2001), and inhibited by NO (via inhibition of caspase-1 activity) (Kim, Talanian et al. 1998) and PGE_2 (direct transcriptional effect)(Suk, Yeou Kim et al. 2001).

4.2 - IL-18 Binding Protein

In an effort to isolate soluble cytokine receptor subunits to facilitate identification of IL-18 receptor components, Novick *et al.* concentrated the proteins in human urine samples and ran them on an IL-18 affinity column (Novick, Kim et al. 1999). In doing so, they identify a protein (IL-18 binding protein (IL-18BP)) with high affinity and specificity for IL-18, but with antagonistic effects on IL-18 and LPS (but not ConA) induced IFNγ production in *in vitro* cultures (Novick, Kim et al. 1999). Administration of this protein at the time of LPS challenge to LPS primed mice reduces serum IFNγ

levels 6 hrs following LPS challenge by >90% (Novick, Kim et al. 1999). Further characterization of this IL-18 binding activity revealed it is a member of the Ig superfamily but was not a soluble IL-18R subunit, has no discernable transmembrane domain or GPI-anchor site and displays minimal sequence homology with eukaryotic proteins (Novick, Kim et al. 1999). The highest homology seen is with Pox-virus encoded proteins suggesting that suppression of IL-18 activity may be a strategy for successful viral infection (Novick, Kim et al. 1999). Interestingly, the Ig-domain of the IL-18BP is homologous to the IL-1 binding domain of the decoy IL-1R (IL-1R type II) (Novick, Kim et al. 1999).

The murine version of this protein has been sequenced and cloned, and displays significant sequence homology with human IL-18BP. In humans, are 4 different splice variants are produced (IL-18BPa to IL-18BPd) while in mice there are only 2 (IL-18BPc and IL-18BPd) (Novick, Kim et al. 1999; Kim, Eisenstein et al. 2000). It is interesting to note that only human IL-18BPa and IL-18BPc can bind and neutralize hIL-18 activity, yet both murine isoforms of IL-18BP have mIL-18 neutralizing activities (Kim, Eisenstein et al. 2000). IL-18BP binds only to mature IL-18, not pro-IL-18 (Kim, Eisenstein et al. 2000). In both species, mRNA for IL-18BP is strongly expressed in the spleen (Novick, Kim et al. 1999). In humans, message is constitutively observed in the small intestine, prostate, colon and PBMCs and IL-18BPa is the principal isoform (Novick, Kim et al. 1999; Veenstra, Jonak et al. 2002). While the cellular source of IL-18BP is still unclear *in vivo*, in *in vitro* LPS stimulated PBMC, monocytes are the principal producers (Veenstra, Jonak et al. 2002).

IFNγ strongly upregulates both message and protein for IL-18BPa in human colonic cell lines and primary samples (Muhl, Kampfer et al. 2000; Paulukat, Bosmann et al. 2001; Hurgin, Novick et al. 2002; Veenstra, Jonak et al. 2002). While IL-2, IL-12 and IL-15 also induce the production of IL-18BP *in vitro* and *in vivo*, this is abrogated in the absence of IFNγ (Veenstra, Jonak et al. 2002). Th2 cytokines (IL-4, IL-10, IL-1β) have no effect on IL-18BP expression (Veenstra, Jonak et al. 2002). Examination of the promoter for IL-18BP reveal it is constitutively under the control of IRF-1 and C/EBPβ, both increased after exposure to IFNγ (Hurgin, Novick et al. 2002). Consistent with the IFNγ dependence of IL-18BP production in humans, circulating IL-18BP levels are dramatically reduced in IRF-1 -/- mice (Fantuzzi, Reed et al. 2001).

Thus, there is clearly an additional level of control which regulates IL-18 production and IL-18 bioactivity. As the levels of IL-18BP in the serum of healthy humans is reportedly ~2.5 ng/ml (Novick, Schwartsburd et al. 2001) and the binding affinity of IL-18 is such that an equimolar concentration of IL-18 and IL-18BP results in a 50% decreased in IL-18 activity (Kim, Eisenstein et al. 2000), this likely represent a very powerful regulatory control on the activity of IL-18. It is particularly noteworthy that IFNγ seems to be the most potent stimulus to promote IL-18BP production identified to date. As IL-18, particularly in combination with IL-12, is such a potent inducer of IFNγ production, the subsequent induction of IL-18BP may represent a negative feedback mechanism which serves to prevent unrestricted induction of IFNγ potentially leading to excessive inflammation and/or autoimmune disease. Indeed, IL-18BP administration has been shown to ameliorate collagen induced arthritis, contact dermatitis, colitis and acute graft versus host disease suggesting its importance in controlling the overall intensity of a Th1-dominated immune response

(Plater-Zyberk, Joosten et al. 2001; Zecchina, Novick et al. 2001; Sivakumar, Westrich et al. 2002; Plitz, Saint-Mezard et al. 2003)

4.3 - IL-18 Receptor

The IL-18 receptor consists of two subunits, IL-18Rα and IL-18Rβ (Torigoe, Ushio et al. 1997; Born, Thomassen et al. 1998; Debets, Timans et al. 2000). These two subunits were initially discovered as orphan members of the IL-1 receptor family and named IL-1 receptor related protein (IL-1Rrp) and IL-1 receptor accessory protein-like (IL-1R AcPL) due to their high sequence homology with the two subunits which make up the functional IL-1 receptor (Torigoe, Ushio et al. 1997; Born, Thomassen et al. 1998). However, neither IL-1α, IL-1β nor IL-1 receptor antagonist bind to the IL-18 receptor complex (Debets, Timans et al. 2000). Conversely, IL-18 does not bind to the IL-1 receptor complex (Debets, Timans et al. 2000). Although IL-18 can bind IL-18Rα alone, there is no signal transferred to the interior of the cell in the absence of IL-18Rβ (Born, Thomassen et al. 1998; Hoshino, Tsutsui et al. 1999). High affinity binding of IL-18 requires the simultaneous expression of both IL-18Rα and IL-18Rβ (Born, Thomassen et al. 1998; Hoshino, Tsutsui et al. 1999; Debets, Timans et al. 2000). Thus, the presently accepted model of IL-18 and IL-18R binding is that IL-18Rα mediates initial binding with IL-18 which triggers conformational changes in the IL-18/IL-18Rα complex. This exposes sites allowing the recruitment of the IL-18Rβ subunit which increases affinity of binding and facilitates IL-18R mediated signalling (Wu, Sakorafas et al. 2003).

IL-18 responsiveness has been reported in a great number of cells including murine Th1 cells, NK cells, B cells, mast cells and NKT cells (Ahn, Maruo et al. 1997; Tomura, Zhou et al. 1998; Xu, Chan et al. 1998; Hoshino, Tsutsui et al. 1999; Hoshino, Wiltrout et al. 1999; Yoshimoto, Tsutsui et al. 1999 Yoshimoto, 2003 #172; Debets, Timans et al. 2000; Leite-De-Moraes, Hameg et al. 2001; Smeltz, Chen et al. 2001). Invariably, IL-12 upregulates IL-18 receptor expression on T, NK and B cells (Adachi, Kawai et al. 1998; Xu, Chan et al. 1998; Yoshimoto, Takeda et al. 1998), through STAT4 activation and its induction of IFNγ production (Nakahira, Tomura et al. 2001). The latter is required to maintain both IL-12Rβ2 and IL-18Rα expression on differentiating T cells in the presence of IL-4 (Smeltz, Chen et al. 2002). In contrast, stimulation of the TCR, exposure to IL-2 or IL-18 downregulates the expression of IL-18Rα (Xu, Chan et al. 1998; Yoshimoto, Takeda et al. 1998; Smeltz, Chen et al. 2001).

Studies examining the kinetics of IL-18Rα expression during T cell differentiation reveal that Th2, but not Th1 cells downregulate the expression of the IL-18Rα chain (Xu, Chan et al. 1998; Debets, Timans et al. 2000; Smeltz, Chen et al. 2001). While Th2 cells downregulate expression of IL-18Rα, they still express detectable levels of IL-18Rβ (Debets, Timans et al. 2000). Furthermore, the expression of IL-18Rα and IL-18 driven IFNγ production was high, medium and low in $CD4^-CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^-$ populations respectively (Tomura, Maruo et al. 1998). This finding, suggesting that IL-18 may have a greater role in innate rather then adaptive immunity, has not been further explored.

Binding of the IL-18R, causes the activation of MyD88 (Adachi, Kawai et al. 1998), Tyk2 (Shimoda, Tsutsui et al. 2002), IRAK (Kanakaraj, Ngo et al. 1999) NF-κB (Matsumoto, Tsuji-Takayama et al. 1997) and AP-1 (Barbulescu, Becker et al. 1998). Thus, the synergistic actions of IL-12 and IL-18 in the induction of IFNγ (see below) are likely a result of both reciprocal upregulation of their receptors, and novel signalling pathways to induce the IFNγ production.

4.4 - Biologic Activites of IL-18

IL-18 shares many biologic activities with IL-12, primarily exerted upon T cells. IL-18 is a potent inducer of IFNγ production and proliferation of activated, but not naive T cells (Okamura, Tsutsi et al. 1995; Kohno, Kataoka et al. 1997; Robinson, Shibuya et al. 1997; Hoshino, Yagita et al. 2000). IL-18 synergizes with IL-12 to enhance IFNγ production by T cells, particularly Th1 cells (Okamura, Tsutsi et al. 1995; Kohno, Kataoka et al. 1997; Robinson, Shibuya et al. 1997; Hoshino, Yagita et al. 2000). However, in contrast to IL-12, IL-18 *cannot* alone induce the differentiation of naive T cells into IFNγ producing Th1 cells (Robinson, Shibuya et al. 1997). However, if present with IL-12, IL-18 potentiates the development of IFNγ producing Th1 cells (Robinson, Shibuya et al. 1997). In contrast, IL-18 has no effect on established Th2 cells *in vitro* (Robinson, Shibuya et al. 1997).

IL-18 also synergizes with IL-12 to increase NK cell cytotoxicity and IFNγ production (Tsutsui, Nakanishi et al. 1996; Takeda, Tsutsui et al. 1998), B cell IFNγ production and class switch to IgG_{2a} (Yoshimoto, Okamura et al. 1997) and DC IFNγ production (Fukao, Matsuda et al. 2000). IL-18

deficient mice, despite having normal IL-12 levels, synthesize substantially less IFNγ following LPS challenge (Takeda, Tsutsui et al. 1998), suggesting an important role for IL-18 in endotoxin mediated responses particularly liver damage seen in these models (Netea, Fantuzzi et al. 2000).

4.5.0 - IL-18 as a regulator of type 2 immunity

4.5.1 - Administration of IL-12 + IL-18 as a negative regulator of type 2 immunity

The classical observations of IL-18 (detailed above) would suggest it is an important promoter of type 1 immunity, and thereby a negative regulator of type 2 immune responses. Indeed, numerous studies have supported the concept of IL-18, particular in the presence of IL-12, as a negative regulator of type 2 immune responses. Co-administration of IL-12 and IL-18 abrogate the increases in IgE production normally seen in response to *Nippostrongylus brasiliensis* infection (Yoshimoto, Okamura et al. 1997), treatment with anti-IgD (Yoshimoto, Okamura et al. 1997), or OVA (alum) immunization (Pollock, Conacher et al. 2003). In a model of allergen induced airway hyperresponsiveness, IL-12 + IL-18 treatment at airway challenge decreases serum IgE levels, airway hyperreactivity, eosinophilia, IL-4 and IL-5 levels in BAL fluid and type 2 cytokine production in antigen restimulated cultures of draining lymph node cells (Hofstra, Van Ark et al. 1998). In these studies, however, administration of IL-18 alone causes no inhibition of type 2 immune responses (Yoshimoto, Okamura et al. 1997; Hofstra, Van Ark et al. 1998; Pollock, Conacher et al. 2003).

4.5.2 - Administration of IL-18 as an adjuvant: a promoter or inhibitor of type 2 immunity?

However, several other studies examining the role of administration of IL-18 have demonstrated substantial inhibition of type 2 immunity. Immunization with a plasmid encoding a fusion product of OVA and IL-18 results in significantly lower IL-4 synthesis upon *in vitro* OVA restimulation than immunization with an OVA containing plasmid alone (Kim, Cho et al. 2001). Such a fusion product also reverses established airway hyperresponsiveness (Maecker, Hansen et al. 2001). Administration of IL-18 expressing adenovirus vector to the airways of mice with developing or established airway hyper-responsiveness prevents or abrogates the observed rise in airway responsiveness while decreasing IL-4, IL-5 and IL-13 production from Ag-stimulated cultures of draining lymph nodes (Walter, Wong et al. 2001).

In contrast, there are numerous reports suggesting that IL-18 also has the capacity to dramatically increase the intensity of type 2 immune responses. Stimulation of *in vitro* matured basophils or those purified from mice infected with *Nippostrongylus brasiliensis* and treated with IL-18 + IL-3 results in release of IL-4 in the absence of IgE crosslinking (Yoshimoto, Tsutsui et al. 1999). In spleen cell cultures from IL-2 treated C57Bl/6, but not untreated C57Bl/6, mice the presence of IL-18 results in enhanced IL-13 production from both T and NK cells and the IL-13 enhancing effect is even greater in IL-2 treated IFNγ -/- mice (Hoshino, Wiltrout et al. 1999). NKT cells cultured in the presence of IL-18 following either *in vivo* or *in vitro* stimulation with α-galactosylceramide or anti-CD3 produce more IL-4 than those stimulated in the absence of IL-18 (Leite-De-Moraes, Hameg et al. 2001). Finally, culture of naive T cells from BALB/c mice in the presence of IL-18 facilitates anti-CD3 driven production of IL-4, which is sufficient to induce the subsequent development of IL-4

producing Th2 cells (Xu, Trajkovic et al. 2000). While IL-18 increases anti-CD3 driven production of IL-5 and IL-13 from naive T cells from C57Bl/6, CBA and BALB/c mice (considered to have low, medium and high Th2 biases respectively), only BALB/c mice demonstrate increased IL-4 production (Xu, Trajkovic et al. 2000).

In a murine model of ragweed induced airway responsiveness, IL-18 administration timed to coincide with both allergen sensitization and challenge, or with challenge alone decreases lung hyperreactivity and eosinophilia (associated with increased local IFNγ production), yet oddly increases the Ag-stimulated *in vitro* IL-4 and IL-5 production (Wild, Sigounas et al. 2000). However, if this measurement of airway reactivity and eosinophilia is delayed for 2 weeks, a significant *increase* in reactivity and eosinophilia is observed suggesting that there are temporal aspects important in IL-18 regulation of type 2 immunity (Wild, Sigounas et al. 2000).

4.5.3. - Administration of IL-18 to naive animals

Administration of IL-18 alone also has significant effects on the development of type 2 immunity. High doses of IL-18 (65 μg over 13 days), given in the absence of any other immunological stimuli induces increased IgE production, and the appearance of IL-4 and IL-13 in the serum, while lower doses (<10 μg over 13 days) have no significant effect on IgE production (Wild, Sigounas et al. 2000; Yoshimoto, Mizutani et al. 2000; Yoshimoto, Min et al. 2003). This effect is observed in both C57Bl/6 and BALB/c mice (Wild, Sigounas et al. 2000; Yoshimoto, Mizutani et al. 2000; Yoshimoto, Min et al. 2003). The increases in serum IgE are abrogated in CD1 -/- mice, STAT6 -/-

mice and in mice treated with anti-CD4 to deplete T cells *in vivo* demonstrating that both conventional, and NKT cells play important, non-redundant roles in IgE induction (Yoshimoto, Mizutani et al. 2000; Yoshimoto, Min et al. 2003). Similar increases in IgE production are also seen in IL-18 or caspase-1 transgenic mice (which display increased serum IL-18 levels) (Yoshimoto, Mizutani et al. 2000; Konishi, Tsutsui et al. 2002).

4.5.4 - Endogenous IL-18 as a regulator of type 2 immunity

In the absence of endogenous IL-18 (a result of neutralizing antibodies or genetic knockout), BAL fluid eosinophilia is more rapidly induced, being enhanced early in the response, (Kumano, Nakao et al. 1999; Campbell, Kunkel et al. 2000), but indistinguishable from control mice at 72 hours (Campbell, Kunkel et al. 2000). Airway responsiveness in IL-18 deficient mice, measured in anaesthetized, artificially respirated animals is either double (Campbell, Kunkel et al. 2000) or indistinguishable (Kumano, Nakao et al. 1999) from control mice. In a parasite model, IL-18 -/- mice clear infection with *Trichinella spiralis* infection more rapidly than control animals, a response associated with increased production of IL-4, IL-10, and particularly, IL-13 (Helmby, Takeda et al. 2001). Finally, treatment of mice which normally clear *T. spiralis* infection with exogenous IL-18, slows clearance of the parasite by limiting the differentiation of mast cells and their ability to produce IL-4 (Helmby and Grencis 2002).

4.6 - Summary

Thus, IL-18 has the capacity to promote type 1 inflammatory immune responses and large amounts of IFNγ production. In striking contrast, however, there are clearly some not yet well understood situations which allow IL-18 to act as a powerful promoter of the development of type 2 immune responses. However, given the numerous tight regulatory mechanisms which exist on the production and responsiveness to IL-18 (requirements for pro-peptide processing, restricted receptor expression, constitutive availability IL-18BP, and the possible differential roles of different isoforms of IL-18BP) the ability of endogenously produced IL-18 to attain such levels in the serum is questionable. Thus, the physiological role that IL-18 plays in regulation of type 2 immune responses is presently unclear.

5.0 - "IL-12 like" cytokines

5.1 - IL-23

5.1.1 - IL-23: Structure and regulation

The observation of differing disease susceptibility in p35 -/- and p40 -/- mice is frequently attributed to potential agonistic actions of IL-12 p40 homodimer (Piccotti, Chan et al. 1997; Decken, Kohler et al. 1998; Brombacher, Dorfmuller et al. 1999; Kopp, Kieffer et al. 2001). However these studies can not rule out the possibility that IL-12 p40 binds a protein subunit other than p35 and thus mediates additional biological activity. Using computational screening methods to look for proteins possessing similarities to members of the IL-6 family facilitated the identification of a novel cytokine

gene, p19 (~19 kDa) (see Table 1 for summary) (Oppmann, Lesley et al. 2000). While this protein is not secreted by p19 expressing transfectants, and displays no biological activities when purified from cellular lysates, co-expression of a number of IL-6R family members allowed identification of a novel p19/IL-12 p40 heterodimer (IL-23) secreted in supernatants of co-transfectants (Oppmann, Lesley et al. 2000).

The tissue distribution of p19 mRNA expression is limited to polarized murine Th1 and Th2 T cells, LPS activated murine macrophages, both resting and viral infected human macrophages (Pirhonen, Matikainen et al. 2002) and human CD40L stimulated, monocyte-derived DCs (Oppmann, Lesley et al. 2000). Both human and murine DCs produce IL-23 protein following activation with TNFα, LPS and CD40L (Oppmann, Lesley et al. 2000). Studies examining the regulation of IL-23 production are few, but the presence of IFNβ during the differentiation of monocyte derived dendritic cells increases basal expression of p19 while dramatically blunting the increases in p19 expression normally seen after DC activation with LPS (van Seventer, Nagai et al. 2002). Furthermore, exposure of resting DC to IL-12 was shown to upregulate p19 production (Belladonna, Renauld et al. 2002). To date, there are no reports examining IL-23 production in antigen driven systems. The ability of DCs to produce IL-23 following CD40 ligation suggests, however that CD40L expressing, antigen activated T cells may have the capacity to trigger IL-23 synthesis.

5.1.2 - IL-23 function

Like IL-12, IL-23 acts on T cells to induce proliferation and IFNγ production in both murine and human, anti-CD3 stimulated T cell cultures (Oppmann, Lesley et al. 2000). Interestingly, the principal effects of IL-23 are on murine memory ($CD45RB^{low}$) and human memory (CD45RO) T cell subsets whereas IL-12 acts principally on naive T cells subsets ($CD45RB^{high}$ in mice and CD45RA in humans) (Oppmann, Lesley et al. 2000). Although IL-23 induces IFNγ production and STAT4 activation specifically in memory T cells, the degree of IFNγ production and STAT4 activation was consistently greater following exposure to IL-12 than following exposure to IL-23 (Oppmann, Lesley et al. 2000). IL-23 also induces IL-12 production from resting human DC (Belladonna, Renauld et al. 2002). Taken together with the observation reported above that IL-12 induces p19 expression, this suggests the existence of a positive feedback mechanism controlling the production of both IL-12 and IL-23.

5.1.3 - IL-23 receptor

The receptor for IL-23 includes the IL-12Rβ1 subunit which alone is sufficient for IL-23 binding, but not intracellular signalling (Oppmann, Lesley et al. 2000; Parham, Chirica et al. 2002). A novel subunit of the IL-23 receptor complex (called IL-23R) mediates signalling, bears a high degree of homology with IL-12Rβ2 and is found on Th1 and Th2 clones, NK cells and weakly expressed on DCs (Parham, Chirica et al. 2002). Memory T cells express high levels IL-23R but little IL-12Rβ2 (Parham, Chirica et al. 2002). Signalling through the IL-23R also results in the activation of JAK1, STAT1, STAT3 and STAT4 (Parham, Chirica et al. 2002). However, unlike IL-12 which chiefly

activates STAT4, signalling through IL-23R principally results in phosphorylation of STAT3 (Parham, Chirica et al. 2002).

The discovery of IL-23, which has similar actions as IL-12 but acts primarily upon memory T cells has exciting implications on the control of Th1 responses. While IL-12 (and IL-27, see below) may be critical in the *induction* of type 1 immunity, the principal role of IL-23 may be the maintenance of type 1 immunity. It is particularly interesting that IL-23R is expressed on polarized Th2 cells as well as Th1 cells. As IL-12Rβ1, the other subunit of a functional IL-23 receptor is not downregulated upon differentiation into a Th2 effector cell, it will be important to determine what effects (if any) IL-23 has on Th2 cells *in vitro* and *in vivo*. Furthermore, as IL-23 is comprised of IL-12 p40 disulfide bonded to a novel cytokine subunit, p19, it may be subject to the same antagonism by IL-12 p40 homodimers, since p40 and $(p40)_2$ is produced and secreted in vast excess of the combined quantities of both IL-12 and IL-23 that are produced in the cell (Kopp, Lenz et al. 2003).

5.2 - IL-27

5.2.1 - IL-27: Structure and regulation

IL-27 is another recently described member of the IL-12 family which consists of two novel cytokine genes: p28, which has homology with IL-12 p35, and Epstein-Barr virus induced gene 3 (EBI3), which has homology with IL-12 p40 (see Table 1 for summary) (Pflanz, Timans et al. 2002). EBI3 was previously identified as a protein expressed in high levels at the fetal-maternal interface where it

was secreted as a homodimer or in a heterodimer with IL-12 p35. Neither of these dimers have demonstrable activity (Devergne, Hummel et al. 1996; Hashimoto, Suzuki et al. 2000; Devergne, Coulomb-L'Hermine et al. 2001). Transfection of murine and human p28 (73% identical) into cells lines reveals that the murine, but not the human protein, is secreted at low levels into the supernatant but with no discernible activity (Pflanz, Timans et al. 2002). Co-transfection of EBI3 allows secretion of high levels of p28 into the tissue culture supernatant and depletes intracellular levels of p28 (Pflanz, Timans et al. 2002). Expression of both subunits is required to allow secretion of the heterodimer (Pflanz, Timans et al. 2002). mRNA for both subunits is only found in myeloid cells: particularly human monocytes and monocyte derived DC and murine macrophages (Pflanz, Timans et al. 2002). Production of IL-27 is noted in CD40L and LPS stimulated DC cultures, and *precedes* the production of IL-12 suggesting that IL-27 might exert important biological effects distinct from and *before* those of IL-12 (Pflanz, Timans et al. 2002). Again, the capacity of CD40L to trigger IL-27 production again suggests that antigen specific T cells may have the capacity to trigger IL-27 synthesis through a CD40 dependent pathway.

5.2.2 - IL-27 function

As IL-27 is similar in structure and sequence to IL-12, the effects of IL-27 on T cells are of principal interest. Like IL-12 and IL-23, it induces proliferation and IFNγ production from anti-CD3 stimulated cultures of T cells (Pflanz, Timans et al. 2002). However, the actions of IL-27 are most potent on naive T cell populations, but require co-stimulation with either IL-12 or anti-CD28, signals

with which IL-27 synergized (Pflanz, Timans et al. 2002). IL-27 has no effect on Th2 cytokine production (Pflanz, Timans et al. 2002).

Recently, an EBI3 -/- mouse was generated to study the effects of IL-27 deficiency on the immune response. Contrary to expectations, stimulation of spleen cells from EBI3 -/- mice with anti-CD3 and anti-CD28 results in significantly higher IFNγ production and significantly lower IL-4 synthesis (Nieuwenhuis, Neurath et al. 2002). This IL-4 deficiency is a result of lower NKT cell levels in the spleen and liver of EBI3 -/- mice (Nieuwenhuis, Neurath et al. 2002). Both *in vitro* and *in vivo* stimulation with αGalCer results in significantly lower IL-4 production (Nieuwenhuis, Neurath et al. 2002). IFNγ production in αGalCer treated cells or whole EBI3 -/- animals is initially slightly below that seen in control animals, but the levels are eventually indistinguishable between the two strains of mice (Nieuwenhuis, Neurath et al. 2002). Thus, although IL-27 has an apparent role in promoting the Th1 immune response by synergizing with IL-12, it potentially contributes to the generation of NKT cells and thus may also contribute to the generation of type 2 immunity.

5.2.3 - IL-27 receptor

The receptor for IL-27 has not yet been completely identified. However, it is known that IL-27 signals through the orphan receptor previously identified as WSX-1 or TCCR, identified on the basis of its high degree of homology with the IL-12R subunits (Sprecher, Grant et al. 1998; Chen, Ghilardi et al. 2000). While WSX-1 represents the signalling subunit of the receptor, it alone does not confer IL-27 binding (Takeda, Hamano et al. 2003). Signalling through this receptor facilitates STAT1

activation which in turn mediates T-bet activation (Takeda, Hamano et al. 2003). As T-bet causes the upregulation of IL-12Rβ2 which is commonly associated with Th1 differentiation, it has been proposed the IL-27 actually acts on naive T cells to induce IL-12 responsiveness, necessary for subsequent differentiation into a Th1 effector cell (Takeda, Hamano et al. 2003). This may provide an explanation for the observation that T-bet activation precedes the upregulation of the IL-12Rβ2 (Mullen, High et al. 2001). These data suggests that IL-12 may not be the critical mediator of T bet induction it was previously thought to be.

5.3 - Summary

Taken together the identification of IL-23 and IL-27 have suggested potential additional steps in the development of Th1 effector cells and thus type 1 immunity. In the present model, IL-27 produced by a professional, antigen presenting and activated APC signals a naive T cell through the IL-27R (consisting of WSX-1 and at least one other, as yet unidentified component). In response to this signal, the T cell upregulates STAT1, T-bet and then IL-12Rβ2 allowing the T cell to respond to APC produced IL-12. Exposure of the T cell to IL-12 at this intermediate stage of differentiation leads to upregulation of the IL-18R subunits and subsequent responsiveness to APC produced IL-18. In turn, this leads to the activation of STAT4, AP-1 and IRAK pathways and promotes T cell IFNγ production. As the T cell reaches the final stages of its maturation, the expression of IL-12Rβ2 is eventually downregulated and replaced with the IL-23R, which maintains STAT3 and STAT4 activation and facilitates extended IFNγ production, promoting the maintenance of the Th1 response.

6.0 - Treatment of allergic diseases

6.1 - Pharmacotherapy

Management of allergic disease focuses primarily on preventing allergen exposure through allergen avoidance or providing symptomatic relief. Although allergen avoidance can be accomplished in certain circumstances, avoidance of allergens like grass pollen or house dust mite allergens cannot easily be accomplished due to seasonal environmental release, or the ubiquitous nature of these allergens. Where avoidance is impractical, pharmacological control of disease symptoms is often relied upon. For asthmatic patients, inhaled corticosteroids (ICS; eg Beclomethasone, Budesonide) are the mainstay of treatment. The mechanism of action of ICS therapy is not fully understood, but they are potent anti-inflammatory drugs which act at numerous levels to limit induction of inflammatory cytokines, adhesion molecules, and enzymes normally seen following allergen exposure and mast cell activation (reviewed in Barnes 2001). Importantly, long term use of ICS is relatively safe provided it is used at the lowest dose required to effect symptom relief. In more severe cases of asthma, or in instances where there is not suitable control of symptoms, additional drugs are "added on" to the existing ICS therapy. These include β_2 agonists that bind to the β_2 adrenergic receptors and trigger bronchodilation. β_2 agonists are used as both daily medications to control asthma symptoms (long-acting β_2 agonists; eg salmeterol, formoterol) or as short term rescue medication taken at the time of an exacerbation to rapidly control symptoms and restore breathing (short acting β_2 agonists; eg albuterol, terbutaline). Leukotriene modifiers (eg montelukast,

zafirlukast) are also used as an add-on therapy, but overall, these appear to be less effective than long-acting β_2 agonists for controlling asthma. Finally, anti-histamines (eg loratadine, fexofenadine) have been used to treat all sorts of allergic diseases for many years (Simons and Simons 1994) and function primarily to block H1-receptor mediated signaling, thereby reducing the ability of mast cell degranulation leading to allergic inflammation.

The range of powerful drugs in use today, either alone, or in combination, supply adequate relief from symptoms for the allergic patient, but it is important to understand that these interventions do nothing to cure the underlying cause of the allergic disease: the type 2 dominated immune response to environmental allergens. Cessation of drug therapy is associated with a return of inflammation and, thus, a return of symptoms.

There is presently a great deal of uncertainty as to whether long-term use of drugs as a treatment for asthma is able to reverse pre-existing damage done by chronic inflammation of the airway. However, there is a growing sentiment that early treatment of relatively minor symptoms and good control of mild disease can prevent the subsequent progression to more severe, life-threatening forms of disease.

6.2 - Allergen immunotherapy

Drugs are capable of controlling the symptoms and improving overall patient quality of life, but there remain very few treatment options that address the underlying immune response causing the allergic

disorder. Allergen immunotherapy attempts to clinically tolerize an individual through exposure to increasingly large quantities of allergen, and when successful, results in increased type 1 character of immune cells in the nasal mucosa and skin (Varney, Hamid et al. 1993; Durham, Ying et al. 1996; Wachholz, Nouri-Aria et al. 2002), and a decrease in immediate skin prick test reactivity and allergen specific IgE levels (Durham, Kay et al. 1995; Pichler, Helbling et al. 2001; Bodtger, Poulsen et al. 2002). These may result in the decreased symptom scores, medication use and increase in overall quality of life observed following immunotherapy (Durham, Walker et al. 1999; Pichler, Helbling et al. 2001; Walker, Pajno et al. 2001; Bodtger, Poulsen et al. 2002). While some also report a shift towards type 1 dominance in PBMC populations (Ebner, Siemann et al. 1997; Benjaponpitak, Oro et al. 1999), this is not a universal finding (Moverare, Elfman et al. 2000; Wachholz, Nouri-Aria et al. 2002). In addition to the alterations seen in the balance of type 1 and type 2 immunity at the mucosal surfaces, there is a concomitant increase in allergen specific serum IgG_4 levels (Ewbank, Murray et al. 2003; Fellrath, Kettner et al. 2003) which many have suggested represent an increase in "blocking antibodies" which are ultimately the cause of improved clinical status. Yet others argue that an observed increase in $CD4^+CD25^+$, IL-10 producing regulatory T cells are responsible for the clinical benefit of allergen immunotherapy (Akdis, Blesken et al. 1998; Akdis and Blaser 1999; Francis, Till et al. 2003; Jutel, Akdis et al. 2003).

While specific allergen immunotherapy remains the only "curative" therapy which attempts to address the underlying cause of allergic disease, it has many shortcomings. For example, it does not work in all patients or for all allergens, requires numerous injections over the course of years, and has an inherent risk of triggering potentially fatal anaphylactic reactions, particularly in patients with

asthma. Most importantly, despite observed beneficial changes in numerous immunological parameters, the underlying mechanism remains a mystery.

7.0 - Murine model of immediate hypersensitivity

In this thesis, we utilize a common murine model of immediate hypersensitivity. In this model, exogenous protein antigen (in this case ovalbumin (OVA)) is adsorbed to aluminum hydroxide adjuvant (alum) and administered intraperitoneally to mice. The ensuing immune response to OVA is characterized by high levels of OVA-specific IgE and low levels of OVA-specific IgG_{2a} in the serum. This type 2 dominated immune response is also seen in OVA-restimulated cultures of spleen cells from OVA-alum immunized animals with production of high levels of IL-4, IL-5 and IL-13 and low levels of IFNγ.

Furthermore, it has been found that this type 2 dominated immune response to OVA can be both prevented and abrogated by treatment of mice with glutaraldehyde polymerized OVA (OA-POL). Treatment of mice with OA-POL, either before (HayGlass, Gieni et al. 1991; Gieni, Yang et al. 1993) or following (HayGlass and Stefura 1990; HayGlass and Stefura 1991) immunization with OVA (alum) results in high levels of OVA-specific IgG_{2a}/IgG_{2c} and dramatically lower levels of IgE (< 5% of the levels typically seen in immunized but untreated mice). This shift in the balance of type 1 versus type 2 antibody production is mirrored by a shift in the balance of type 1 versus type 2 cytokine production following OVA restimulation of spleen cells *in vitro* (Yang, Gieni et al. 1993). Furthermore, the type 1 dominated immune response is stable in the face of repeated boosts with

OVA (alum) (HayGlass, Gieni et al. 1991; Gieni, Yang et al. 1993), dependent on $CD4^+$ T cells (HayGlass and Strejan 1983) and OVA specific as immune responses to keyhole limpet hemocyanin (KLH) are unaffected by OA-POL (HayGlass and Stefura 1991). Thus we have the ability to examine the endogenous control of type 2 immune responses in both type 1 and type 2 dominated OVA-specific immune responses.

8.0 - Summary of project

This work was initiated as a result of studies from our lab, and others, reporting that PBMC from non-allergic individuals demonstrated an allergen specific proliferative response equal to that seen in allergic individuals, but that the cytokine profile produced by non-allergic individuals was dramatically shifted towards a type 1, IFNγ dominated profile (Li, Simons et al. 1996). This was contrary to the dogma of the time, which stated that non-allergic individuals were unable to respond to allergen, and thus were protected by means of immunological ignorance. The demonstration of allergen specific, type 1 dominated immune responses in non-atopic individual suggested that there are endogenous factors which could prevent the development of a type 2 dominated allergic response and promote the development of a protective type 1 dominated immune response to allergens. The aim of this thesis is to identify the *endogenous* factors which are important in limiting the induction of allergic responses.

8.1 - Hypothesis 1: Endogenously produced IFNγ is a negative regulator of type 2 immunity

IFNγ is a critical effector molecule in type 1 dominated immune responses and inhibits the proliferation of Th2 clones and IgE synthesis *in vitro*. Remarkably, despite numerous studies examining the potential of recombinant IFNγ to modulate type 2 immunity, the role of endogenous IFNγ in limiting induction of type 2 immunity has not been throughly explored. In light of its demonstrated ability to actively suppress type 2 activity, we hypothesized that IFNγ would be a critical negative regulator of type 2 immunity. To examine this, we made use of IFNγ -/- mice, treated under both type 1 and type 2 inducing conditions and examined subsequent Ab production and the production of a *panel* of type 2 cytokines. We examined the role of IFNγ in inhibiting both developing *and* established type 2 immune responses.

8.2 - Hypothesis 2: Endogenously produced IL-12 inhibits type 2 immunity

IL-12 is the quintessential cytokine involved in promoting the development of Th1 cells, and thus, largely by extension, was assumed to simultaneously inhibit the development of type 2 immune responses *in vivo* (O'Garra 1998). While the administration of exogenous IL-12 yields transient inhibition of type 2 immune responses, the role of endogenously produced IL-12 in limiting the induction and severity of allergic responses has not been throughly explored. We hypothesized that endogenous IL-12 actively inhibited the development of type 2 immunity. To test this, we made use of IL-12 -/- mice immunized under both Th1 and Th2 inducing conditions and assessed the balance of type 1 versus type 2 immunity by examining T cell cytokine production, *in vivo* Ab production, and IL-12 responsiveness. We examined the importance of IL-12 in regulating both *de novo* and pre-existing type 2 immune responses.

8.3 - Hypothesis 3: IL-18 is a negative regulator of the type 2 immune response

IL-18 was originally identified as an important co-factor in induction of optimal IFNγ production, and type 1 immunity. More recently however, the ability of IL-18 to promote type 2 cytokine production and support Th2 cell differentiation is widely reported. The circumstances under which IL-18 can actively promote or inhibit type 2 immunity are not fully understood. We hypothesized that the principal role of IL-18 is as an inhibitor of the type 2 immune response. To explore this hypothesis, we examined endogenous IL-18 production, particularly in the absence of endogenous IL-12, and subsequently determined the capacity of increased IL-18 levels to limit the intensity of type 2 cytokine production.

8.4 - Hypothesis 4: OA-POL does not require B cells to prevent induction of type 2 cytokine production

OA-POL, a model chemically modified allergen, is approximately 800 times the molecular weight of native OVA, and antibodies raised against OVA have a markedly lower affinity for OA-POL (HayGlass and Strejan 1983). Due to this low affinity, B cells should have a reduced capacity to phagocytose OA-POL. Thus we hypothesized that B cells were not required for inducing the stable type 1 dominated immune response seen following pre-treatment with OA-POL. To test this hypothesis, B cell deficient mice (μMT) were treated with nothing or OA-POL prior to immunization

with OVA (alum) and the capacity of OA-POL to inhibit type 2 cytokine was compared to that seen in C57Bl/6 controls.

8.5 - Hypothesis 5: OA-POL increases T cell responsiveness to IL-12/IL-18

Redirection of a type 2 dominated immune response that is typically associated with an allergic phenotype, to a balanced or type 1 dominated profile more associated with clinical tolerance would be of great utility in the treatment of allergic disease. While such a shift has been observed in patients who respond successfully to allergen immunotherapy, the mechanism behind such a shift is largely unknown. We hypothesized that immune redirection resulted from increased responsiveness to endogenously produced IL-12 and IL-18, leading to increased IFNγ production. To examine this hypothesis we made use of OA-POL in a murine model of immune redirection and examined both biological responsiveness to, and expression of the receptors for IL-12 and IL-18 in OA-POL treated versus untreated animals.

8.6 - Global objectives

The cause of allergic disease is increasingly well understood (an excessive, inappropriate Th2 dominated immune response to environmental allergens), yet the endogenous factors which are responsible for *preventing* the induction and maintenance of allergic disease are unclear. In summary, the overall objective of this thesis is to directly examine if endogenous IL-12, IFNγ and IL-18 have the capacity to limit the induction and intensity of type 2 immune responses.

Identification of critical negative regulators of type 2 immunity would provide potentially useful therapeutic targets for the treatment of allergic disease.

III - Materials and Methods

1.0 - Mice

C57Bl/6 mice were bred at the University of Manitoba animal breeding facility (Winnipeg, MB) or purchased from Charles River Canada (St. Constant, PQ). IL-12 p35 -/-, IL-12 p40 -/-, IFNγ -/- and μMT mice (B cell deficient) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Manitoba breeding facility (Winnipeg, MB). All mice were used in accordance with guidelines set forth by the Canadian Council on Animal Care.

2.0 - Preparation of reagents for *in vivo* use

2.1 - Aluminum hydroxide (alum) adjuvant

To prepare alum for immunizations, 2 drops of 0.2% phenol red was added to 20 ml of 10 % Aluminum Potassium Sulfate ($AlK(SO_4)_2$). To this was added 0.5N NaCl until the solution began to turn pink (~20 mls). This solution was allowed to sit for 10 min, and then centrifuged at 170g for 3 minutes. The supernatant was discarded and the precipitate was resuspended in 40 ml of 0.15N NaCl. This procedure was repeated 2 times and the pellet was resuspended in 40 ml of 0.15 N NaCl. This solution consisted of $Al(OH)_3$ at 10-12 mg/ml.

To prepare antigen for immunization, antigen was mixed with the $Al(OH)_3$ solution and allowed to adsorb for 10 minutes at room temperature. To this solution was added 2 volumes of Hank's balanced salt solution (Flow Laboratories, McLean VA) to neutralize the pH of the solution. 0.5 ml of this solution containing 0.2 or 2.0 μg of OVA (five times recrystallized, ICN Biochemicals, Montreal, PQ) and 2.0 mg of $Al(OH)_3$ was administered intra-peritoneally (i.p.) to mice.

2.2 - CFA/HKL

Complete Freund's Adjvant (CFA) was prepared by mixing mineral oil containing *Mycobacterium tuberculosis* strain H37 Ra (Difco, Detroit MI) with an equal volume of PBS containing OVA at a concentration of 666.6 μg/ml. This mixture was emulsified and 0.3 ml (containing 100 μg OVA) was injected i.p. to immunize animals.

For immunization of mice with adjuvants containing heat killed *Listeria monocytogenes* (HKL), one volume of mineral oil (Incomplete Freund's Adjuvant (Difco) was emulsified with an equal volume of PBS containing OVA at a concentration of 666.6 μg/ml and HKL (a gift of Dr. M. Stevenson, McGill University, Montreal, PQ) at a concentration of 3.3×10^7 bacteria/ml. 0.3 ml of this mixture containing 100 μg of OVA and 1.0×10^7 heat killed *Listeria monocytogenes* was injected i.p.

2.3 - OA-POL preparation

Polymerized OVA (OA-POL) was prepared by adding, dropwise over 10 minutes, a 0.15 N NaCl solution containing 6% glutaraldehyde (Sigma Chemical Company, Oakville, ON) to a 25 mg/ml solution of OVA dissolved in a 0.1 M Acetate buffer at pH 5.5 to achieve a final molar ratio (glutaraldehyde to OVA) of 200:1. This mixture was stirred at room temperature for twenty minutes and allowed to sit for an additional 4.5 hours at room temperature. After dialysis and gel filtration on a Biogel A-50m column [2.5 x 90 cm bed size, effective separation range 1.0×10^5 to 5.0×10^7 Da, in borate buffered saline, pH 8.3], OA-POL was collected as a single, sharp, symmetric peak with an relative molecular mass of 3.5×10^7. The concentration of OA-POL in solution was determined by Lowry assay. This approach to allergen polymerization results in a highly homogeneous product unlike previous techniques which produced mixtures of varied molecular weight, with varied immunological effects (Patterson, Suszko et al. 1973; Johansson, Miller et al. 1974; Attallah, Kuroume et al. 1975; Foucard and Johansson 1976; HayGlass and Strejan 1984)

2.4 - *Trichinella spiralis* extract preparation

Trichinella spiralis extract was prepared by Dr. T. Dick (University of Manitoba). The extract was prepared by homogenizing L1 muscle larvae and centrifuging the homogenate at 100,000 g for 1 hour. The supernatant was sterile filtered and this was termed *T. spiralis* extract. A *Trichinella* specific immune response was generated by injection of 50μg of this extract alone (for cytokine responses), or in the presence of alum (Ab responses).

3.0 - Animal Handling

3.1 - Immunizations

Immunizations were administered i.p. on day 0 and consisted of 2 μg OVA (alum) in 0.5 ml or 100 μg OVA (CFA or HKL) in 0.3 ml to generate type 2 or type 1 dominated immune responses respectively. Mice were boosted i.p. on day 28 with 100 μg of OVA in saline or with 2 μg or OVA in alum. In OA-POL abrogation studies, mice were given 0.2 μg OVA (alum), the lowest dose of OVA required to consistently generate OVA-specific IgE responses. Mice were also administered 100 μg OVA or OA-POL in saline on days 0, 2, and 4 to stimulate *in vitro* cytokine production in the absence of adjuvant. In some experiments, mice were administered 1.0 μg of LPS (from *E. coli* 0111-B4, Sigma Chemical Company) i.p.

3.2 - OA-POL treatment

To prevent *de novo* IgE synthesis, mice were treated with 100 μg OA-POL in saline on days -14, -12 and -10 prior to immunization with 2.0 μg OVA (alum) on day 0. To abrogate existing OVA-specific IgE production, mice were first immunized with 0.2 μg OVA (alum) followed by treatment with three courses of OA-POL treatment during weeks 3, 5 and 7. One "course" of OA-POL treatment consists of three injections of 100 μg OA-POL in saline given over five days. Two weeks following the final OA-POL injection, mice were boosted with 0.2 μg OVA (alum).

3.3 - Bleeding

Mice were bled from the tail prior to immunization to determine baseline total Ig levels. Mice were bled on days 10 and 14 after immunization, times which correlate with peak primary IgE and IgG responses respectively. 7 and 14 days after booster injections, mice were bled for analysis of all Ig isotypes routinely examined. After administration of LPS, mice were bled between 4 hours and 5 days later for assessment of serum IL-18 levels. Blood was stored at 4°C overnight and centrifuged the following day to harvest serum. Sera were stored at -70°C until needed and analysed individually.

3.4 - Anti-NK1.1 treatment

An anti-murine-NK1.1 monoclonal Ab producing cell line, PK136 was obtained from ATCC (Rockville, MD) and grown in UltraDOMA protein free (UPF) media (BioWhittaker, Walkersville, MD). Tissue culture supernatants were collected and concentrated using a Centriprep Centrifugal Filter Unit (Millipore, Nepean ON). To deplete NK cells *in vivo*, mice were given 0.3 mg of anti-NK1.1 (clone PK136) on two consecutive days immediately prior to sacrifice. This dosage of anti-NK1.1 is sufficient to deplete NK cells *in vivo* ((Wang, Ellison et al. 1998), and data not shown).

4.0 - Assessment of Ig production

4.1 - OVA-specific IgE

OVA-specific IgE levels were determined by passive cutaneous anaphylaxis (PCA). Briefly, female Sprague-Dawley rats (bred and raised at the University of Manitoba breeding facility) were injected intradermally with 0.1 ml of a series of two-fold dilutions of the mouse serum to be assayed. After 48 hours, rats were challenged intra-venously (i.v.) with 1 ml of a 0.15 N NaCl solution containing 2.4 mg OVA and 0.6% Evan's blue dye. The reciprocal of the highest dilution of serum which gives a positive reaction (blue spot > 3mm in diameter) was the final PCA titer. Geometric means of duplicate analysis, not differing by more than one two-fold dilution, are presented.

4.2 - OVA-specific IgG_1/IgG_{2c}

OVA-specific IgG levels were determined by an enzyme linked immunosorbent assay (ELISA). ELISA plates (Corning Life Sciences Products, Corning NY) were coated with OVA at 20 μg/ml in carbonate buffer (0.05 M, pH 9.6) overnight at 4°C. Plates were blocked for 45 minutes at 37°C with blocking buffer (1% BSA, 0.05% Tween 20, 0.02% NaN_3 in PBS, pH 7.4). Between all incubations, plates were washed with wash buffer (0.05% Tween 20, 0.02% NaN_3, PBS pH 7.4) using a SkanWasher 300 Version B (Skatron Instruments, Sterling, VA). Eight 2 fold dilutions of serum samples were made down the plate in dilution buffer (0.085% BSA, 0.05% Tween 20, 0.02% NaN_3, PBS pH 7.4). The starting dilutions of samples ranged from 1/50 to 1/10,000. Standards consisting of serial dilutions of pooled immune sera of known OVA-specific IgG_1 and IgG_{2a} titres were included on all plates and plates were incubated at 4°C overnight. Biotinylated goat anti-mouse IgG_1 (25 ng/ml) (Southern Biotechnology Associates, Birmingham, AL) or biotinylated goat anti-mouse IgG_{2c} (60 ng/ml) (Southern Biotechnology Associates) in dilution buffer were added for

detection of IgG_1 and IgG_{2c} respectively. Plates were incubated at 4°C overnight. The following day, plates were incubated with a streptavidin-alkaline phosphatase conjugate (Jackson ImmunoResearch, Mississauga, ON) in dilution buffer at 37°C for 45 minutes. ELISAs were concluded by adding p-nitrophenol phosphate (Sigma Chemical Company) in substrate buffer (50 mM $MgCl_2$, 1 M DEA in H_20, pH 9.8). The reaction was allowed to proceed for 30 minutes and the $O.D._{405-690}$ was measured using a Spectra Max 190 ELISA plate reader (Molecular Devices Corporation, Sunnyvale, CA). The dilution of sample at this time which generated an $O.D._{405-695}$ value of 0.5 was considered the end point. The titers of standards were normalized to previously determined values (2,800,000 for IgG_1 and 180,000 for IgG_{2c}) from a standard run on all plates. The same multiplicative factor required to generate the appropriate titre on the standard was also applied to the samples.

4.3 - Isotype specific total Ig assays

Total levels of serum IgG_1, IgG_{2c} and IgE were independently determined by ELISA. All ELISAs were carried out essentially as above. For IgG_1 and IgG_{2c}, plates were coated with sheep anti-mouse IgG (H+L) (Jackson Immuno Research) at 2 μg/ml. The secondary antibody was biotinylated goat anti-mouse IgG_1 of IgG_{2c} (Southern Biotechnology Associates) used at 25 ng/ml or 60 ng/ml for IgG_1 and IgG_{2c} assay respectively. The standard was a purified mouse IgG_1 mAb generated by Dr. G. Lang (University of Manitoba) or a purified mouse IgG_{2c} mAb (BD Pharmingen, Mississauga, ON) for IgG_1 and IgG_{2c} assay respectively. Total IgE levels were determined by coating plates with rat anti-mouse IgE (Southern Biotechnology Associates) at 1 μg/ml. Biotinylated rat anti-mouse IgE

(Serotec, Raleigh, NC) was used at 125 ng/ml as a secondary Ab. The standard used was highly purified anti-DNP mouse IgE, purified from B cell hybridoma 2682 (a gift of Dr. A. Froese, University of Manitoba). The sensitivity of each assay was 0.5 ng/ml for IgG_1, 0.1 ng/ml for IgG_{2c}, and 0.5 ng/ml for IgE.

5.0 - *In vitro* cultures

5.1 - Preparation of spleen cells

Mice were sacrificed by cervical dislocation on day 5 after immunization with OVA (alum) or OVA (CFA/HKL). Alternatively, mice were sacrificed on day 8 after immunization with OVA (saline) or OA-POL (saline) and following treatment on day 6 and 7 with nothing, or anti-NK1.1. Spleens were removed aseptically. Single cell suspensions were prepared in 5% normal calf serum (Armour Pharmaceutical Company, Kankakee, IL) in RPMI 1640 (Invitrogen Corporation, Grand Island, NY) using 7ml Tenbroeck tissue grinders (Wheaton Science Products, Millville, NJ). Spleen cells were then passed through nytex lined funnels (B & SH Thompson & Company Limited, Scarborough, ON) to remove debris and centrifuged at 300 g for 4 minutes. Cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Intergen Company, Purchase, NY). Cell number was determined by counting viable cells in a hemocytometer after dilution in 0.4% trypan blue and 40% acetic acid.

5.2 - Bulk culture of spleen cells

Single cell suspension of homogenized spleens were cultured at a concentration of 7.5 x 10^6 cells/ml RPMI 1640 supplemented with 10% FBS, 300 μg/ml L-glutamine (Invitrogen), 55 μM 2-mercaptoethanoal (Invitrogen), and 1/100 dilution of an antibiotic-antimycotic cocktail (containing 10,000 U/ml penicillin G, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B (Invitrogen)). Cells were cultured in 48 well plates treated for tissue culture (Costar Corporation, Cambridge, MA) or 96 well Microtest III plates (Becton Dickinson, Lincoln Park, NJ) in volumes of 1 ml or 0.2 ml respectively. Cells were stimulated medium alone, OVA (0.03 - 1.0 mg/ml), Concanavalin A (10 μg/ml) (Pharmacia, Upsalla, Sweden), LPS (50 - 500 ng/ml), anti-IgM $F(Ab')_2$ (0.01 - 1.0 mg/ml) (Jackson Immuno Research), *Trichinella spiralis* extract (20 - 100 μg/ml), rmIL-2 (10 U/ml) (Chiron Corporation, Emeryville CA), rmIL-12 (0.1 - 100 ng/ml) (a gift of Dr. M. Gately, Hoffman-LaRoche, Nutley, NJ), rmIL-18 (0.1 - 50 ng/ml) (R&D Systems, Minneapolis MN). To determine if cytokine production was CD4 dependent, an anti-CD4 cocktail (consisting of supernatants from cultures of hybridomas YTS 191.1 and GK1.5 (ATCC)) was added. Tissue culture supernatant was harvested at 24 hours for analysis of IL-2, IL-4 and TARC, 48 hours for analysis of IFNγ and IL-12 p40, 72 hours for analysis of IL-5, IL-13 and IP-10, and at 96 hours for analysis of IL-10.

5.3 - Proliferation assays

Whole spleen cell proliferation was assessed after OVA restimulation for 2 to 6 days. For B cell proliferation, whole spleen cell populations were stimulated with LPS at 500 or 50 ng/ml (Sigma) or anti-mouse IgM $F(ab')_2$ (Jackson ImmunoResearch, Mississauga, ON) at 10 or 1 μg/ml for 3 days.

Cultures were pulsed with 1μ Ci of [^{3}H]thymidine in the culture for the last 24 hours. Thymidine incorporation was measured using a Wallac 1450 MicroBeta" Trilux (EG&G Wallac, Turku, FI).

5.4 - Flow Cytometry

Samples run on a FACScan" flow cytometer (Beckton-Dickinson Immunocytometry Systems, Mountain View, CA) were analysed using CellQuest Software (Beckton-Dickinson Immunocytometry Systems). To determine percentage of mature versus immature B cells, cells were stained with anti-CD19-APC, anti-IgD-FITC, anti-CD23-PE; isotype controls were conjugated with APC, PE or FITC and possessed irrelevant specificities (BD Pharmingen). CD19+ cells (B cells) were then examined for expression of CD23 and IgD to determine maturational status. For detection of NK cells, cells were stained with PE-labelled rat anti-mouse NK1.1 (clones PK136) or PE-labelled rat anti-mouse CD49b (clone DX5) (BD Pharmingen) both methods yielded similar results. The primary gate included lymphocytes as determined by forward versus side scatter.

5.5 - Cell Sorting

To enrich for activated T cells, spleen cells from OVA or OA-POL immunized mice were cultured in the presence of 1.0 mg/ml of OVA for 48 hours and subsequently stained with PE-Cy5 labelled, rat anti-mouse CD4 (BD Pharmingen) and FITC labelled, hamster anti-mouse CD69 (BD Pharmingen). Isotype controls were conjugated with FITC or PE-Cy5 and had irrelevant specificities. Samples were run on an EPICS Altra High Speed FACS (Beckman Coulter, Mississauga, ON) controlled by

EXPO 32 Multi Comp Software (Beckman Coulter). The primary gate was set to include lymphocytes based on forward versus side scatter.

6.0 -Cytokine determination

Murine cytokine production was assessed by ELISA. The following Ab pairs were used for coat/ detection (biotinylated): IL-2 - clone JES6-5H4/clone JES6-1A12 (BD Pharmingen), IL-4 - clone 11B1/clone BVD6-24G2 (BD Pharmingen), IL-5 - clone TRFK 4/clone TRFK 5 (BD Pharmingen), IL-10 - clone SXC1/clone SXC2 (lab purified); IL-12 p40 - clone C15.6/clone C17.8 (BD Pharmingen), IL-13 - clone 38213.11/ polyclonal goat anti-mouse IL-13 (R&D Systems), IL-18 - clone 51817.111/polyclonal goat anti-mouse IL-18 (R&D systems), IFNγ clone XMG1.2/clone R4.6A2 (lab purified), TARC - clone 110904/polyclonal goat anti-mouse TARC (R&D Systems), IP-10 - polyclonal rabbit anti-mouse IP-10 (PeproTech Rocky Hill, NJ) /polyclonal goat anti-mouse IP-10 (R&D Systems). Detection limits were 4 pg/ml for IL-2, 2.4 U/ml for IL-4, 10 pg/ml for IL-5, 0.3 U/ml for IL-10, 16 pg/ml for IL-12 p40, 75 pg/ml for IL-13, 7.8 pg/ml for IL-18, 0.15 U/ml for IFNγ, 3.9 pg/ml for TARC, and 7.8 pg/ml for IP-10.

7.0 - Cytokine receptor mRNA quantification

7.1 - mRNA purification

Cells for analysis of mRNA levels were obtained directly from single cell suspensions (7.5×10^6) at time of sacrifice or following resuspension of 1 ml cultures (initially seeded with 7.5×10^6 cells) after 48 hours of *in vitro* culture. Cell samples were lysed with RLT buffer (Qiagen, Valencia, CA) and stored at -70°C until needed. Samples were thawed and mRNA was purified using an RNEasy mini kit according to manufacturer's instructions (Qiagen).

7.2 - cDNA reaction conditions

mRNA samples were used to make cDNA under the following reaction conditions:

IL-12Rβ2 - 20 µl final volume containing 500ng Oligo dT (Invitrogen), 0.5 mM mixture of all dNTPs (invitrogen), 10 mM DTT (Invitrogen), 40 U RNAse out recombinant RNAse inhibitor (Invitrogen), 200 U of Superscript II RNase H Reverse Transcriptase (Invitrogen) and ~ 500 ng of purified mRNA.

IL-18Rα/IL-18Rβ - 20 µl final volume containing 100 ng of random hexamers (Invitrogen), 0.5 mM mixture of all dNTPs (invitrogen), 10 mM DTT (Invitrogen), 40 U RNAse out recombinant RNAse inhibitor (Invitrogen), 200 U of Superscript II RNase H Reverse Transcriptase (Invitrogen) and ~ 500 ng of purified mRNA. These reactions were incubated at 42°C for 2 hours, and the reaction was stopped by heating to 70°C for 15 minutes.

7.3 - PCR reactions

7.3.1 - IL-12Rβ2

Real-time PCR was carried out using a LightCycler (Roche, Laval, P.Q., Canada). Reactions were set up in microcapillary tubes at following final concentrations: 0.4 μM of each IL-12Rβ2 primer (5′ - AAT TCA GTA CCG ACG CTC TCA; 3′ - ATC AGG GGC TCA GGC TCT TCA (Invitrogen)), 4 mM $MgCl_2$, 1× SYBR Green master mix (Roche) and 2 μl of cDNA. Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantitation, 35 cycles of : 95°C for 15 s, 57°C for 5 s, 72°C for 32 s and then 84°C for 5 s, with a single fluorescence measurement at the end of the 84°C segment. Melting curve analysis was performed on PCR products (60-95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement). The expected size of the PCR product was 399 bases.

7.3.2 - IL-18Rα

PCR Reactions were set up in microcapillary tubes largely as above (differences from IL-12Rβ2 protocol bolded): **1 μM** each of IL-18Rα primer **(5' - GTG CAC AGG AAT GAA ACA GC; 3' - ATT TAA GGT CCA ATT GCG ACG A** (Invitrogen)), 4 mM $MgCl_2$, 1× SYBR Green master mix (Roche) and **1 μl** of cDNA. Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantitation repeated **45** times (95°C for 15 s, **61°C for 5 s**, 72°C for 18 s with a single fluorescence measurement at the end of the **72°C segment**). The expected size of the PCR product was **195 bases**.

7.3.3 - IL-18Rβ

PCR Reactions were set up in microcapillary tubes largely as above (differences from IL-12Rβ2 protocol bolded): **1 μM** each of IL-18Rβ primer (**5' - GGA GTG GGA AAT GTC AGT AT; 3' - CCG TGC CGA GAA GGA TGT AT** (Invitrogen)), **3 mM** $MgCl_2$, 1× SYBR Green master mix (Roche) and 1 μl of cDNA. Cycling conditions were as follows: denaturation (95°C for 10 min), amplification and quantitation repeated 45 times (95°C for 15 s, **63°C for 5 s, 72°C for 18 s** with a single fluorescence measurement at the end of the **72°C segment**). The expected size of the PCR product was **215 bases**.

7.4 - PCR standards and controls

PCR standards were generated by culturing spleen cells from OVA (alum) immunized with anti-CD3 at 62.5 ng/ml and IL-12 at 50 ng/ml. After 48 hours, cells were harvested, lysed and mRNA was purified using an RNeasy Midi Kit according to manufacturer's instructions (Qiagen). These mRNA samples were aliquoted and stored at -70°C. To create standards, some of these mRNA aliquots were used to create cDNA and run on Lightcycler. The concentration assigned to each of these standards, in arbitrary units, represents the reciprocal of the highest dilution which consistently gave a positive PCR result. For each PCR reaction, standard cDNA was made under identical reaction conditions, at the same time as the samples. A series of 7 four-fold dilutions of this cDNA reaction was included in the PCR run to allow relative quantification of mRNA present in the samples. Negative controls

consisting of the normal PCR reaction mix minus cDNA were included in every run and consistently came up negative.

8.0 - Statistical analysis

For each ELISA assay, data were obtained from a titration of at least four dilutions which were compared to the standard curve run on each plate, and reported as mean + SEM. OVA-specific IgE data were $\log_2$ transformed and are reported as geometric means. Comparison between means was typically done using a standard, two-tailed Student's t test, carried out in Microsoft Excel 97. Comparison of means between multiple groups was carried out by ANOVA followed by Tukey's test, also carried out in Microsoft Excel 97. Differences were considered statistically significant when $p < 0.05$.

IV - Results

1.0 - Endogenous IL-12 as a negative regulator of type 2 immunity

IL-12 is the critical factor inducing differentiation of naive T cells into Th1 effector cells, and a potent inducer of IFNγ. Previous studies also ascribe to IL-12, a powerful inhibitory activity on type 2 immune responses. These studies lack comprehensive examination of parameters associated with a type 2 immunity, frequently relying on the measurement of a single type 2 cytokine. Here, we examine the consequence of chronic absence of endogenous IL-12 production on the overall intensity of the generation of type 2 immune responses by measuring a panel of markers for type 2 immunity including serum IgE levels, and *in vitro* type 2 cytokine production, following a variety of different immunization protocols.

1.1 - Type 2 cytokine production is unaltered in OVA (alum) immunized IL-12 -/- mice

To evaluate the impact of IL-12 deficiency on the intensity of type 2 cytokine production during a type 2 dominated immune response, C57Bl/6 and IL-12 -/- mice were immunized with OVA (alum). As shown in Figure 1, cultured spleen cells from both IL-12 p35 -/- and IL-12 p40 -/- mice produced dramatically less IFNγ (~90% reduction) following OVA restimulation *in vitro* compared to identically stimulated C57Bl/6 spleen cells. This residual level of IL-12 independent IFNγ production was consistently seen in both strains of IL-12 deficient mice. The magnitude of OVA-driven IL-4 production, however, was identical in cultures of IL-12 deficient and C57Bl/6 spleen

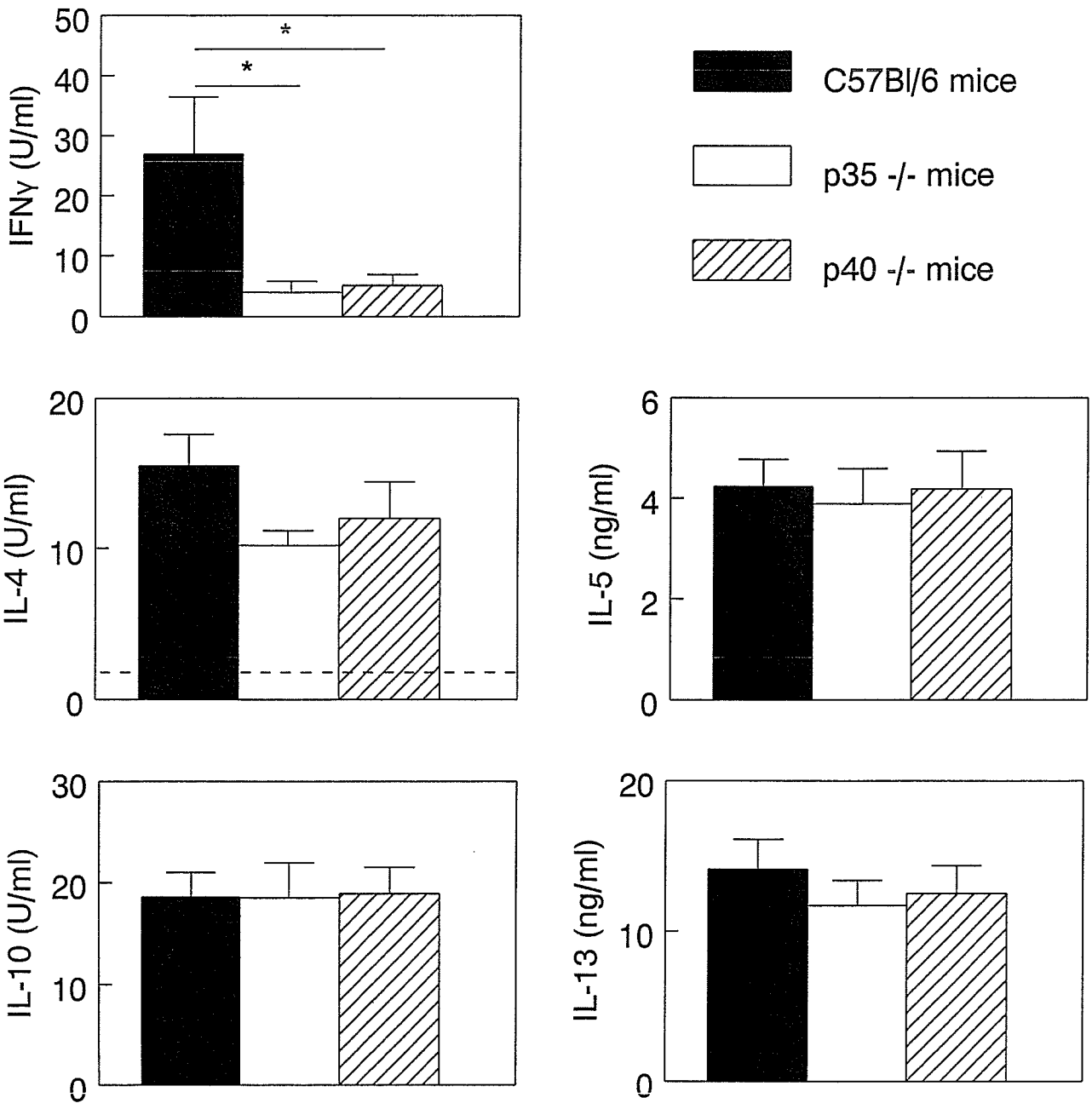

Figure 1: Type 2 cytokine production is identical in OVA (alum) immunized C57Bl/6 and IL-12 -/- mice. Mice were immunized with 2.0 μg OVA (alum) on day 0. Mice were sacrificed on day 5 and spleen cells restimulated with 1.0 mg/ml OVA. Tissue culture supernatants were harvested at 24 (IL-4), 48 (IFNγ), 72 (IL-5, IL-13) and 96 (IL-10) hours. Dotted line indicates detection limit. Mean + SEM shown (n = 24 mice from 6 experiments). Significant differences between C57Bl/6 and knockout strains indicated (ANOVA and subsequent Tukey's test; * $p < 0.001$).

cells (Figure 1). Similar levels of IL-5, IL-10 and IL-13 were also found in OVA restimulated spleen cell cultures from C57Bl/6 and IL-12 -/- mice (Figure 1). Inclusion of blocking anti-CD4 antibodies in these cultures abrogated ≥85% of cytokine production in all three strains indicating the cytokine production was $CD4^+$ T cell dependent (data not shown). Thus, endogenous IL-12 production is crucial for inducing significant IFNγ production but plays no detectable role in modulating the magnitude of developing type 2 cytokine responses.

1.2 - IFNγ production is markedly reduced in OVA (CFA) immunized IL-12 -/- mice

Hypothesizing that the lack of endogenous IL-12 production has a more pronounced effect on type 2 cytokine production during a type 1 dominated immune response, we also immunized C57Bl/6 controls, IL-12 p35 -/- and IL-12 p40 -/- mice with OVA (CFA). In OVA (CFA) immunized C57Bl/6 controls, type 2 cytokine production was markedly lower, compared to OVA (alum) immunization indicating the type 1 dominance of the response (compare Figures 1 and 2). As above, *in vitro* OVA restimulation of spleen cells from both strains of IL-12 -/- mice resulted in IFNγ levels about 10% of that seen in control cultures (Figure 2). However, the production of IL-5, IL-10 and IL-13 was not significantly different in IL-12 p35 -/- or IL-12 p40 -/- mice compared to C57Bl/6 controls (Figure 2). Again, inclusion of blocking anti-CD4 antibodies in these cultures abrogated ≥85% of cytokine production in all three strains (data not shown). These data indicate that the absence of IL-12 has little effect on type 2 cytokine production and that IL-12 is *not* required for preventing hyper-expression of type 2 immune responses.

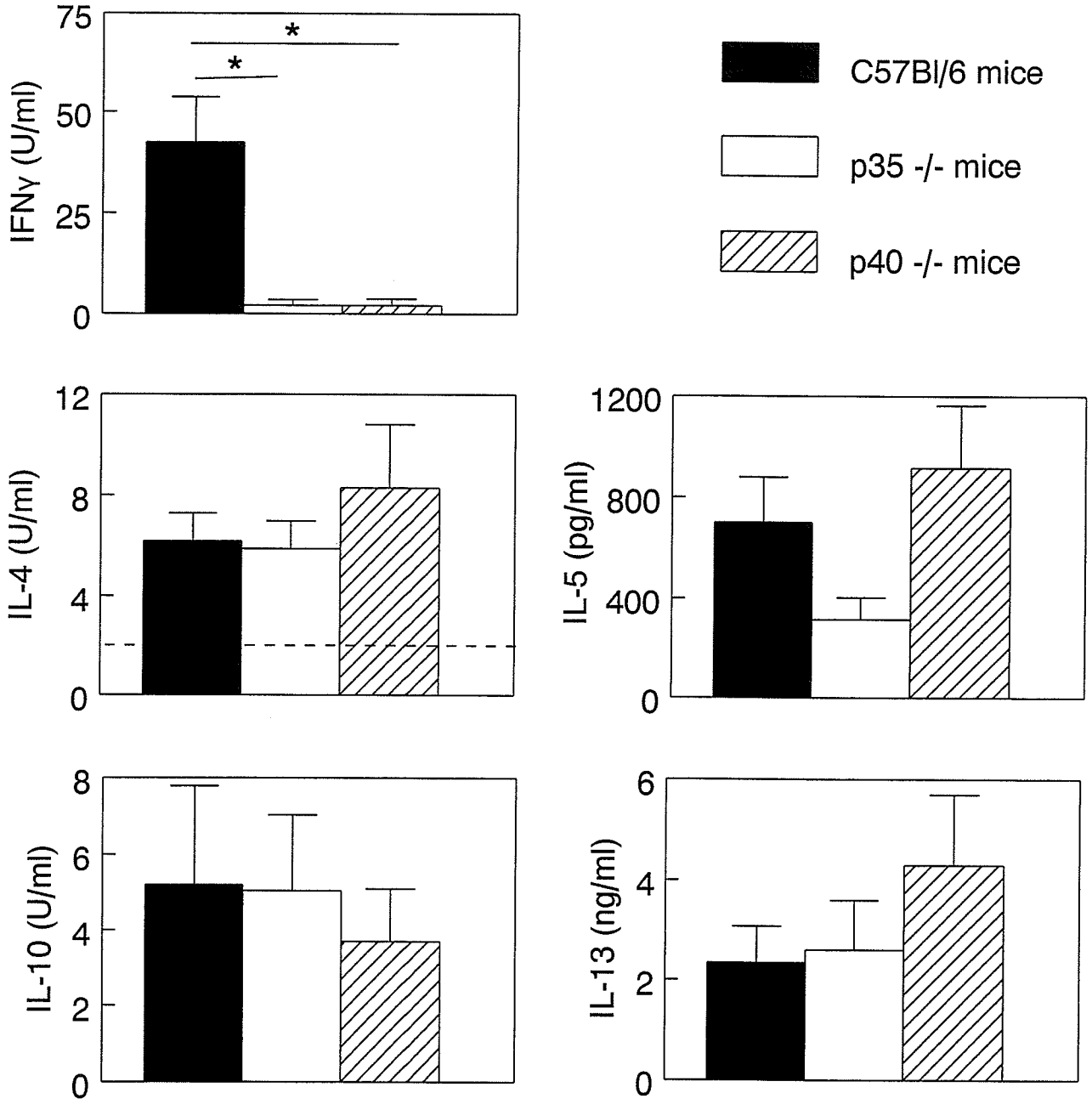

Figure 2: Decreased IFNγ production in OVA (CFA) immunized IL-12 -/- mice. Mice were immunized with 100 μg OVA (CFA) on day 0 and sacrificed on day 5 and spleen cells stimulated with 1.0 mg/ml OVA. Mean + SEM shown (n = 24 mice from 6 experiments). Significant differences between C57Bl/6 and knockout strains indicated (ANOVA and subsequent Tukey's test; * $p < 0.001$).

1.3 - Immunization with *Trichinella spiralis* extract does not result in enhanced type 2 cytokine production in IL-12 -/- mice

To evaluate the impact of IL12 deficiency on development of cytokine responses to an unrelated Ag, mice were immunized with *Trichinella spiralis* sonicate in the absence of adjuvant and sacrificed 5 days later for *in vitro* restimulation. Examination of type 1 and type 2 cytokine responses in *Trichinella spiralis* extract restimulated spleen cell cultures from IL-12 p35 -/- mice revealed markedly reduced IFNγ synthesis (Figure 3). As with OVA (alum) immunization, IL-12 deficient mice demonstrated *T. spiralis* driven IL-4, IL-5 and IL-13 responses equivalent to wild-type controls (Figure 3). Mean IL-10 production was slightly elevated ($p = 0.03$) (Figure 3). This provides additional evidence that there is no bias towards increased type 2 immunity in the absence of endogenous IL-12.

1.4 - IL-12 deficiency does not result in increased type 2 Ab responses

As a complementary way of examining the role that endogenous IL-12 production plays in regulating generation of type 2 immune responses, total IgG_{2c} (the C57Bl/6 equivalent of IgG_{2a}), IgG_1 and IgE levels were determined in naive C57Bl/6, IL-12 p35 -/- and IL-12 p40 -/- mice. As indicated in Figures 4 and 5 (day 0 time points), prior to intentional immunization the mean serum IgG_{2c} concentration was dramatically lower in IL-12 p40 -/- mice. As IgG_{2c} is a type 1 associated Ab, this decrease is likely due to decreased IFNγ production in response to environmental or food

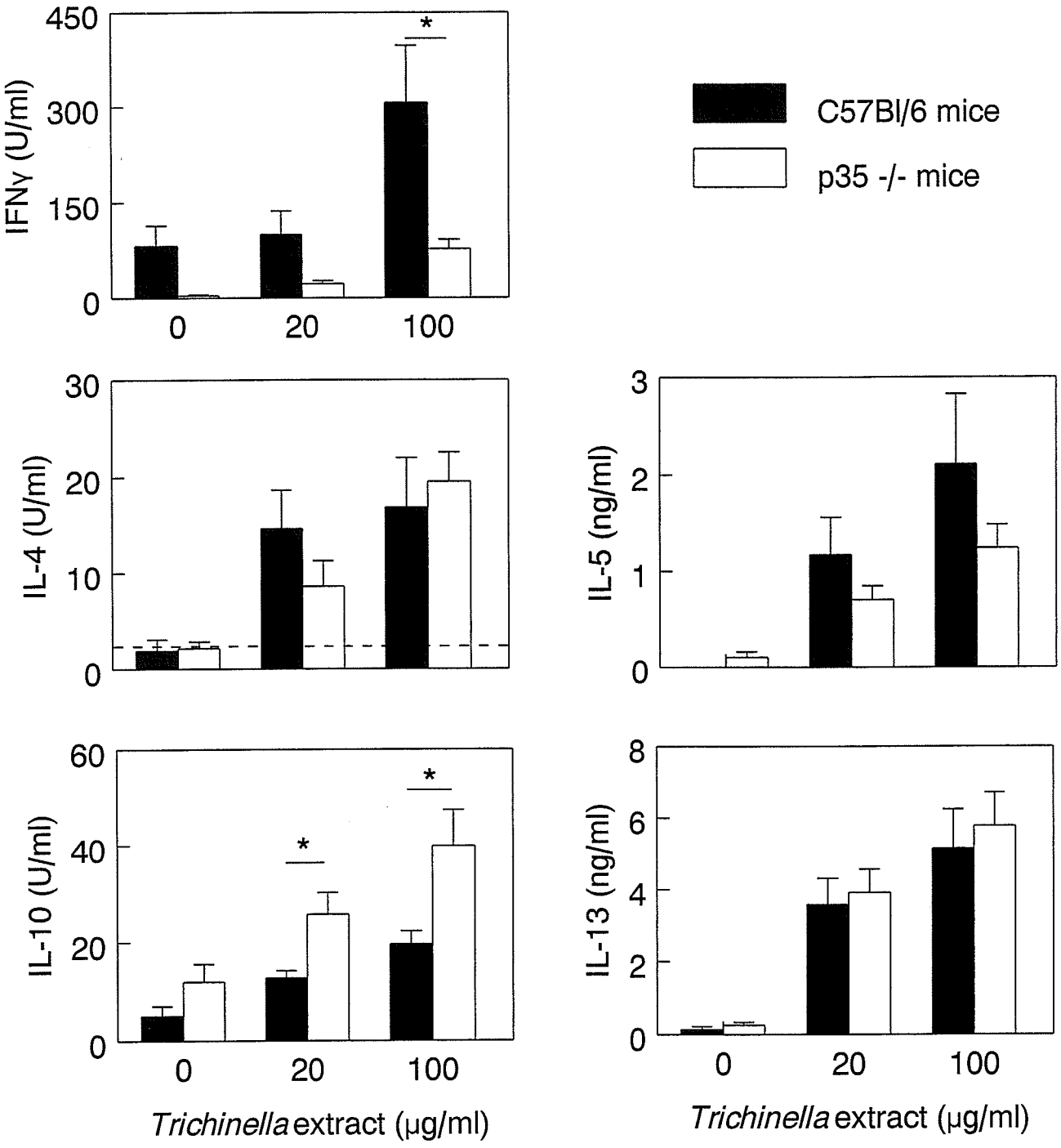

Figure 3: Diminished IFNγ production in *Trichinella spiralis* extract immunized C57Bl/6 and IL-12 -/- mice. Mice were immunized with 50 µg *Trichinella spiralis* extract in saline on day 0. Mice were sacrificed on day 5 and spleen cells were restimulated with *T. spiralis* extract at the indicated concentrations. Dotted line indicates detection limit. Mean + SEM shown (n = 9 mice from 2 experiments). Significant differences between C57Bl/6 and p35 -/- mice indicated (Student's t test; * $p < 0.05$).

allergens. Examining type 2 Ab responses, total serum IgE levels in resting naive mice were not enhanced in either of the IL-12 deficient groups (49.8, 46.8 and 10.2 ng/ml for C57Bl/6, IL-12 p35 -/- and p40 -/- respectively, $p > 0.05$; Figures 4 and 5;). Similar results were found when IgG_1 was measured (171, 183 and 114 μg/ml, $p > 0.05$).

Following immunization under type 1 inducing conditions (Figure 4), total and OVA-specific IgG_{2c} responses were reduced by 50-80% in the IL-12 deficient mice compared to C57Bl/6 controls. Total and OVA-specific IgE levels in both primary and secondary immune responses were similar or, in the case of secondary responses, slightly lower (not higher, as originally hypothesized) than those mounted by C57Bl/6 controls. IgG_1 production was virtually identical in all groups of OVA (CFA) immunized mice (Figure 4).

In independent experiments, mice were immunized under conditions favouring OVA-specific type 2 Ab production. Thus, total and specific serum IgE levels in OVA (alum) immunized mice were markedly higher (~10-fold) than IgE levels seen following immunization with OVA (CFA)(Figure 4 versus Figure 5). In contrast, following immunization with OVA (alum), specific IgG_{2c} responses were 80-100 fold weaker (Figure 4 vs Figure 5). As above, the intensity of IgE and IgG_1 responses did not differ between the three strains of mice examined (Figure 5). As expected, IgG_{2c} levels were significantly diminished in the absence of IL-12 (Figure 5). These data again argues against a generalized enhancement of type 2 activation in the absence of IL-12 production.

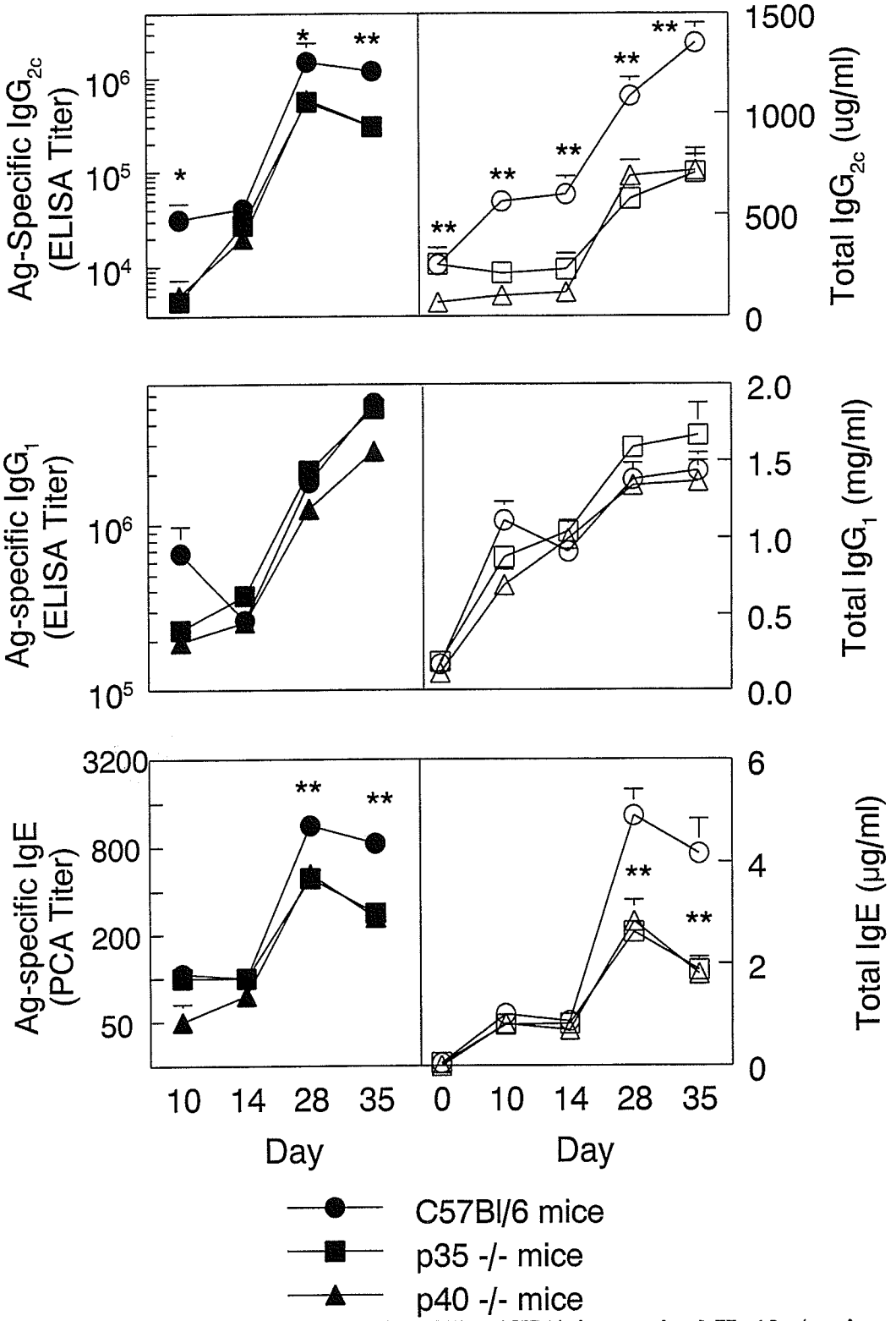

Figure 4: Decreased IgG_{2c} production in OVA (CFA) immunized IL-12 -/- mice. Mice were immunized with 100 µg OVA (CFA). Total (open symbols) and OVA-specific (closed symbols) Ig levels were measured on the indicated days. Mean + SEM shown (n = 20 mice from 5 experiments). Significant differences between C57Bl/6 and knockout strains indicated (ANOVA and subsequent Tukey's test;* $p < 0.05$; ** $p < 0.005$).

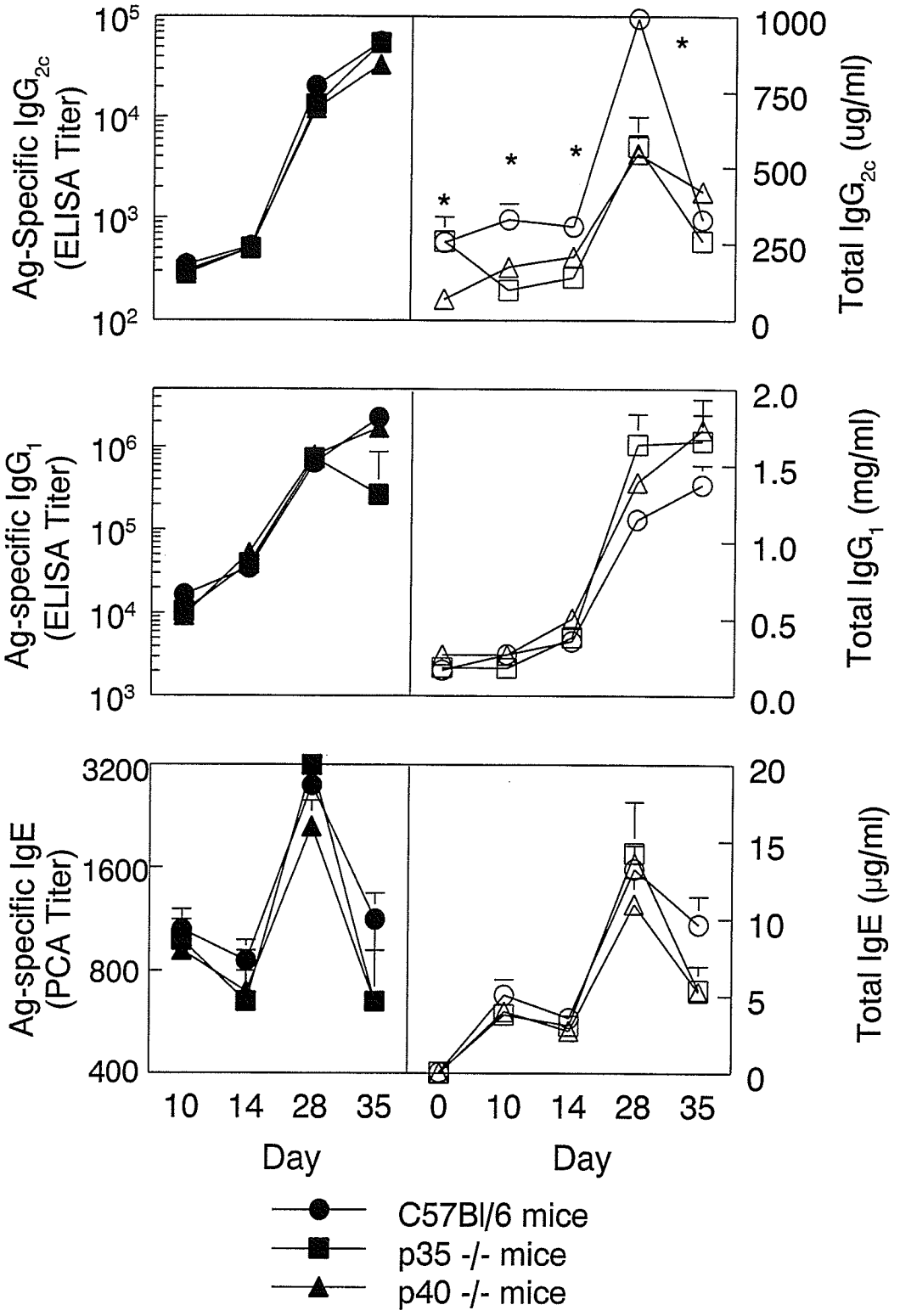

Figure 5: Decreased IgG_{2c} production in IL-12 -/- mice. Mice were immunized with 2.0 µg OVA (alum). Total (open symbols) and OVA-specific (closed symbols) Ig levels were measured. Mean + SEM shown (n = 20 mice from 5 experiments). Significant differences between C57Bl/6 and knockout strains indicated (ANOVA and subsequent Tukey's test; $p < 0.01$).

1.5 - Type 2 Ab responses are not increased in IL-12 -/- mice following immunization with *Trichinella spiralis* extract

As certain parasites are known for their ability to induce the production of non-specific IgE, we also examined Ab responses induced following *Trichinella spiralis* (alum) immunization. IL-12 p35 -/- mice immunized with parasite extract in the presence of alum demonstrated total IgG_{2c} levels significantly lower than those seen in control mice (Figure 6). Such a decrease was observed during both primary and secondary immune responses. The kinetics, duration and intensity of both primary total IgE and total IgG_1 responses were, however, indistinguishable between control and IL-12 deficient mice (Figure 6). Thus, regardless of the type 1 or type 2 dominance of the elicited immune response, lack of endogenous IL-12 does not result in enhanced type 2 Ab production.

1.6 - IL-12 deficient mice do not display diminished responsiveness to IL-12

IFNγ (Szabo, Dighe et al. 1997), and IL-12 itself (Hyodo, Matsui et al. 1999; Chakir, Camilucci et al. 2000) alter IL-12 responsiveness of T cells by modulating expression of the IL-12Rβ2 subunit. Moreover, upon differentiation into Th2 cells, naive T cells lose IL-12Rβ2 expression and become refractory to Il-12 stimulation. Given that IL-12 -/- mice produce no IL-12, and exhibit a 90% reduction in IFNγ production, we examined IL-12 responsiveness in IL-12 deficient mice. Mice were immunized with OVA (alum), sacrificed on day 5 after immunization and spleen cells were cultured in the presence of 100 pg/ml rIL-12 (± 10U/ml rIL-2) with and without OVA. In the absence of rIL-12, the mean OVA-dependent IFNγ response in C57Bl/6 mice was sevenfold that of either IL-12

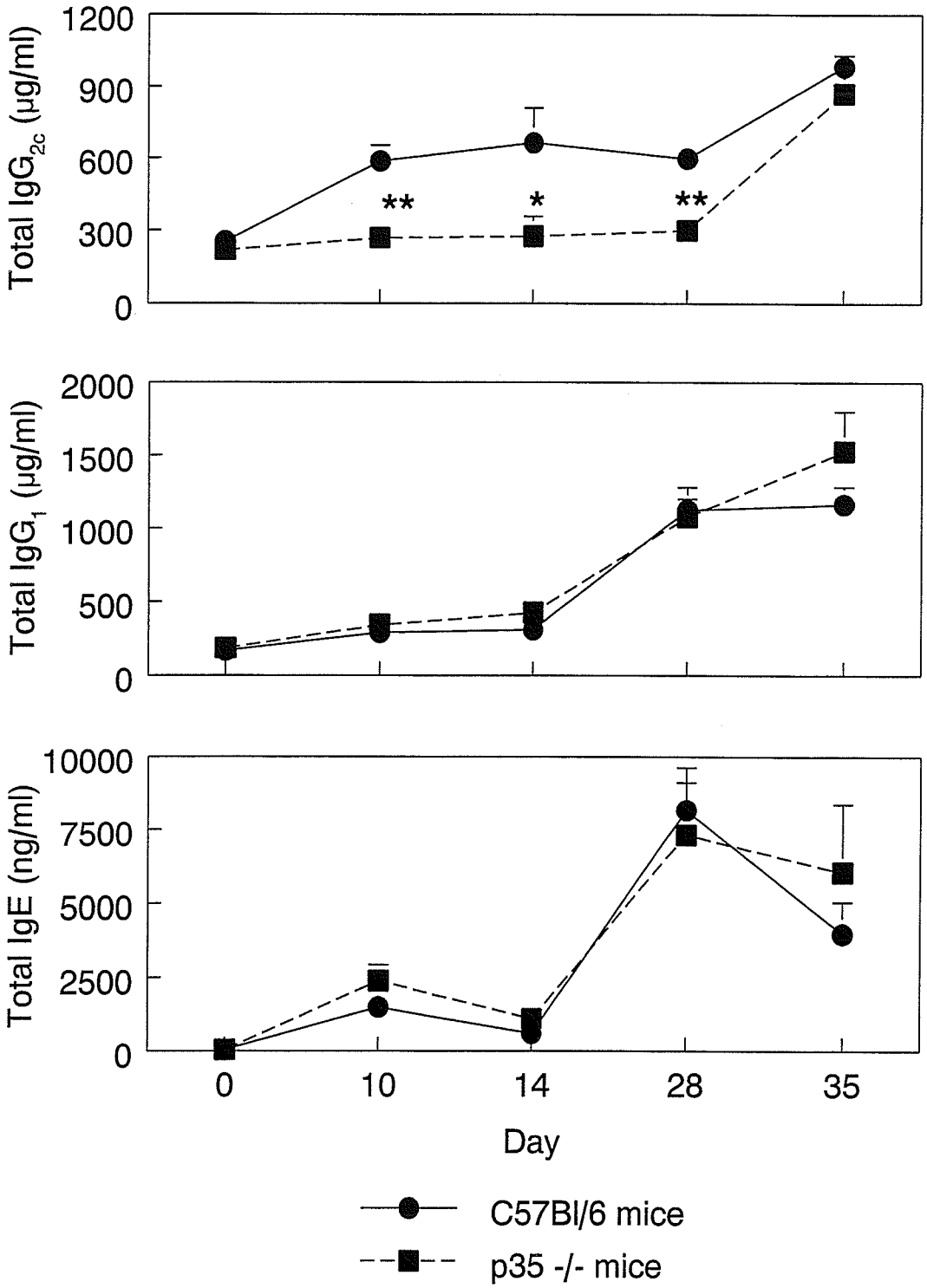

Figure 6: Diminished IgG_{2c} production in IL-12 -/- mice immunized with *Trichinella spiralis* extract. Mice were immunized with 50 µg *Trichinella spiralis* extract (alum) and bled on indicated days for quantification of total Ig levels. Mean + SEM shown (n = 12 mice from 3 experiments). Significant differences between C57Bl/6 and p35 -/- mice indicated (Student's t test; * $p < 0.05$; ** $p < 0.005$).

deficient strain (Figure 7). Addition of rIL-12 (± rIL-2) resulted in increased IFNγ responses of similar intensity in C57Bl/6 and IL-12 deficient strains (Figure 7). Finally, the intensity of the Ag-independent IFNγ responses observed, primarily attributable to NK cell activation (Rempel, Wang et al. 1997; Gately, Renzetti et al. 1998) were also indistinguishable in C57Bl/6 and both strains of IL-12 -/- mice (Figure 7). Th2 cells actively downregulate the expression of IL-12Rβ2, and therefore possess a reduced capacity to produce IFNγ upon stimulation with IL-12. The observation that C57Bl/6 and IL-12 -/- mice demonstrated similar intensity of IL-12 driven IFNγ production, provides further evidence that there is not an increased outgrowth of Th2-like cells in the absence of endogenous IL-12 production.

1.7 - Summary

IL-12 deficient mice display no evidence of significantly increased production of IgE or type 2 cytokine synthesis regardless of the phenotype (type 1 or type 2) of the immune responses induced to exogenous protein antigen. Furthermore, the type 2 immune response against a complex antigenic extract of the parasite *Trichinella spiralis* was similarly unenhanced in the absence of endogenous IL-12 production. In contrast, there was a consistent and substantial decrease in the intensity of type 1 antibody (IgG_{2c}) and cytokine (IFNγ) production. Additional evidence arguing against the hyperexpression of type 2 immunity in the absence of IL-12 comes from the observation that spleen cells from control and IL-12 -/- mice produce equivalent levels of IFNγ in response to exogenous rIL-12. Thus, the data indicate that endogenous IL-12 is a potent promoter of type 1 immunity, but plays, at best, a redundant role in negative regulation of type 2 immunity.

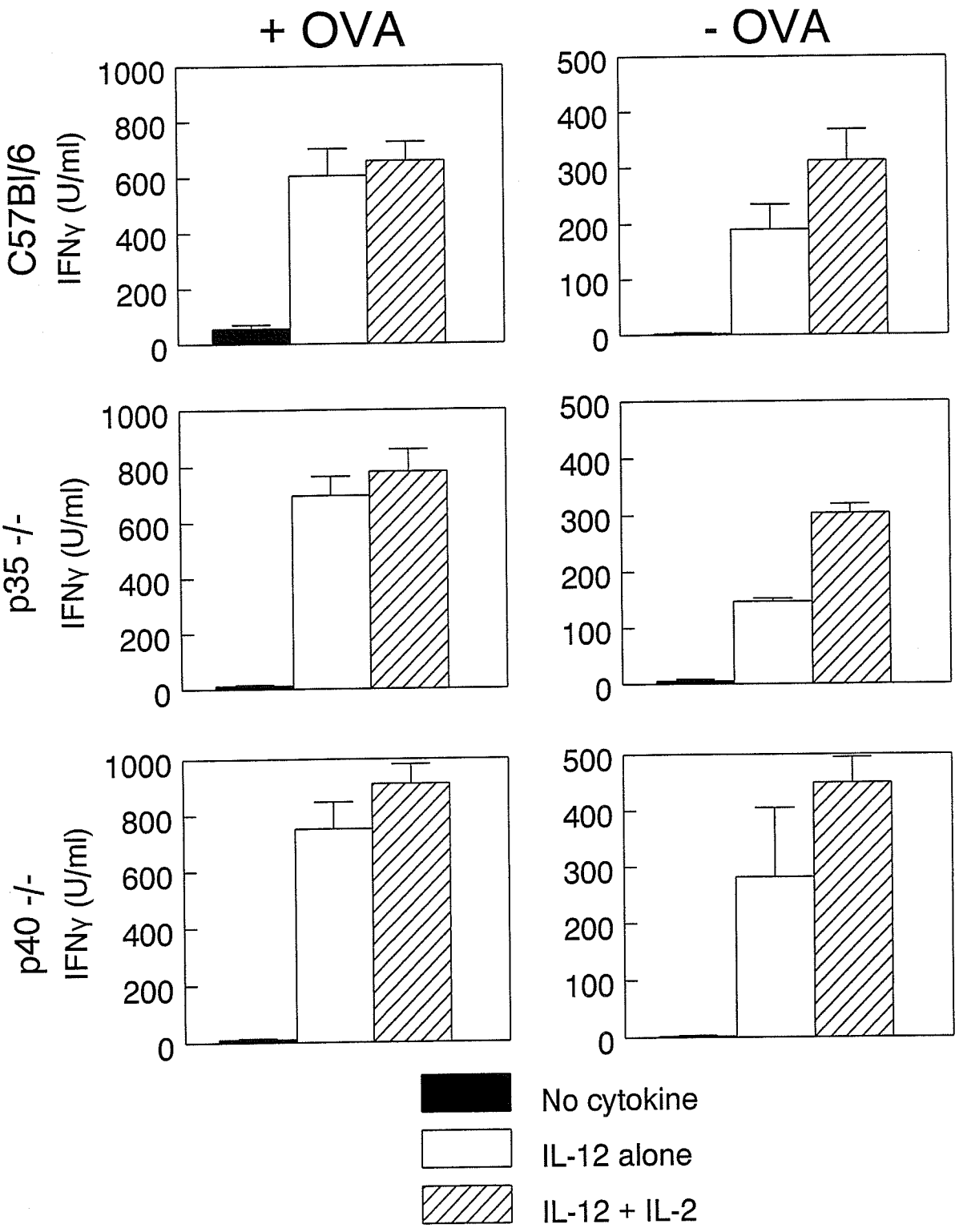

Figure 7: Undiminished IL-12 responsiveness in IL-12 -/- mice. C57Bl/6, p35 -/- and p40 -/- mice were immunized with OVA (alum). Spleen cells were cultured +/- OVA, with rIL-12 (100 ng/ml) or with IL-12 + IL-2 (10 U/ml). Tissue culture supernatants were harvested at 48 hours and the amount of IFNγ present in supernatants was determined. Mean + SEM shown (n = 7 mice from 2 experiments).

2.0 - Endogenous IFNγ as a negative regulator of type 2 immunity

IFNγ is a critical effector molecule of type 1 immunity. It inhibits Th2 cell proliferation and differentiation (Mosmann and Coffman 1989), and inhibits IgE production (Maggi, Parronchi et al. 1992). Administration of exogenous IFNγ has protective effects in some murine models of allergy and asthma (Lack, Renz et al. 1994; Lack, Bradley et al. 1996; Dow, Schwarze et al. 1999; Kang, Kim et al. 1999). In contrast, others argue IFNγ promotes disease, particularly airway hyperresponsiveness (Hessel, Van Oosterhout et al. 1997; Hofstra, Van Ark et al. 1998). Surprisingly, in studies designed to examine the role of endogenous IFNγ in limiting the allergic response, the impact of endogenous IFNγ production on type 2 cytokine and chemokine production, arguably one of the most important controls on the overall development of type 2 immunity, was *not* examined (Coyle, Tsuyuki et al. 1996; Bruselle, Kips et al. 1997; Hessel, Van Oosterhout et al. 1997; Hofstra, Van Ark et al. 1998). Due to its demonstrated type 2 inhibitory activities, we hypothesized that endogenous IFNγ is an important negative regulator of type 2 cytokine production, and by extension, a potent inhibitor of type 2 immunity.

2.1 - Type 2 cytokine and chemokine production is enhanced in spleen cells from OVA (alum) immunized IFNγ -/- mice.

To directly evaluate the capacity of IFNγ to limit expression of type 2 cytokines *in vivo*, control C57Bl/6 and IFNγ -/- mice were immunized with 2 μg of OVA (alum) to induce a strong type 2 dominated immune response. Spleen cells from OVA (alum) immunized IFNγ -/- mice produced

significantly more IL-4 following *in vitro* OVA restimulation than those from C57Bl/6 controls ($p<0.005$, Figure 8). Similarly, 60-75% increased IL-5 and IL-13 production was observed in tissue culture supernatant from OVA-stimulated IFNγ -/- spleen cell cultures ($p<0.005$, Figure 8). This indicates a consistent bias towards increased Th2 cytokine production in the absence of endogenous IFNγ- a finding in marked contrast to that seen in the absence of IL-12 subunits. Addition of anti-CD4 mAb resulted in ≥85% reduction in cytokine synthesis in both strains indicating that the cytokine production was $CD4^+$ T cell dependent (data not shown).

IL-10 production is strongly associated with type 2 immunity. Unlike IL-4, IL-5 and IL-13, IL-10 is produced in significant amounts by APC. Thus, as an alternative indicator of immune responsiveness, we examined IL-10 production in IFNγ -/- and C57Bl/6 mice. IL-10 production was consistently 50-120% elevated in IFNγ deficient mice compared to controls (Figure 8). In the absence of antigen stimulation, no difference between the levels of type 2 cytokines produced in control and IFNγ -/- cultures was evident. This indicates that type 2 cytokine expression is not spontaneous in IFNγ -/- mice.

As an alternative approach to assessing the balance of type 1 versus type 2 immunity in IFNγ deficient mice, *in vitro* OVA-stimulated production of chemokines IP-10 and TARC was examined in spleen cell cultures from OVA (alum) immunized IFNγ -/- and control C57Bl/6 mice. IP-10 synthesis, induced by IFNγ, is associated with type 1 immune responses (Farber 1997), while TARC production associates strongly with type 2 responses (Bonecchi, Bianchi et al. 1998).

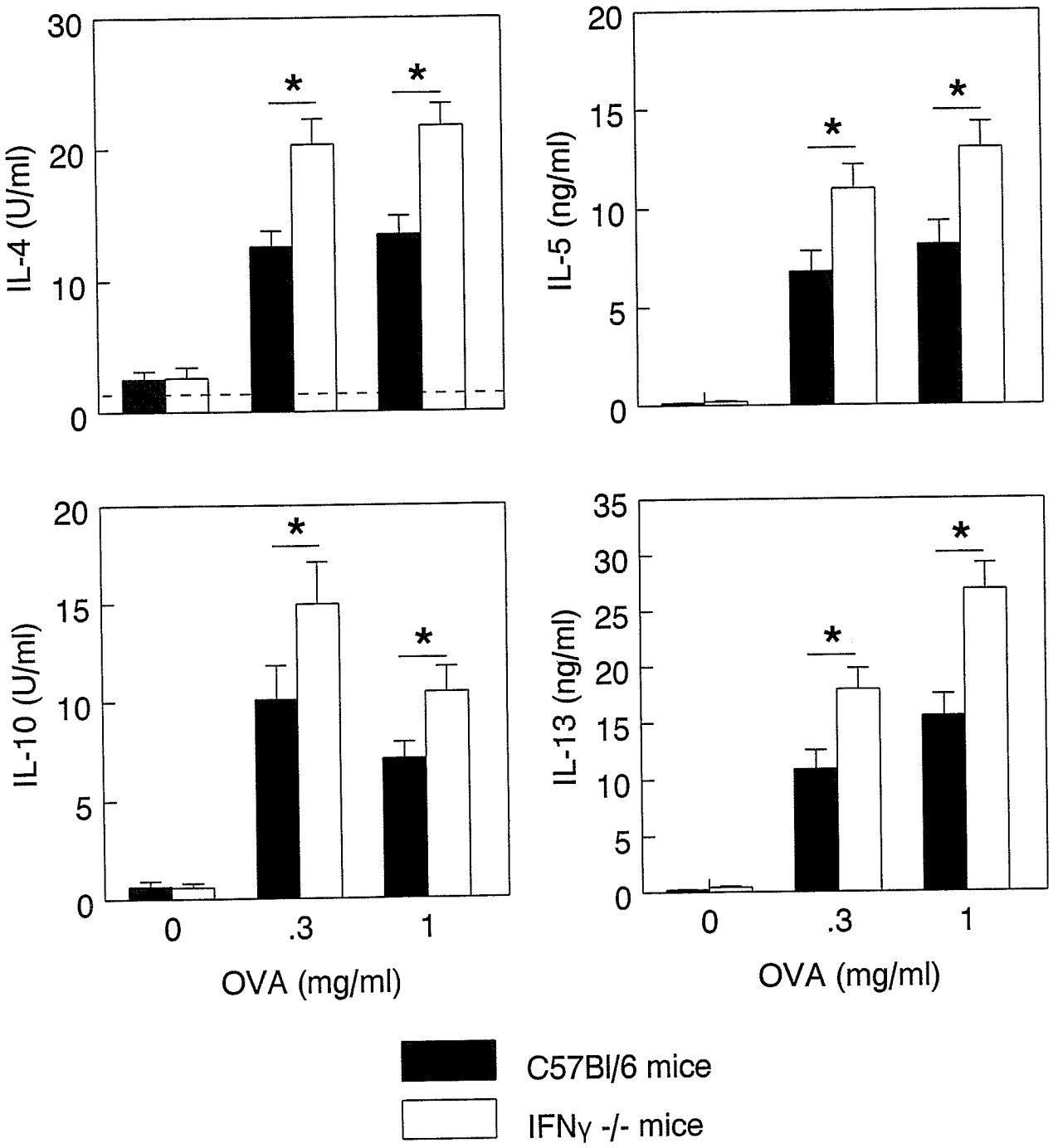

Figure 8: Increased type 2 cytokine production in IFNγ -/- mice. Mice were immunized with OVA (alum), sacrificed on day 5, and spleen cells cultured in the presence of 1.0 mg/ml OVA. Dotted line indicates detection limit. Mean + SEM shown (n = 30 mice from 6 experiments). Significant differences between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * $p < 0.005$).

IP-10 production in spleen cell cultures from IFNγ -/- mice was consistently less than 5% of that seen in C57Bl/6 mice ($p < 0.0001$, Figure 9). Conversely, under all OVA-stimulated conditions examined, TARC production was markedly elevated in IFNγ -/- mice ($p < 0.0001$, Figure 9). This pronounced shift towards type 2 chemokine production in IFNγ -/- mice (ie, from IP-10:TARC ratios of 460:1 to 8:1 in C57Bl/6 and IFNγ -/- mice, respectively) provides further evidence that IFNγ is a non-redundant negative regulator of the development of type 2 responses in vivo.

2.2 - Type 2 cytokine production is significantly increased in spleen cell cultures from OVA (HKL) immunized IFNγ -/- mice

To determine the impact of IFNγ deficiency on the development of Th2 cytokine responses in a model of Th1 biased immunity, we also immunized mice with OVA (HKL) and measured *in vitro* cytokine production. Type 2 cytokine responses following OVA restimulation of spleen cells from OVA (HKL) immunized IFNγ -/- and C57Bl/6 controls were, as anticipated, much weaker, and frequently fell near the level of detection, making it difficult to detect differences. However, for those sufficiently robust to be accurately quantitated (primarily IL-5 and IL-13), levels in IFNγ -/- cultures were consistently ~2 fold higher than those achieved in normal C57Bl/6 mice ($p < 0.05$, Figure 10).

In contrast to previous studies examining IFNγ deficient mice in allergic models which failed to examine Ag-driven type 2 cytokine production (Coyle, Tsuyuki et al. 1996; Bruselle, Kips et al. 1997; Hessel, Van Oosterhout et al. 1997; Hofstra, Van Ark et al. 1998), these data clearly indicate

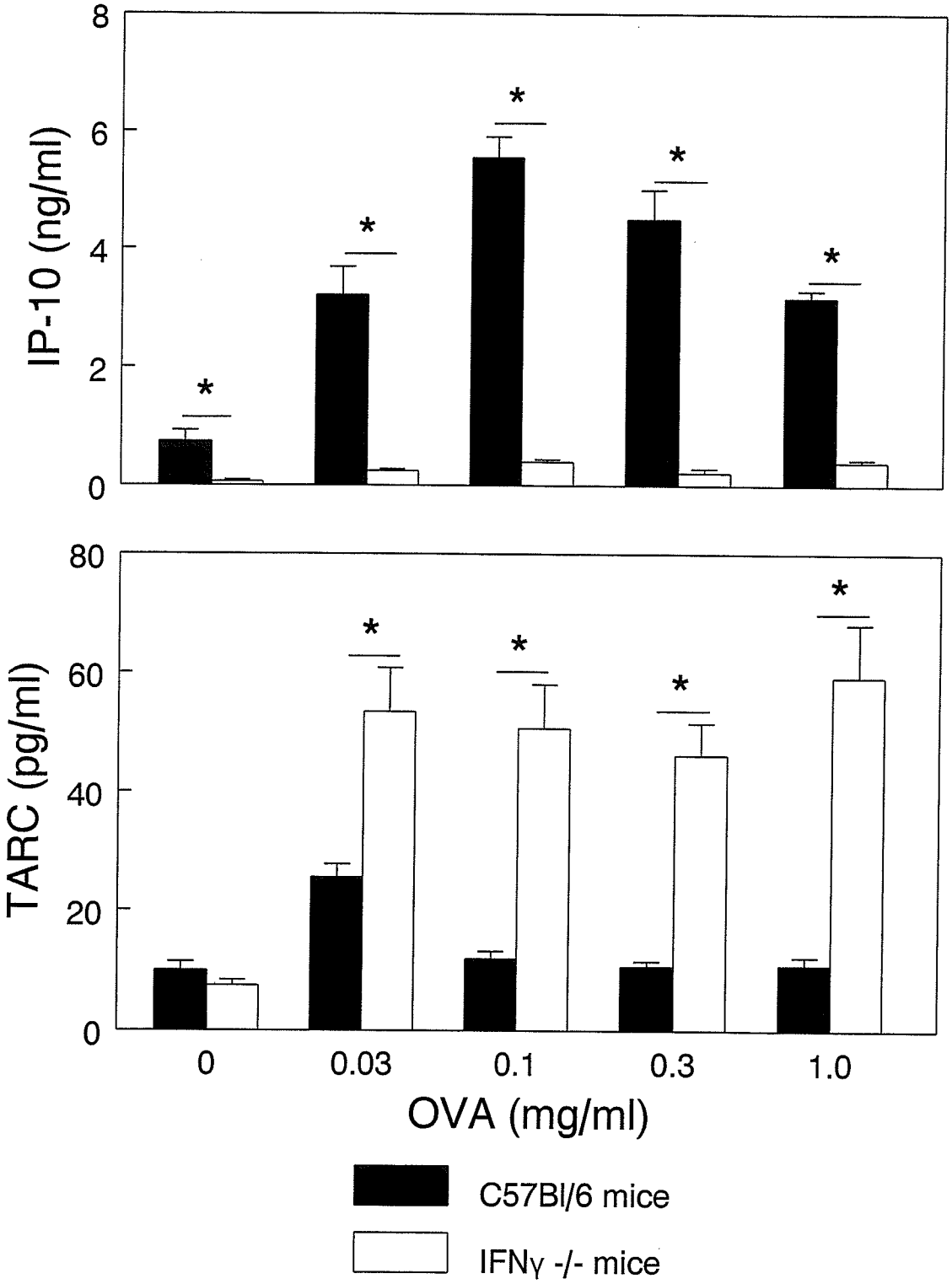

Figure 9: Increased type 2 chemokine production in the absence of endogenous IFNγ. Mice were immunized with OVA (alum), sacrificed on day 5 and spleen cells were cultured with OVA at the indicated concentrations. Tissue cultures were harvested at 24 (TARC) and 72 (IP-10) hours for chemokine determination. Mean + SEM shown (n = 16 mice from 2 experiments). Significant differences between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * $p < 0.0001$).

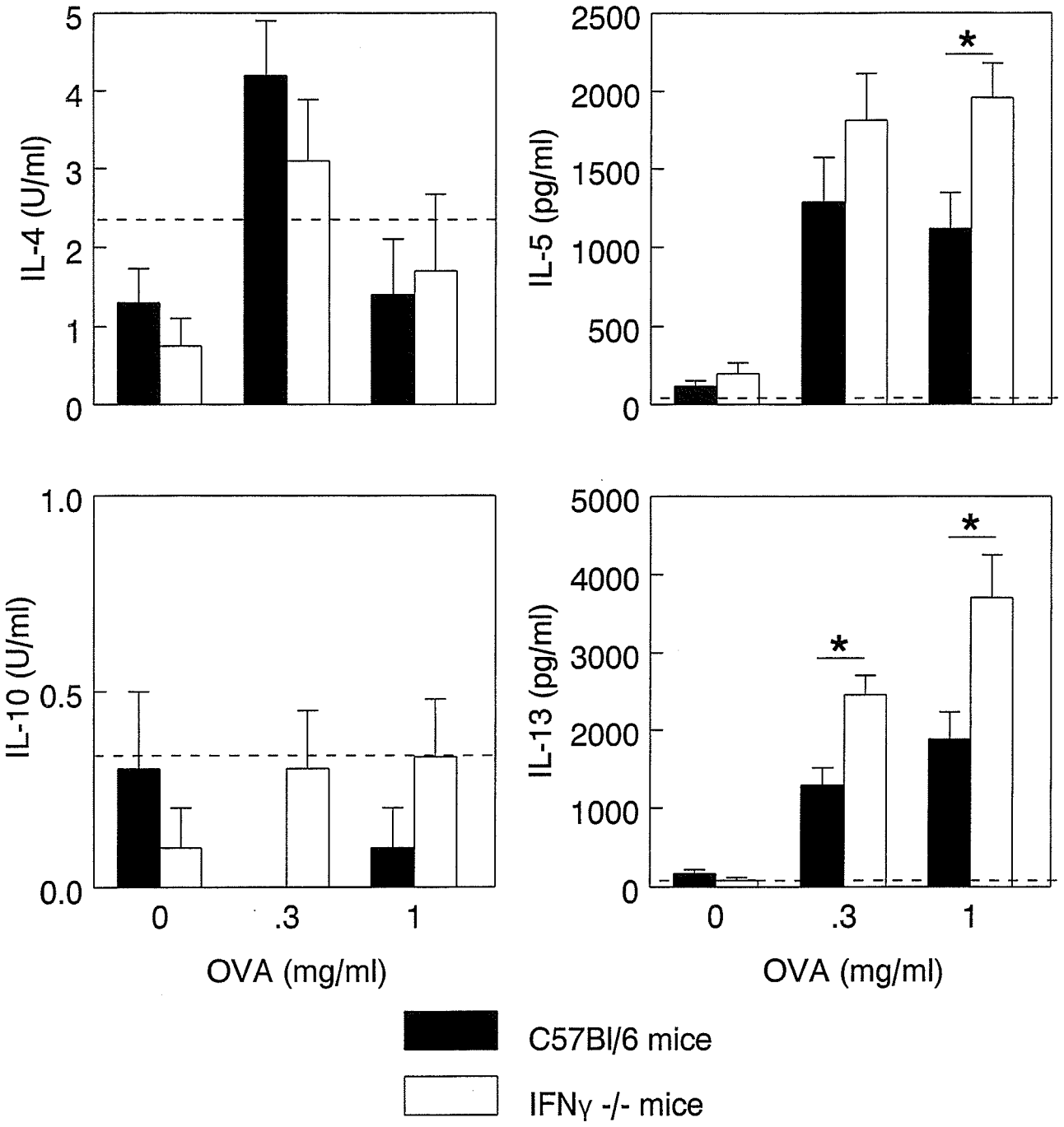

Figure 10: Increased IL-5 and IL-13 expression in OVA (HKL) immunized IFNγ -/- mice. Mice were immunized with 100 μg OVA (HKL) on day 0, sacrificed on day 5 and spleen cells cultured with OVA at the indicated concentrations. Dotted line indicates detection limit. Mean + SEM shown (n = 13 mice from 3 experiments). Significant differences between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * $p < 0.05$).

that antigen specific immunity induced in IFNγ deficient environments results in cytokine responses skewed towards a more Th2-like profile. Thus endogenous IFNγ production is required for optimal negative regulation of type 2 cytokine responses.

2.3 - Ig production in is globally reduced in OVA immunized IFNγ -/- mice, regardless of immunization protocol

As antibody class switching is exquisitely sensitive to the *in vivo* balance of cytokine synthesis, examination of the comparative levels of total and OVA-specific serum IgE, IgG_1, and $IgG2_c$ was used as an independent approach to assess the balance of type 1 versus type 2 immunity in C57Bl/6 and IFNγ -/- mice. As indicated in Figures 11 and 12, prior to immunization, total IgE and IgG_1 levels were unchanged or marginally elevated whereas total IgG_{2c} levels were significantly lower in IFNγ -/- mice (Figure 11 and 12, day 0 time point). Following OVA (alum) immunization, C57Bl/6 mice exhibit type 2 dominated responses characterized by high levels of OVA-specific and total IgE (Figure 11). Total and OVA-specific IgG_{2c} levels were ~ 75% and 50% lower respectively in the absence of endogenous IFNγ synthesis. Surprisingly, a similar decrease was also seen in total and OVA specific IgE and IgG_1 levels in IFNγ -/- mice (Figure 11). The mechanism underlying this counterintuitive finding is addressed below.

Mice immunized with OVA in the presence of heat killed *Listeria monocytogenes* (HKL) adjuvant displayed ~10-fold lower IgE and 10-200 fold higher IgG_1 and IgG_{2c} responses than following OVA (alum) immunization (compare Figures 11 and 12), indicating type 1 dominated, OVA specific immune responses. Even under such conditions, IFNγ deficient mice displayed significantly lower

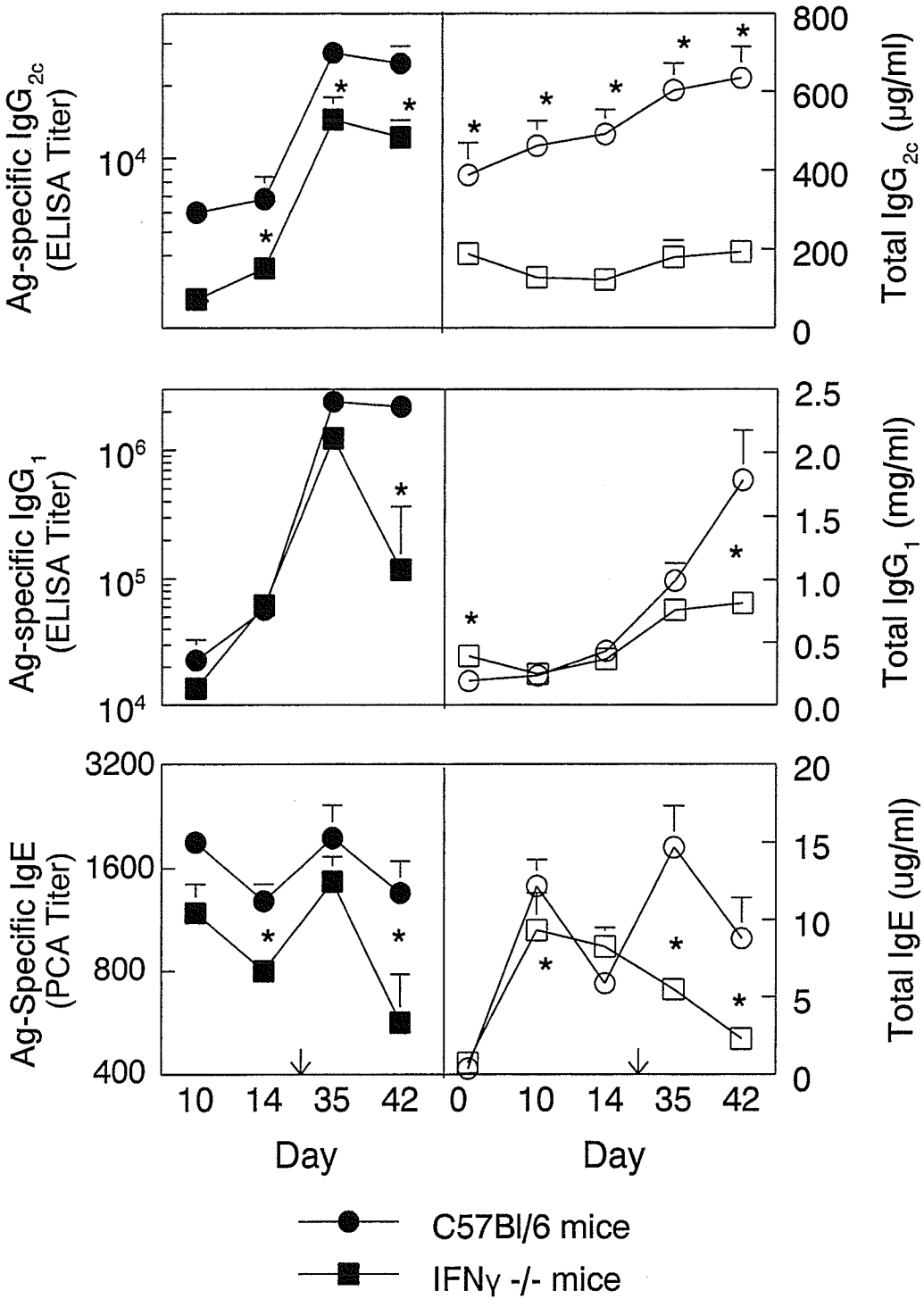

Figure 11: Globally depressed Ig production in OVA (alum) immunized IFNγ -/- mice. Mice were immunized with OVA (alum), and boosted with OVA (saline) on day 28. Mice were bled on the indicated days for determination of total (open symbols) and OVA-specific (closed symbols) Ig production. Mean + SEM shown (n = 30 mice from 5 experiments). Significant differences between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * p values in the range of ≤ 0.05 to $p < 0.0001$).

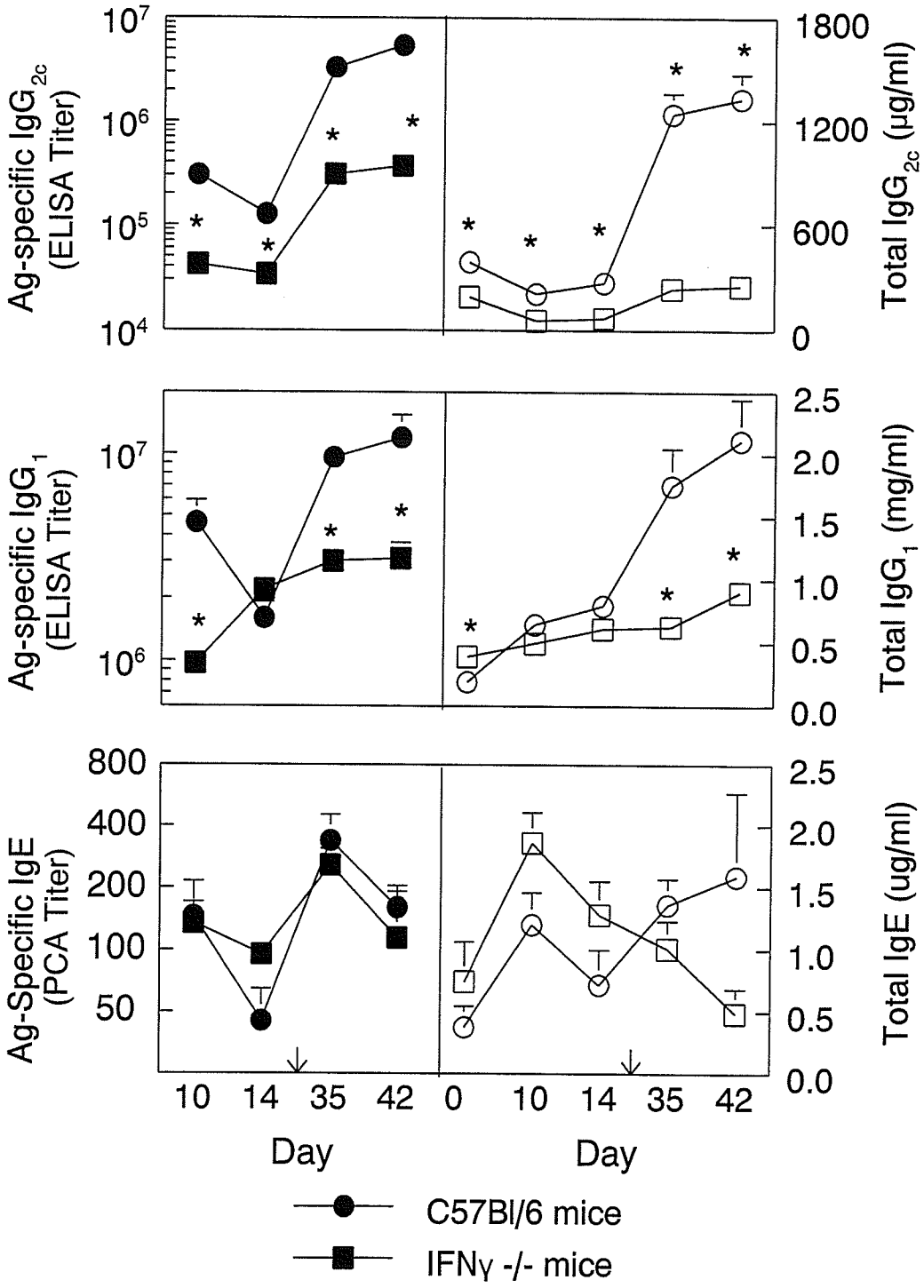

Figure 12: Diminished Ig production in OVA (HKL) immunized IFNγ -/- mice. Mice were immunized with OVA (HKL), and boosted with 100 µg OVA (saline) on day 28. Mice were bled on the indicated days for determination of total (open symbols) and OVA-specific (closed symbols) Ig production. Mean + SEM shown (n = 14 mice from 3 experiments). Significant differences (measured by) between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * p values in the range of < 0.05 to $p < 0.0001$).

IgG_1 and IgG_{2c} responses, with consistent reductions in both OVA-specific and total levels of these isotypes (Figure 12). Thus, IFNγ is required for optimal prevention of excessive type 2 cytokine and chemokine production, but it is also necessary for optimal induction of antibody responses - both Th1 and Th2 dependent.

2.4 - B cell function and maturational status are not diminished in IFNγ -/- mice

In light of enhanced type 2 cytokine production seen in IFNγ deficient mice, diminished Ab synthesis, particularly that of IgE, was unexpected. In the absence of endogenous IFNγ, immature B cells are found in the lymph nodes leading to the suggestion that autocrine secretion of IFNγ is required for proper homing of immature B cells to the spleen to undergo their final maturation (Flaishon, Hershkoviz et al. 2000). In the lymph node, exposure of immature B cells to Ag results in B cell death and clonal deletion (Monroe 1996). We therefore wished to determine if the low levels of Ig production seen in IFNγ -/- animals was a result of fewer mature B cells due to inappropriate homing of immature B cells to lymph nodes. Using flow cytometry and gating on $CD19^+$ lymphocytes, we analysed the expression of phenotypic markers to distinguish mature ($CD19^+$, $CD23^+$, IgD^+) from immature ($CD19^+$, $CD23^-$, IgD^-) B cells. We found that there was no difference in the percentage of immature B cells in the spleen of IFNγ -/- mice and likewise there was no alteration in the percentage of immature B cells in the bone marrow (Table 2). These findings suggest that the development of B cells is not compromised in IFNγ -/- mice.

To examine the functional capacity of B cells from IFNγ -/- and control mice to respond to BCR crosslinking, proliferation assays using LPS and anti-IgM $F(ab')_2$ were performed. B cells from IFNγ

Table 2: Percentage of mature and immature B cells in spleen and bone marrow of IFNγ -/- and control mice[a]

Mice	Spleen		Bone Marrow	
	Mature[b]	Immature[c]	Mature	Immature
C57Bl/6	92.1 ± 0.85	2.52 ± 0.50	69.9 ± 2.15	16.9 ± 1.25
IFNγ -/-	92.2 ± 1.00	2.76 ± 0.85	71.0 ± 1.25	17.1 ± 1.41

a - Results compiled from 8 mice in two separate experiments

b - Mature B cells defined as $CD19^+$,$CD23^+$,IgD^+

c - Immature B cells defined as $CD19^+$,$CD23^-$,IgD^-

-/- mice did not demonstrate reduced proliferative responses to either stimulus (Figure 13), arguing that the functional response of B cells is not diminished in the absence of endogenous IFNγ. Thus the defect in antibody synthesis noted in IFNγ deficient mice was not a result of altered B cell maturation or an inability to respond to BCR crosslinking.

2.5 - Altered T cell activation and proliferation kinetics in IFNγ -/- mice.

Given that T cell help is required to initiate and maintain efficient Ab synthesis, an alteration in the kinetics or magnitude of T cell proliferation may have a significant impact on the amount of B cell help available, and hence, on Ig synthesis. To address this hypothesis, we examined IL-2 production and T cell proliferation in IFNγ -/- mice. As shown in Figure 14a, IL-2 synthesis was significantly enhanced in OVA restimulated cultures of IFNγ deficient spleen cells. Consistent with enhanced IL-2 synthesis, T cell proliferation was slightly elevated in IFNγ -/- cultures at day 2 (Figure 14b). However, peak OVA-driven proliferative responses (day 6) were reduced by 50% in IFNγ -/- mice ($p<0.000001$; Figure 14b). Similar results were noted in cultures from OVA (HKL) immunized mice (data not shown).

Collectively the data examining IFNγ -/- T and B cell proliferation indicate that despite previously reported trafficking of immature B cells to lymph nodes (Flaishon, Hershkoviz et al. 2000), there is no alteration of B cell responsiveness to B cell receptor crosslinking or LPS stimulation or a decreased number of

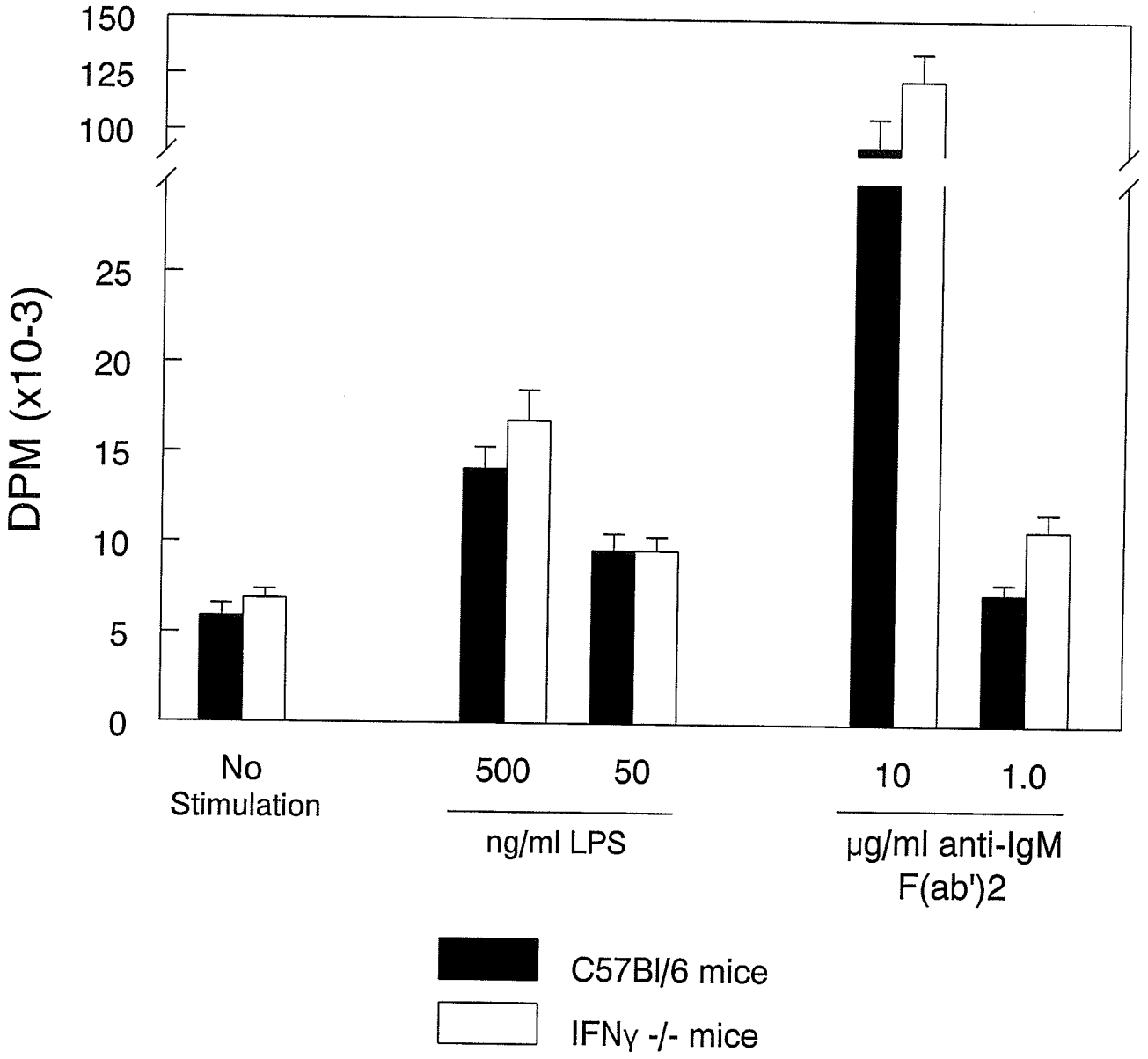

Figure 13: B cell function is not compromised in IFNγ -/- mice. Mice were immunized with 2.0 µg OVA (alum) and sacrificed on day 5. B cell proliferation was assessed after 3 days of stimulation with LPS or anti-murine IgM $F(ab')_2$ and 10hours exposure to $[^3H]$thymidine. Mean + SEM shown (n = 8 mice from 2 experiments).

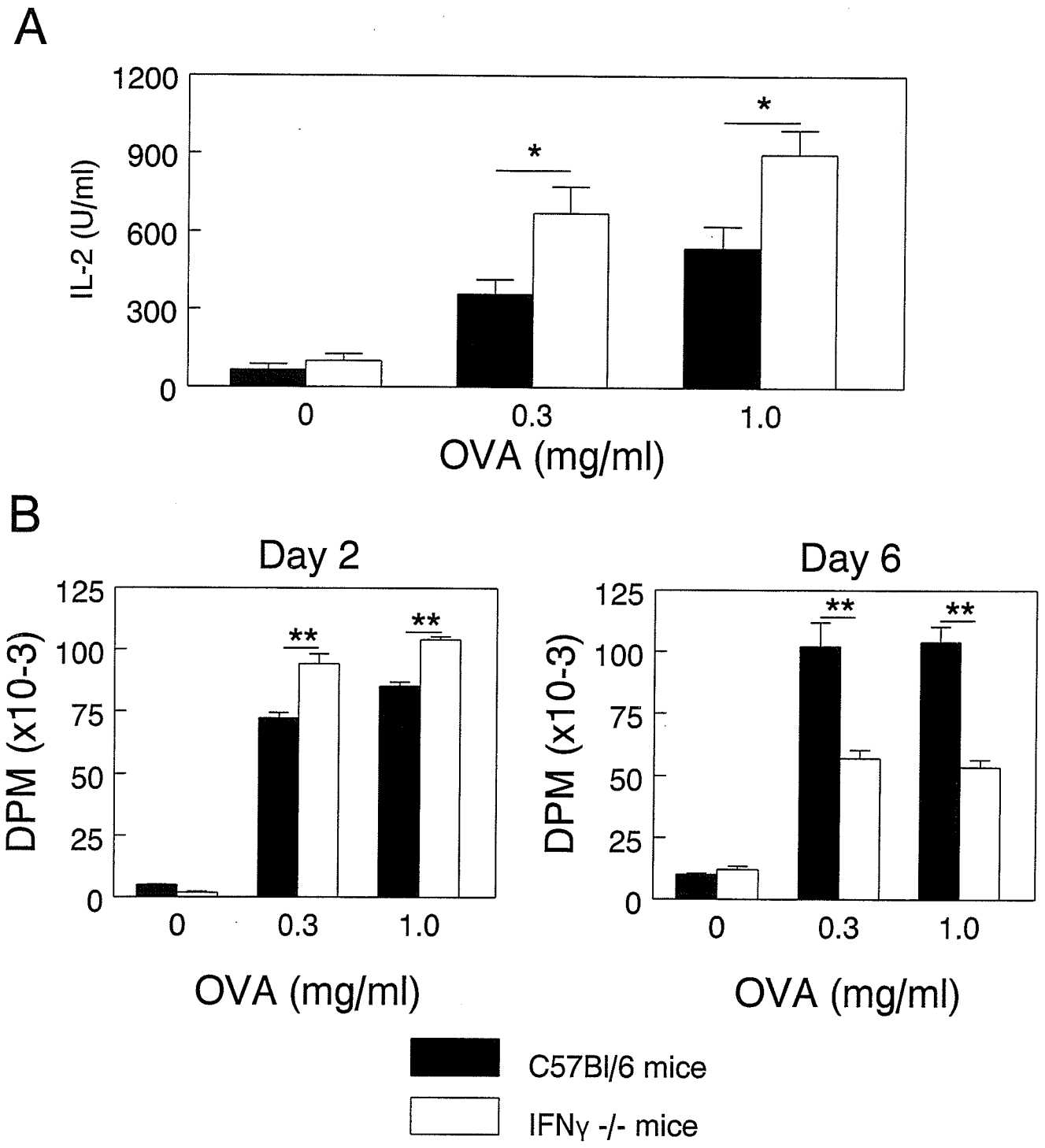

Figure 14: Altered T cell proliferation in IFNγ -/- mice. Mice were immunized with OVA (alum), sacrificed on day 5, and spleen cells cultured in the presence of OVA at the indicated concentrations. (A) IL-2 production was measured in 24 hour tissue culture supernatants. (B) T cell proliferative responses were measured after 2 or 6 days of culture after pulsing with [^{3}H]thymidine for 10 hours. Mean + SEM shown (n = 8 mice from 2 experiments). Significant differences between C57Bl/6 and IFNγ -/- mice indicated (Student's t test; * $p < 0.05$, ** $p < 0.000001$).

mature B cells in the spleen or bone marrow of IFNγ -/- mice. Our examination of T cell proliferative responses are in agreement with the findings of Dalton *et al.* who report enhanced proliferation of IFNγ -/- cells in an MLR (Dalton, Pitts-Meek et al. 1993). Extending these findings however, we report that there is a significant *decrease* in T cell proliferation at time of maximal Ag-induced T cell proliferation in normal mice (Figure 14). We hypothesize that if similar kinetics of T cells proliferation are observed *in vivo* (increased T cell proliferation soon after activation followed by a 50% reduction in T cell proliferation) may provide for fewer activated T cells expressing the necessary costimulatory molecules to support B cell Ig production.

2.6 - Summary

IFNγ deficient mice demonstrate significantly greater of type 2 cytokine and chemokine production following immunization under both type 1 and type 2 inducing conditions. It is interesting that in IFNγ -/- mice, there is also a significant decrease in overall Ab production, a defect not related to maturational or functional deficiencies in the B cell compartment. Collectively these data argue that, in contrast to IL-12, IFNγ is required for both optimal expression of type 1 cytokine synthesis, and for negatively regulating the intensity of type 2 cytokine responses.

3.0 - IL-18 as a negative regulator of type 2 immunity

Given the surprising findings of unenhanced type 2 immune responses in IL-12 -/- mice, but the demonstrated importance of IFNγ in controlling these same responses, we hypothesized that residual

IFNγ production in IL-12 -/- mice was responsible for controlling type 2 immunity. IL-12 is a major inducer of IFNγ (indeed IFNγ levels in IL-12 -/- mice are 10% of those seen in control mice; Figure 1, 2 and 3) and IL-18 was initially discovered by virtue of its IFNγ inducing activity, generally synergizing with IL-12 to induce very high levels of IFNγ production. As IL-12-/- IL-18-/- doubly deficient mice demonstrate a greater reduction in IFNγ production than either IL-12-/- or IL-18-/- mice (Takeda, Tsutsui et al. 1998), we hypothesized that IL-18 was responsible for controlling the residual IFNγ production in IL-12 -/- and, by extension, was also preventing the hyper-expression of type 2 immunity in IL-12 -/- mice.

3.1 - IL-12 -/- mice mount an excessive and prolonged serum IL-18 response following LPS treatment

Increased IL-18 expression in IL-12 -/- mice could compensate, to a degree, for a lack of endogenous IL-12 production. To test the global capacity of IL-12 -/- mice to mount IL-18 responses, serum IL-18 levels were determined in IL-12 deficient (p35 -/-) and C57Bl/6 control mice challenged with 1 μg LPS. As shown in Figure 15, IL-18 levels in both p35 -/- and C57Bl/6 control mice were readily detectable and indistinguishable prior to LPS administration. Following administration of LPS, IL-18 serum levels in C57Bl/6 mice rose slightly at 4 hours after injection, returning to baseline levels by day 4. In contrast, p35 -/- mice displayed serum IL-18 levels 200-250% of normal controls by 4 hours post-challenge, remaining substantially higher until day 4 after injection (Figure 15). Thus, in the absence of endogenous IL-12, IL-18 responses are enhanced in both intensity and duration.

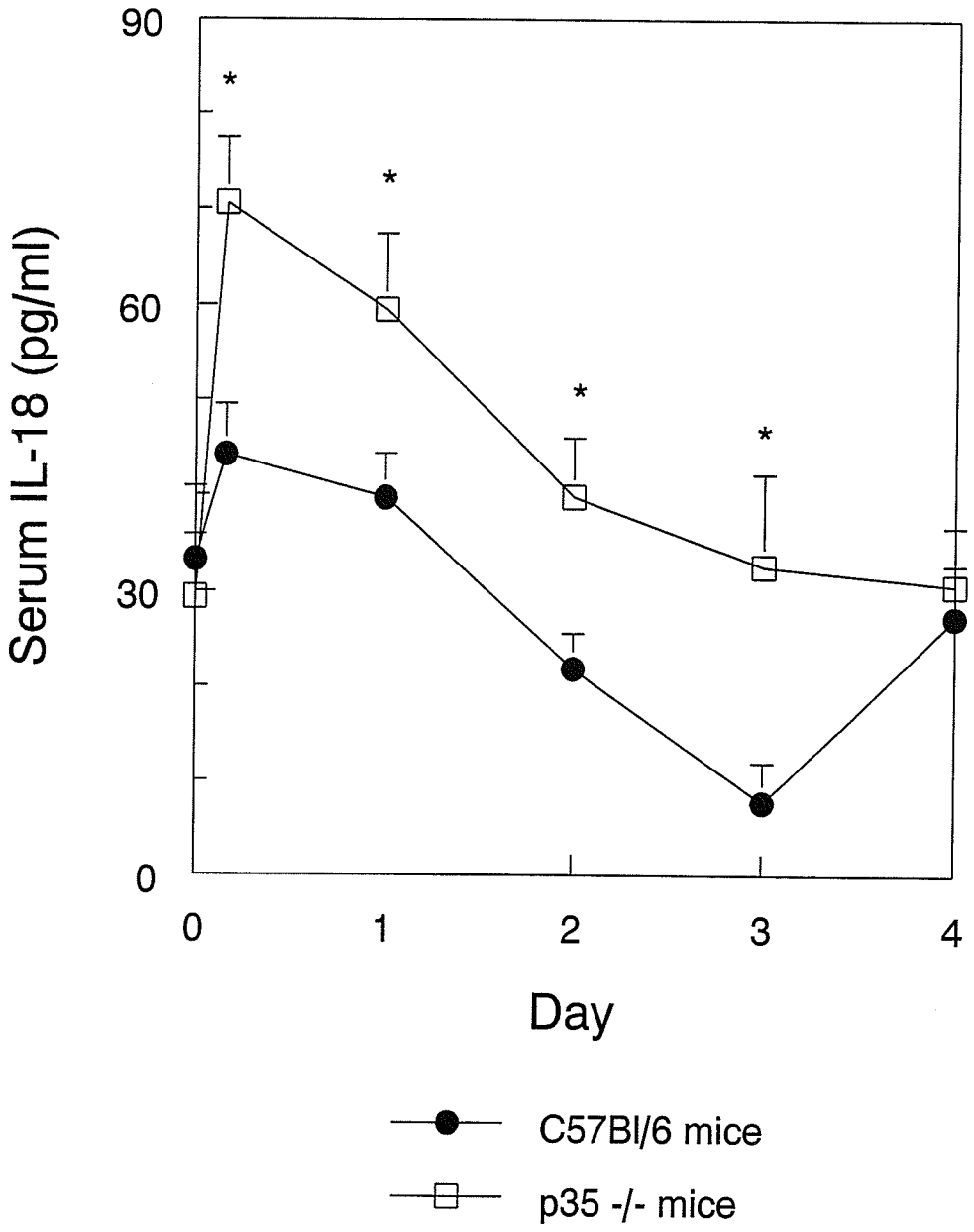

Figure 15: Increased IL-18 production in IL-12 -/- mice. Mice were given 1 μg LPS on day 0. Mice were bled at indicated times for quantification of serum IL-18 levels. Mean + SEM shown (n = 14 mice from 3 experiments). Significant differences between C57Bl/6 and p35 -/- mice indicated (Student's t test; * p values in the range of < 0.05 to $p < 0.005$).

3.2 - rIL-18 dramatically inhibits OVA stimulated IL-4 production in an IFNγ independent manner

In light of enhanced serum IL-18 levels in IL-12 deficient mice, we directly examined the capacity of IL-18 to inhibit the development of type 2 immunity. As IL-4 is pivotal in eliciting type 2 immune responses, we examined IL-4 responses in spleen cell cultures from OVA (alum) immunized C57Bl/6 mice restimulated with antigen B rIL-18 and/or IL-12. Addition of IL-18 (Figure 16a) or IL-12 (Figure 16b) resulted in similar (60%) inhibition of IL-4 production (closed symbols, $p=0.001$). Since IL-12 and IL-18 synergize in inducing IFNγ and mutually upregulate receptor expression for the other cytokine (Yoshimoto, Takeda et al. 1998), we sought to determine if inhibition of IL-4 production by IL-12 and/or IL-18 was further enhanced in the presence of both cytokines. However, stimulation with OVA in the presence of IL-12 and IL-18 did not result in inhibition of IL-4 greater than that seen with either cytokine alone (Figure 16c).

As both IL-12 and IL-18 possess IFNγ inducing capabilities, we next examined the IFNγ dependence of the observed IL-4 inhibition. Following addition of IL-18, there was virtually identical inhibition (60%, $p < 0.0001$) of IL-4 in IFNγ +/+ (Figure 16a, closed symbols) and IFNγ -/- (open symbols) cultures. This indicates that IL-18 can limit production of IL-4 in an IFNγ independent manner. In contrast, the capacity of IL-12 to inhibit IL-4 production was substantially weaker in the absence of IFNγ (Figure 16b, open symbols). Clearly IL-18 has potent inhibitory effects on IL-4 production, both through its capacity to induce IFNγ and, as demonstrated here for the first time, through IFNγ independent mechanisms.

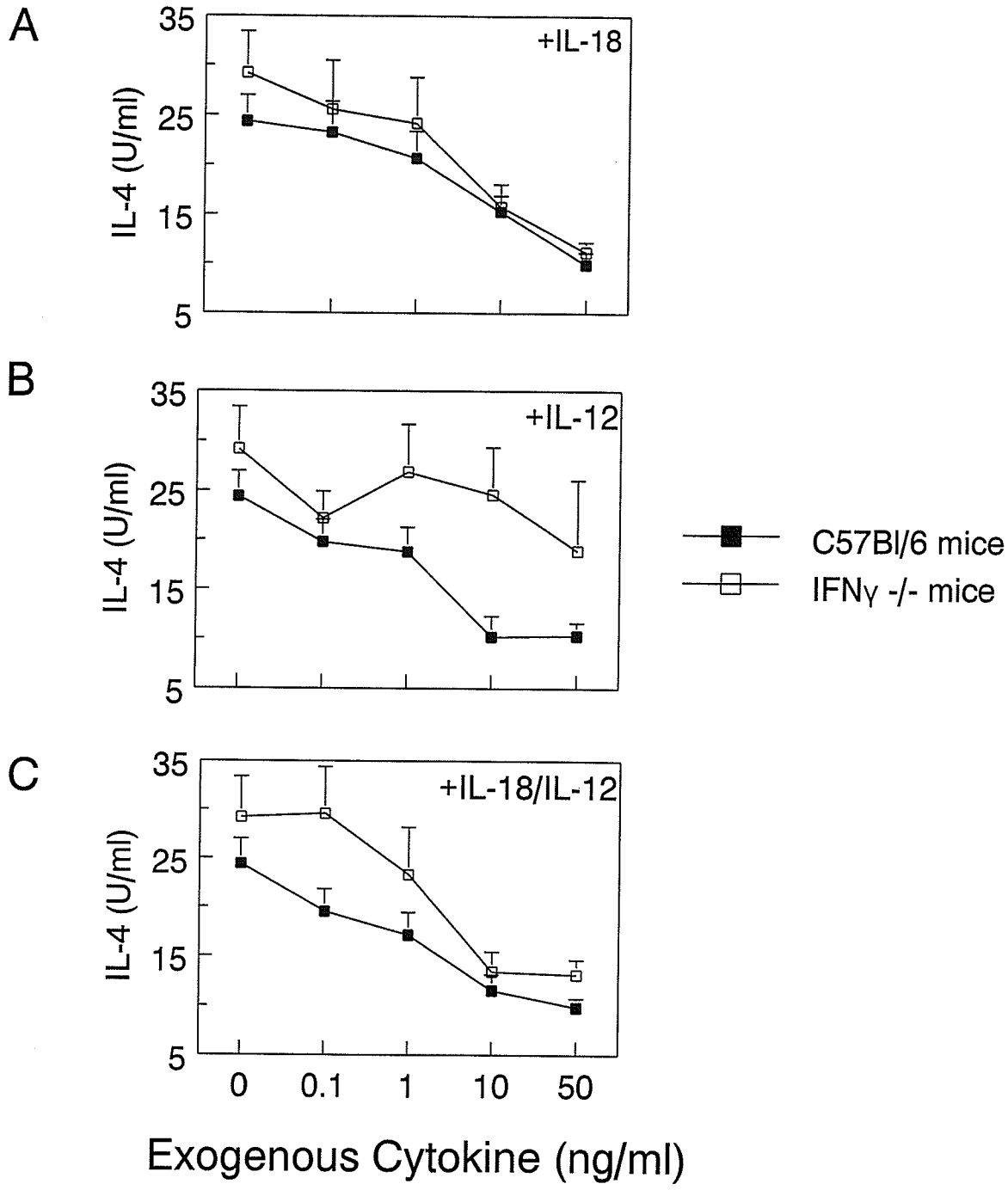

Figure 16: IL-18 inhibits IL-4 production through IFNγ independent mechanisms. Spleen cells from OVA (alum) immunized mice were cultured with OVA (1 mg/ml) and (A) IL-18 alone, (B) IL-12 alone, or (C) IL-12 + IL-18 at the indicated concentrations. Tissue culture supernatants were harvested at 24 hours for analysis of IL-4 synthesis. Mean + SEM shown (n = 16 mice from 4 experiments).

3.3 - rIL-18 does not significantly inhibit the production of other type 2 cytokines

Finally, to determine the capacity of IL-18 to inhibit other components of type 2 immunity, Ag-stimulated IL-5, IL-13, and TARC recall responses were assessed. IL-5, IL-13 and TARC production was unaffected by the presence of exogenous IL-18 in both IFNγ +/+ and IFNγ -/- cultures (Figure 17a). Inhibition of IL-5 and IL-13 synthesis in the presence of IL-12 was pronounced in IFNγ +/+ cultures (closed symbols; Figure 17b). In contrast, 100 to 500 fold greater IL-12 concentrations were required for equivalent inhibition in IFNγ -/- mice (open symbols; Figure 17b). In the absence of endogenous IFNγ, rIL-12 had no significant effect on TARC levels (Figure 17b). Simultaneous addition of IL-12 and IL-18 to cultures did not result in synergistic inhibition of type 2 responses (Figure 17c). Thus IL-12 inhibits type 2 cytokine and chemokine production chiefly through the activities of induced IFNγ. In contrast, the inhibitory effects of IL-18 are IFNγ independent, and are specific for IL-4.

3.4 - Summary

IL-12 deficient mice display significantly lower IFNγ levels than control mice, and yet display no elevation in the severity of type 2 immune responses. Here we demonstrate significantly increased IL-18 production in IL-12 -/- mice. Furthermore, the demonstration that IL-18 can specifically, and IFNγ-independently, inhibit IL-4 production, suggests the possibility that IL-18 is a potent inhibitor of type 2 immunity

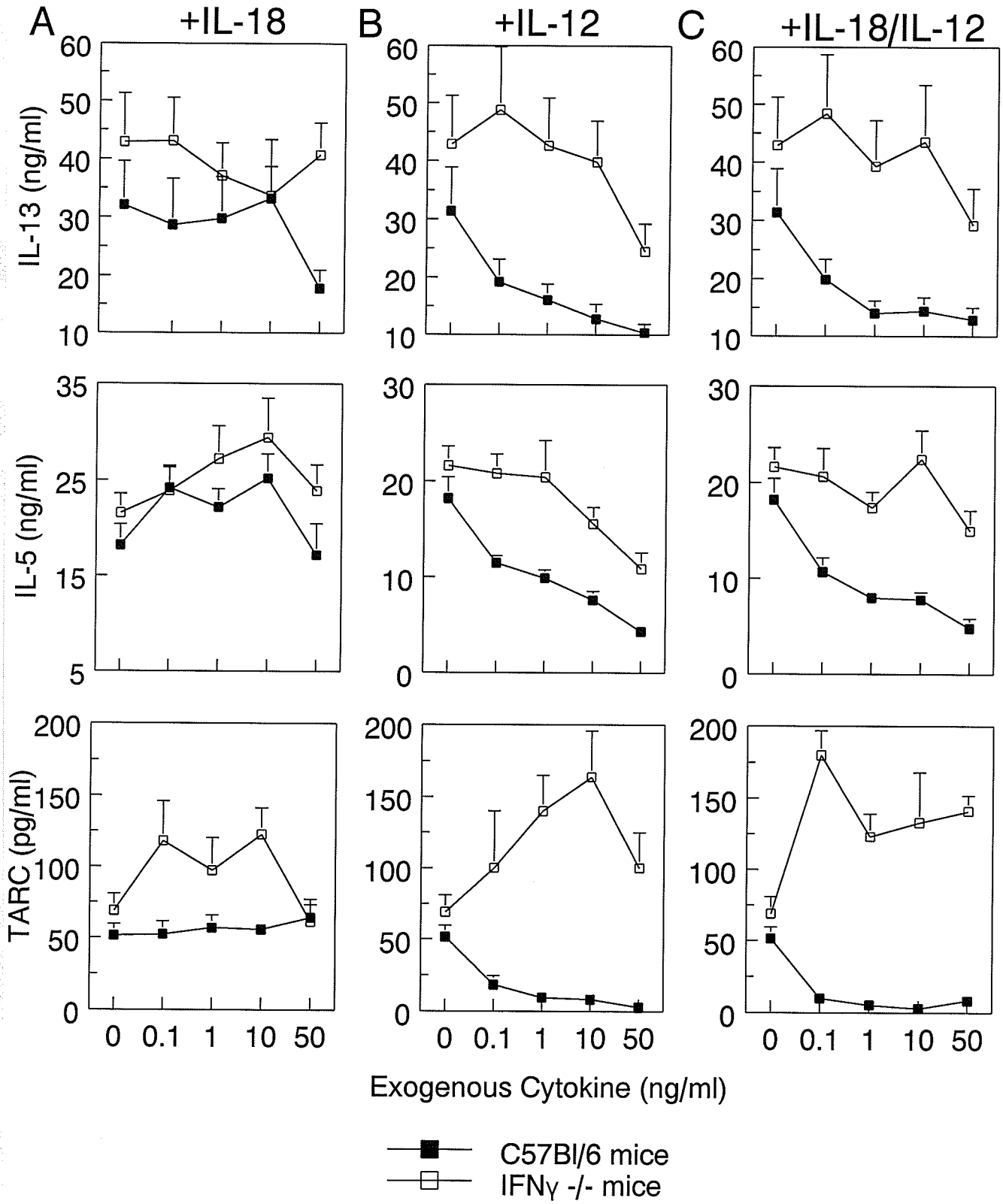

Figure 17: IL-18, in contrast to IL-12, does not inhibit IL-5, IL-13 or TARC synthesis. Spleen cells from OVA (alum) immunized mice were cultured with OVA (1 mg/ml) and (A) IL-18 alone, (B) IL-12 alone, or (C) IL-12 + IL-18 at the indicated concentrations. Mean + SEM shown (n = 16 mice from 4 experiments).

4.0 - Immune redirection following treatment with chemically modified allergen

OA-POL is a high molecular weight, glutaraldehyde polymerized version of ovalbumin, ($M_R \sim 800$ times that of native OVA). Previous studies have indicated that treatment of mice with OA-POL has the capacity to redirect type 2 immune responses (associated with allergy) to a more type 1 dominated immune profile (associated with clinical tolerance). This immune redirection is similar to that seen in the mucosal tissues of patients undergoing successful allergen specific immunotherapy. While, specific immunotherapy is successful in some patients, and with certain allergens, the mechanisms which mediate the observed immune redirection is not yet understood. Therefore, in the subsequent sections, we use OA-POL as a model system to explore the endogenous factors which are critical for stably preventing induction of IgE synthesis and redirecting existing type 2 dominated immune responses.

4.1 - Treatment with chemically modified allergen can prevent *de novo* type 2 Ab responses

To confirm that pre-treatment of mice with chemically modified allergen has the capacity to redirect *de novo* Ab responses, mice were treated with nothing, or with 100 μg OA-POL in saline 14, 12, and 10 days before immunization with 2.0 μg OVA (alum). As expected, untreated mice demonstrated high levels of OVA-specific and total IgE production, but relatively low levels of OVA-specific and total IgG_{2c} following OVA (alum) immunization (Figure 18). In contrast, IgE responses in OA-POL pre-treated mice were 30-50% of those seen in control animals (Figure 18). Even following subsequent boosts with 2.0 μg OVA (alum), OA-POL treated mice demonstrated significantly lower

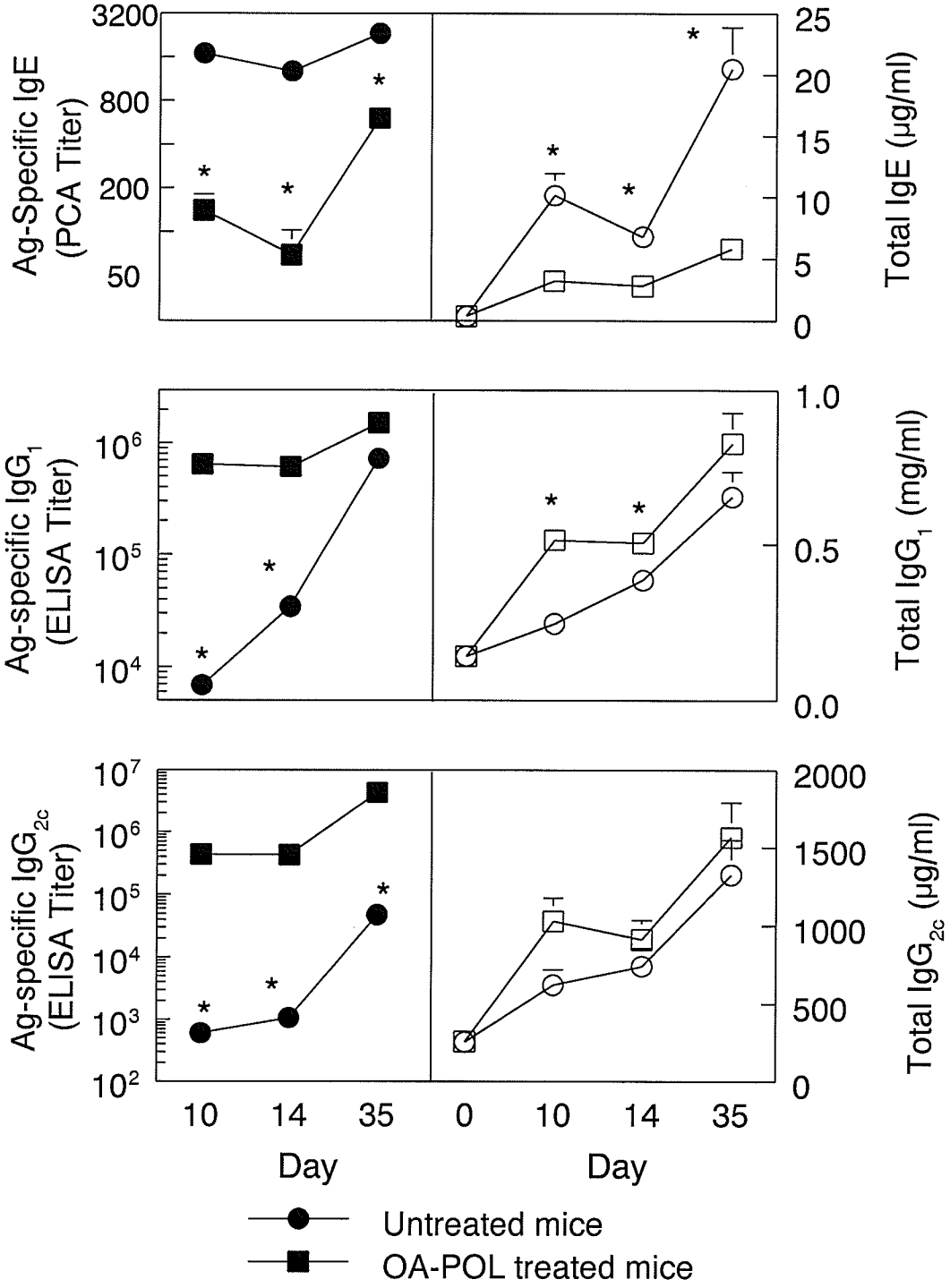

Figure 18: OA-POL treatment dramatically decreases *de novo* IgE responses in C57Bl/6 mice. C57Bl/6 mice were untreated or treated with 100 µg OA-POL (saline) on days -14, -12 and -10, and immunized with OVA (alum) on days 0 and 28. Mice were bled on the indicated days for determination of total (open symbols) and OVA-specific (closed symbols) Ig levels. Mean + SEM shown (n = 24 mice from 6 experiments). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test;* p values in the range of $p<0.05$ to $p<1 \times 10^{-11}$).

IgE levels (total and OVA-specific) following OVA (alum) immunization than untreated animals, demonstrating the stability of the type 1 dominated immune response generated following OA-POL treatment (Figure 18). Concomitant with marked decreases in IgE production following OA-POL treatment, there was a dramatic rise in the production of OVA-specific IgG_{2c} levels (up to 750 fold) and slight increases in total IgG_{2c} levels (Figure 18). IgG_1 levels were also consistently higher following OA-POL treatment and subsequent OVA (alum) immunization (Figure 18). These data confirm that OA-POL has the ability to dramatically redirect developing Ab responses from type 2 dominated to type 1 dominated.

4.2 - OA-POL treatment abrogates existing IgE production

While treatment with OA-POL prior to immunization clearly prevents the induction of a substantial IgE response, even following repeated immunization under type 2 conditions, the more relevant situation (in that it more closely resembles the situation in human disease where treatment will rarely be prophylactic) is the abrogation of previously established IgE responses. To confirm previous reports or successful redirection of existing IgE responses, mice were immunized with 0.2 μg OVA (alum) to induce a type 2 dominated immune response. Mice were then treated with nothing, or three courses of OA-POL (saline) over the span of five weeks. Two weeks after the final treatment with OA-POL, mice were boosted with OVA (alum). As shown in Figure 19, immunized but untreated C57Bl/6 mice produced high levels of both OVA-specific and total IgE indicating a very strong type 2 character to the immune response. In mice treated with OA-POL, recall IgE levels were significantly lower, being 5-40% of those seen in mice which did not receive OA-POL

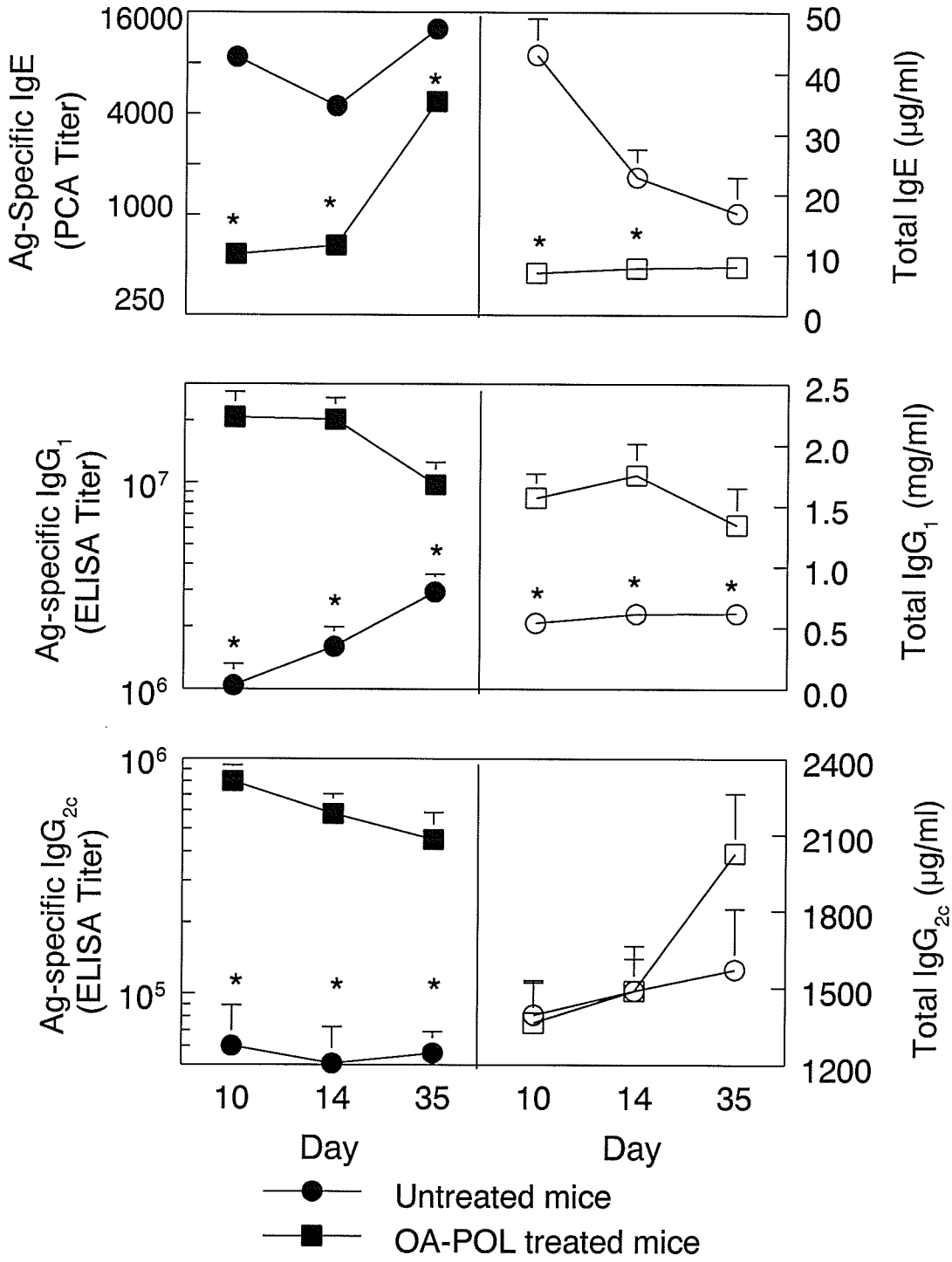

Figure 19: OA-POL treatment abrogates established IgE responses in C57Bl/6 mice. C57Bl/6 mice were immunized with 0.2 µg OVA (alum) and treated with nothing or OA-POL. Mice were boosted with 0.2 µg OVA (alum) on days 0 and 28. Mice were bled on the indicated days for determination of total (open symbols) and OVA-specific (closed symbols) Ig levels. Mean + SEM shown (n = 12 mice from 3 experiments). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * p values in the range of $p<0.05$ to $p<1 \times 10^{-11}$).

treatment (Figure 19). As previously reported, OVA-specific IgG_1, total IgG_1 levels and OVA-specific IgG_{2c} levels were markedly enhanced following treatment with OA-POL (Figure 19).

4.3 - Summary

We confirm here the previous reports that OA-POL administration has the capacity to facilitate redirection of both *de novo* and existing IgE responses by >90% while simultaneously increasing IgG_{2c} production by several orders of magnitude. Thus, we used OA-POL treatment as a model of successful immune redirection in subsequent experiments designed to explore the mechanisms of OA-POL mediated changes in the phenotype of the OVA-elicited immune response.

5.0 - Role of B cells in OA-POL mediated immune redirection

To examine the importance of B cells in mediating the immune response elicited following administration of chemically modified allergen, we made use of B cell deficient mice. It had previously been shown that OVA-specific Abs have a much lower affinity for OA-POL than native OVA (HayGlass and Strejan 1983). Based on these results, we hypothesized that B cells are not critical APCs involved in the uptake and presentation of OA-POL. Recent reports of a requirement for B cells in other models of immune redirection (Tsitoura, Yeung et al. 2002) prompted us to directly explore this hypothesis.

5.1 - Significant inhibition of type 2 cytokine production is still evident following OA-POL treatment of B cell deficient mice

Prior to examination of the importance of B cells on the effectiveness of OA-POL pre-treatment, we first characterized the cytokine response elicited by OVA (alum) immunization and subsequent *in vitro* OVA restimulation in B cell deficient μMT mice. As shown in Figure 20, OVA-stimulated IL-5 and IL-13 production was modestly reduced in spleen cell cultures from μMT mice (30% and 45% reductions respectively, $p < 0.05$). While there was a trend towards reduced IFNγ production in cultures from μMT mice, this did not reach statistical significance (Figure 20). There was no detectable decrease in the production of IL-4 or IL-10.

To directly examined the impact of B cell deficiency on the capacity of OA-POL to influence the immune response we treated B cell deficient (μMT) mice and control C57Bl/6 mice with 100 μg of OA-POL, or nothing, on days -14, -12 and -10 and subsequently immunized all mice with 2.0 μg OVA (alum). We subsequently analyzed spleen cell cytokine production following *in vitro*, OVA restimulation. As shown in Figure 21, both untreated and OA-POL treated mice (either C57Bl/6 or μMT) produced similar amounts of IFNγ following *in vitro* OVA stimulation. In contrast, treatment of C57Bl/6 mice with OA-POL resulted in a marked inhibition (~60%) of the production of the type 2 cytokines IL-4 and IL-13 (Figure 21). Similarly effective inhibition of type 2 cytokine production was also seen in μMT mice treated with OA-POL. While the differences in OVA stimulated cytokine production in cultures from OVA (alum) immunized C57Bl/6 and μMT mice make direct comparison of the effects of OA-POL treatment difficult, the degree of change in cytokine production

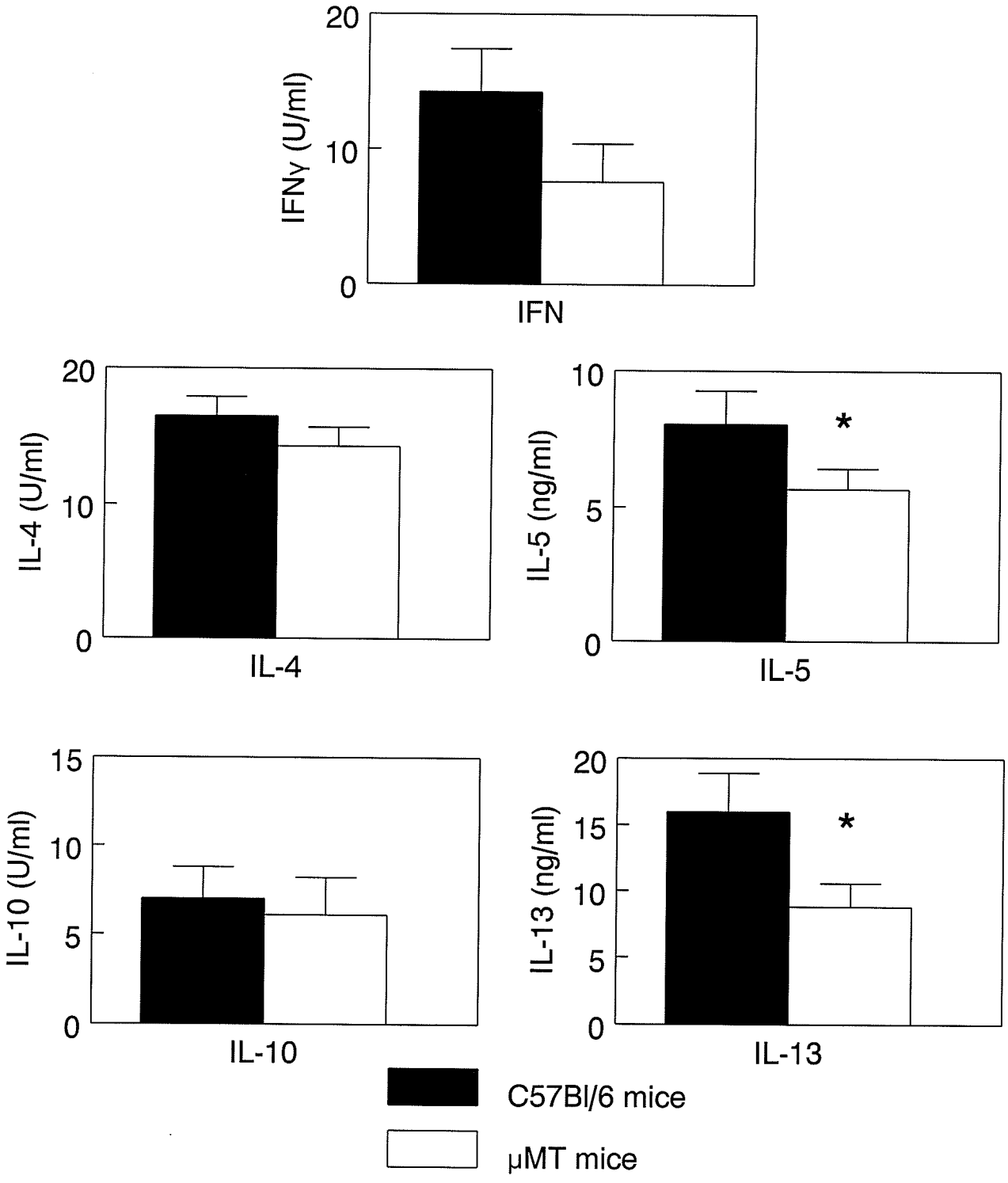

Figure 20: Type 2 cytokine production diminished in μMT mice. Mice were immunized with OVA (alum) sacrificed on day and spleen cells stimulated Mean + SEM shown (n = 8 mice from 2 experiments). Significant differences between C57Bl/6 and μMT mice are indicated (Student's t test * $p < 0.05$).

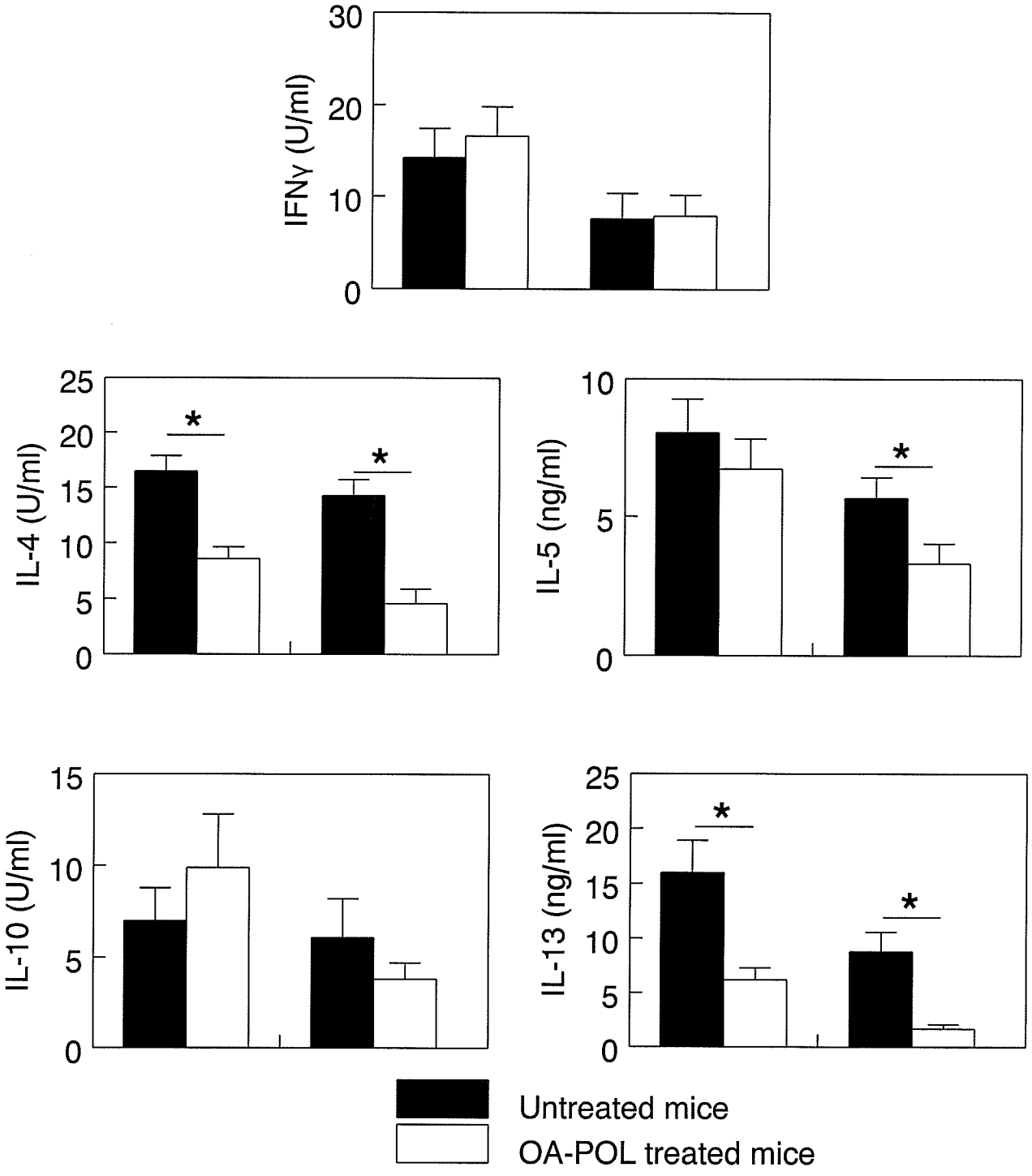

Figure 21: Diminished type 2 cytokine production in OA-POL treated μMT mice. C57Bl/6 and B cell deficient mice were treated with nothing or OA-POL prior to immunization with OVA (alum) on day 0 and sacrificed on day 5. Mean + SEM shown (n = 8 mice from 2 experiments). Significant differences between C57Bl/6 and μMT mice are indicated (Student's t testl * p values in the range of $p<0.05$ to $p< 0.001$).

following OA-POL treatment was similar in the two strains of mice (Table 3). That the magnitude of type 2 cytokine production was equivalent in C57Bl/6 and μMT mice indicates that the absence of B cells has no effect on the capacity of OA-POL to inhibit the induction of type 2 immunity.

5.2 - B cell deficiency does not reduce the magnitude of the shift from type 2 to type 1 dominated cytokine production.

Finally, to determine the balance of type 1 versus type 2 immunity before and after OA-POL administration in the two strains of mice we determined the ratio of IFNγ to IL-4 production in μMT and C57Bl/6 mice. As displayed in Figure 21, under normal OVA (alum) immunization conditions, both strains have an IFNγ:IL-4 ratio < 1.0 characteristic of a type 2 dominated immune response. In contrast, in OA-POL treated animals, this ratio rises above 1, suggesting a more type 1 dominated immune response (Figure 22). The magnitude of the increase in IFNγ:IL-4 ratio following OA-POL treatment is similar in both C57Bl/6 and μMT mice again suggesting B cells are not required for the capture and presentation of OA-POL (Table 3).

5.3 - Summary

Here we demonstrate that OA-POL is equally effective at inhibiting type 2 cytokine production and shifting the immune response from a type 2 dominated immune response to a type 1 dominated one in both C57Bl/6 and μMT mice. Collectively these data argue that B cells are not required for induction of the stable type 1 immune response elicited by administration of chemically modified

Table 3: OA-POL mediated changes in cytokine production in C57Bl/6 and μMT mice

Strain[b]	n/x	Fold Decrease in Cytokine Production[a]				Increase in IFNγ:IL-4 Ratio[b]
		IL-4	IL-5	IL-13	IFNγ	
C57Bl/6	22/4	1.92	1.19	2.57	0.974	2.25
μMT	18/3	2.76	1.71	5.21	0.965	2.86

a - Decrease of *in vitro*, OVA-stimulated cytokine production following OA-POL treatment
b - Increase in IFNγ:IL-4 Ratio following OA-POL treatment
b- μMT mice are on a C57Bl/6 background

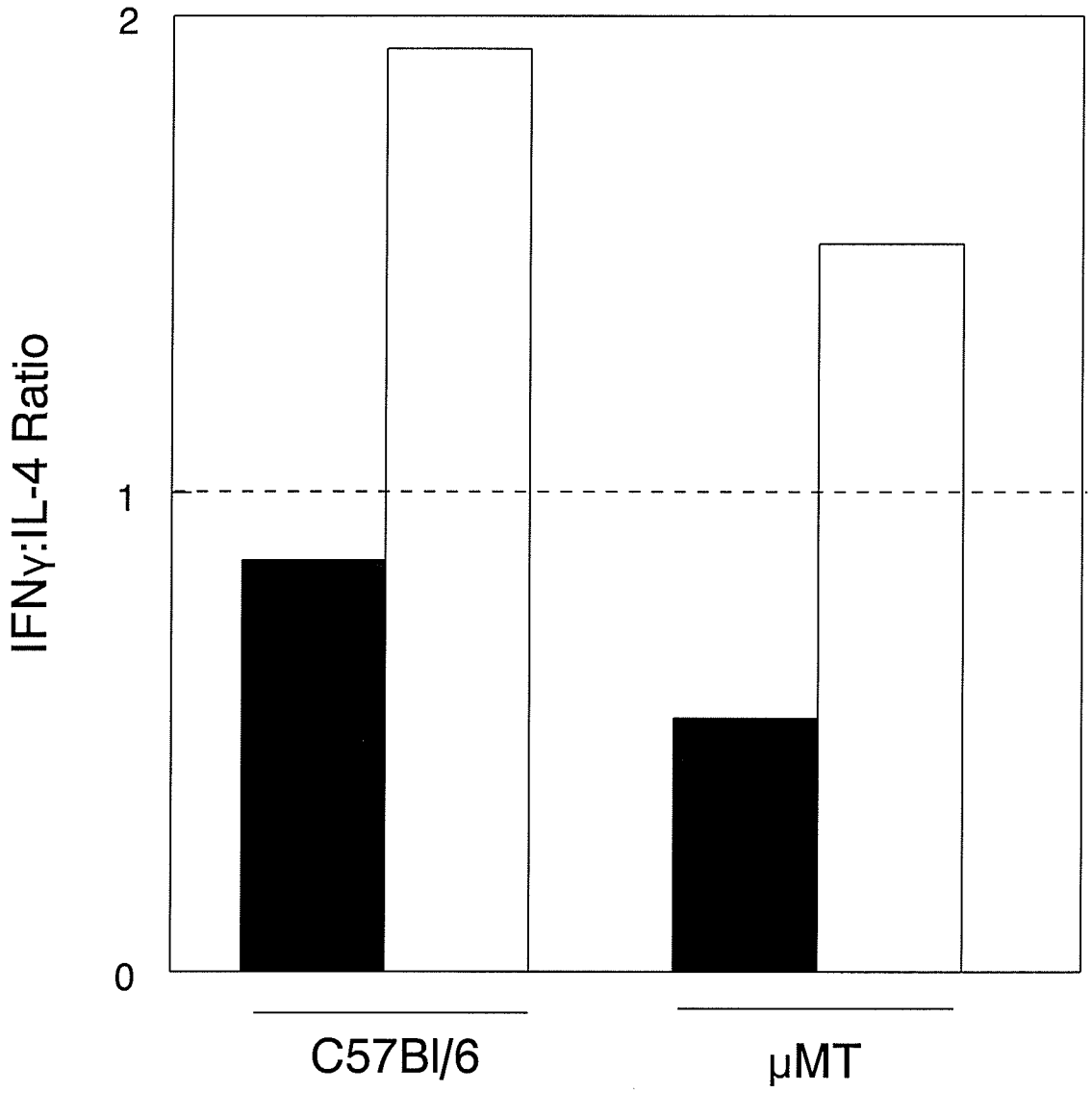

Figure 22: Similar increase in IFNγ:IL-4 ratio in both C57Bl/6 and μMT mice following OA-POL treatment. Comparison of mean IFNγ and IL-4 production from *in vitro*, OVA restimulated spleen cell cultures from C57Bl/6 and μMT mice (n = 8 mice from 2 experiments).

allergen. We hypothesize that dendritic cells and macrophages, because of their ability to process and present very large antigens, may be critical APCs involved in presentation of OA-POL.

6.0 - OA-POL treatment does not stimulate enhanced IL-12 production

Treatment with OA-POL causes a dramatic inhibition of IgE production and concomitant increase in IgG_{2c} levels, regardless of whether OA-POL is administered before, or after attempts to initiate a type 2 dominated immune response. IL-12 is a potent promoter of type 1 immunity and essential for optimal production of IFNγ (IFNγ levels in IL-12 -/- mice are ~10% of those seen in C57Bl/6 controls, see Figure 8 and 9). Previous reports demonstrate that macrophages produce IL-12 following mannan-receptor mediated phagocytosis of large, but not small chitin coated particles (Shibata, Metzger et al. 1997). Since OA-POL is ~800 times the M_r of native OVA, and our previous data argues that B cells are minimally involved in capture and presentation of OA-POL, we hypothesized that OA-POL, (a large glycosylated protein), is taken up preferentially by macrophages and dendritic cells, which are triggered to produce IL-12. To test the capacity of OA-POL treated mice to produce IL-12, mice were treated with 100 μg OVA or OA-POL in saline on days 0, 2 and 4, and sacrificed on day 8 to examine *in vitro* IL-12 p40 production in the presence of media alone, 1 mg/ml OVA, or 1 mg/ml OA-POL (± 150 U/ml rIFNγ).

As shown in Figure 23, cells from OVA (saline) immunized mice cultured in media alone produced 174 ± 28 ng/ml of IL-12 p40. This rose to 334 ± 18 ng/ml when cells were cultured in the presence 150 U/ml of rIFNγ (Figure 23). IL-12 p40 production was not enhanced in spleen cells taken from

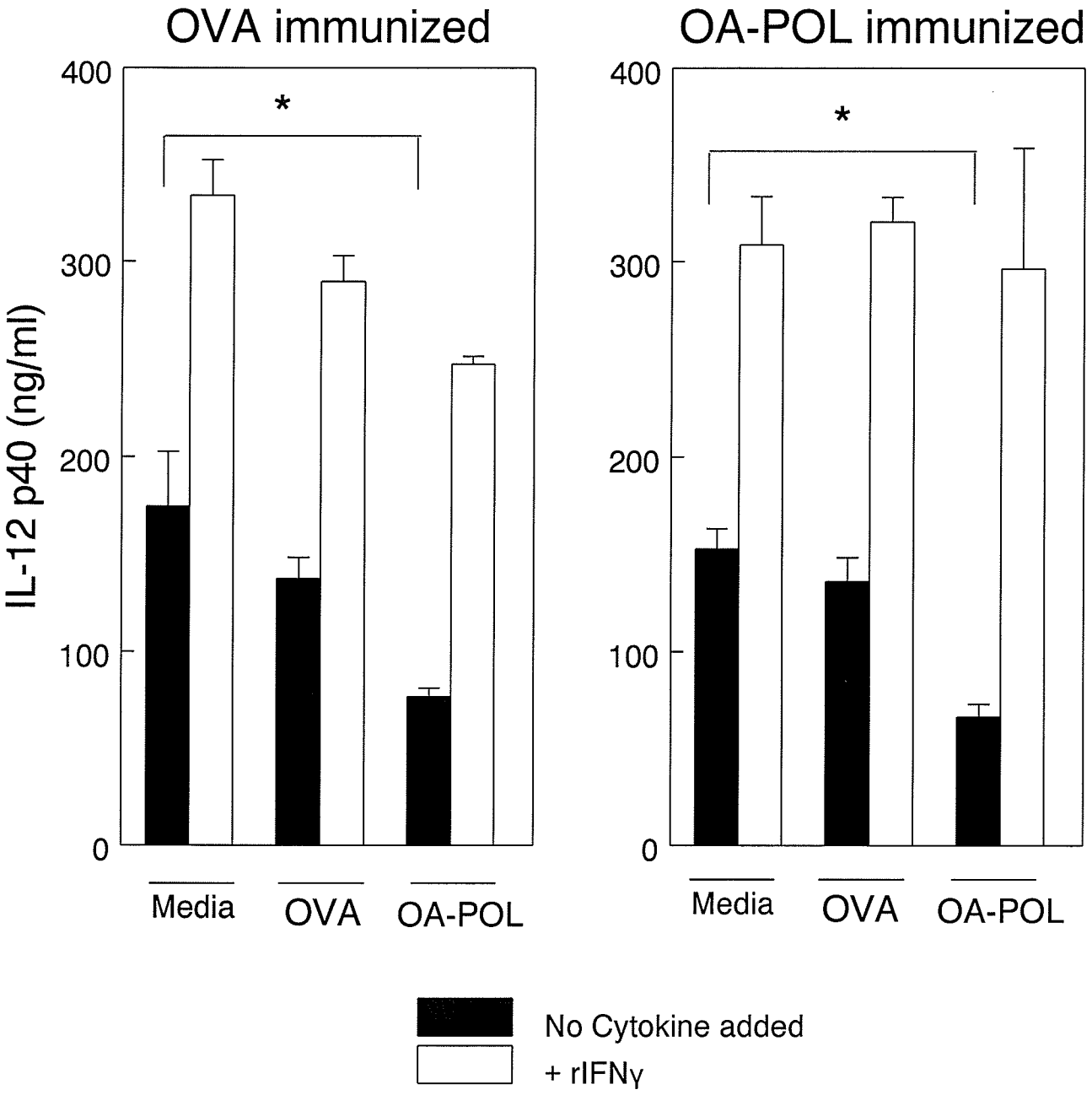

Figure 23: OA-POL treatment does not increase Ag-stimulated IL-12 p40 production. Mice were treated with 100μg OVA (saline) or OA-POL (saline) on days 0, 2 and 4. Mice were sacrificed on day 8 and spleen cells were cultured with 1.0 mg/ml OVA or 1.0 mg/ml OA-POL ± 150 U/ml rIFNγ. Tissue culture supernatants were harvested at 48 hours for analysis of IL-12 p40 production. Mean + SEM shown (n = 4 mice from 1 experiment). Significant differences between p40 production in stimulated and media control cultures are indicated (Student's t test; * $p<0.05$).

OA-POL (saline) immunized mice (152 ± 10 pg/ml and 308 ± 25 pg/ml when cultured with media alone or rIFNγ respectively) ($p > 0.05$, Figure 23). Stimulation of spleen cells with OVA did not increase the amount of IL-12 p40 produced in cultures from OVA and OA-POL (saline) immunized mice to levels above that seen in media stimulated cultures (Figure 23). Stimulation with OVA + IFNγ increased the mean IL-12 p40 production, but there was no greater elevation of IL-12 p40 production in cultures from OA-POL (saline) immunized mice compared to OVA (saline) immunized mice (Figure 23). Taken together these data indicate that OA-POL does not have a greater capacity to stimulate IL-12 production suggesting that OA-POL treatment does not redirect type 2 immune responses by causing generalized increases in IL-12 production. However, the lack of enhancement of IL-12 production reported above may have been a result of the use of inappropriate stimuli to trigger IL-12 production, or an inappropriate reliance on IL-12 p40 as an accurate surrogate for predicting bioactive IL-12 p70 production. Thus, additional methods of determining the importance of IL-12 production following OA-POL treatment are explored below.

7.0 - Role of endogenous IL-12 and IFNγ in OA-POL mediated suppression of type 2 immunity

OA-POL does not detectably increase IL-12 production following *in vivo* or *in vitro* stimulation suggesting that OA-POL might not limit the development of type 2 immune responses via increased production of type 1 inducing monokines. As an alternative approach to examine the role of IL-12 and IFNγ we used IL-12 -/- and IFNγ -/- mice. We hypothesized that both endogenously produced IL-12 and IFNγ are important for the observed inhibition of developing and existing type 2 immune responses seen following OA-POL administration.

7.1 - IL-12 is not required for optimal inhibition of *de novo* IgE production

In subsequent experiments we examined the effect of OA-POL treatment in IL-12 deficient (either p35 -/- or p40 -/- mice) and IFNγ -/- mice. As expected, there was a marked inhibition of OVA-specific IgE at day 10, and a simultaneous increase of day 14 OVA-specific IgG_{2c} levels following OA-POL pre-treatment of C57Bl/6 mice (Figure 24). In the absence of endogenous IL-12 production (p35 -/- and p40 -/- mice) we still saw a dramatic inhibition of OVA-specific IgE following OA-POL treatment but only a modest increase in IgG_{2c} synthesis. Finally, OA-POL treatment resulted of IFNγ -/- in both a weak decrease in OVA-specific IgE and a modest increase in OVA-specific IgG_{2c} in IFNγ -/- mice demonstrating that OA-POL remains effective in both IL-12 and IFNγ deficient animals.

Due to differences in Ab production in OVA (alum) immunized strains (i.e. - comparing OVA (alum immunized C57Bl/6 and IFNγ -/- mice), it is difficult to directly compare the *effectiveness* of OA-POL treatment in each strain. However, a comparison of the fold change in OVA-specific IgE production reveals that the effects of OA-POL treatment are similar in C57Bl/6 and IL-12 p40 -/- mice (10-11 fold inhibition; Table 4) and slightly more effective in p35 -/- mice (>30 fold inhibition; Table 4). In contrast, the inhibition of OVA-specific IgE is much less efficient in IFNγ -/- mice (<5 fold inhibition; Table 4). All knockout strains of mice had dramatically impaired increases in OVA-specific IgG_{2c} as compared to C57Bl/6 controls (C57Bl/6 mice - 400 fold increases, knockout mice - 20 - 55 fold increases; Table 4). Changes in total IgG_{2c} levels following pre-treatment with OA-POL were similar between all strains examined. Collectively, these data suggest that enhanced

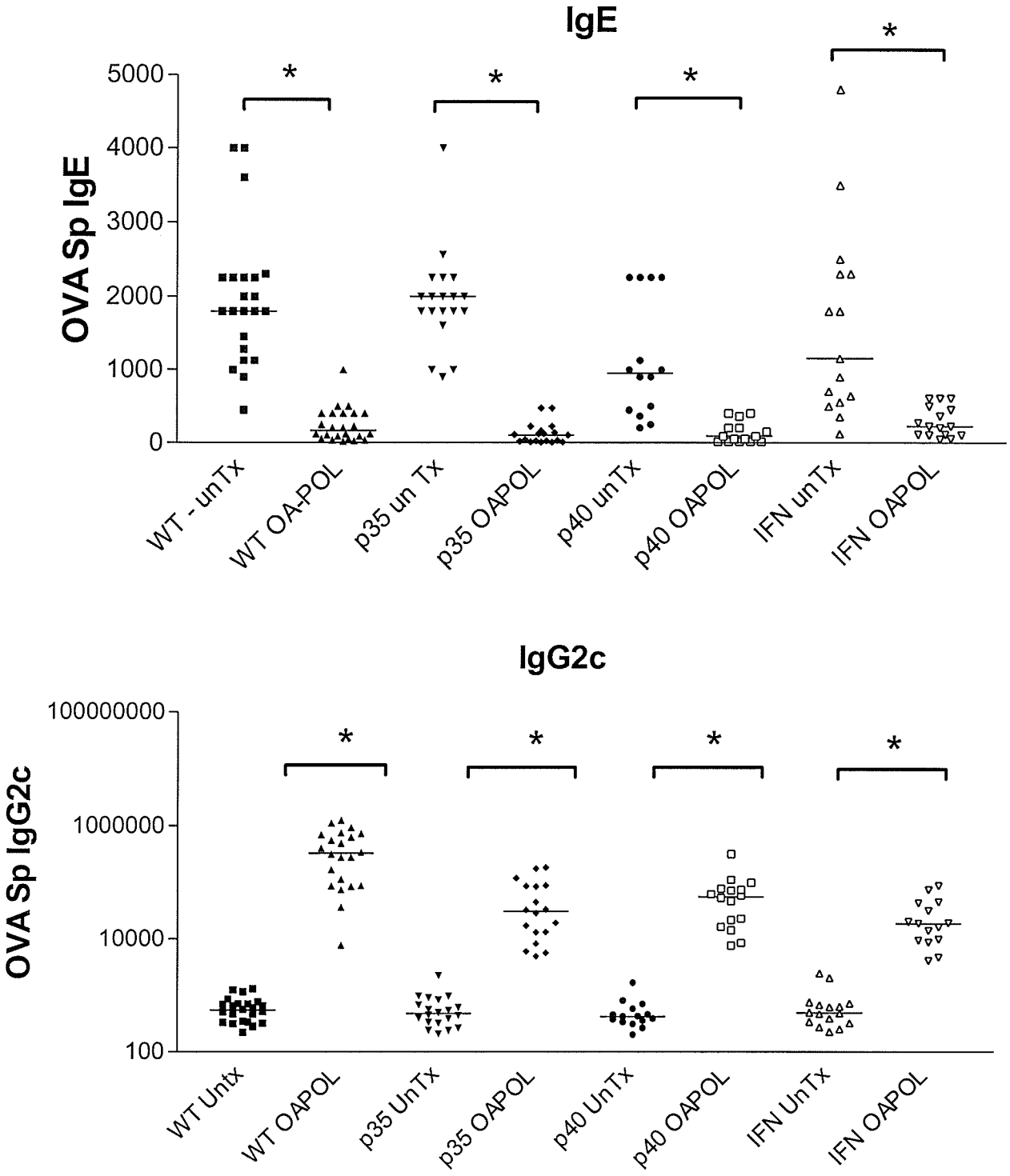

Figure 24: IgE inhibition following OA-POL treatment in IFNγ -/- and IL-12 -/- mice. C57Bl/6, IL-12 p35 -/-, IL-12 p40 -/- and IFNγ -/- mice were treated with nothing or OA-POL prior to immunization with OVA (alum). Mice were bled on days 10 and 14 for analysis of OVA specific IgE and IgG_{2c} respectively. Median indicated by line. Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p< 0.0001$).

Table 4: Fold changes in Ab levels following OA-POL pre-treatment in C57Bl/6, IFNγ -/- and IL-12 -/- mice

Strain[c]	n/x[d]	Fold Decrease in IgE levels[a]		Fold Increase in $IgG2_c$ levels[b]	
		OVA specific	Total	OVA specific	Total
C57Bl/6	24/6	11.8	3.12	400	1.23
IL-12 p35 -/-	20/4	32.5	7.29	43.9	1.38
IL-12 p40 -/-	15/3	10.7	2.58	57.4	0.97
IFNγ -/-	16/4	4.73	2.08	20.7	1.12

a - Decrease in mean IgE responses in OA-POL treated animals as measured at day 10
b - Increase in mean IgG_{2c} responses in OA-POL treated animals as measured at day 14
c - All deficient strains on a C57Bl/6 background
d - "n" mice used over "x" independent experiments

endogenous IL-12 is a redundant regulator of IgE production following treatment with OA-POL. In contrast, IFNγ appears to play an irreplaceable role in inhibiting IgE production.

7.2 - IFNγ required for optimal inhibition of established IgE responses

As abrogation of existing IgE responses is ultimately the more clinically relevant intervention, we examined the effects of OA-POL treatment following immunization with OVA (alum) in IL-12 p35 -/-, IL-12 p40 -/-, and IFNγ -/- mice. As expected, there was substantial inhibition of day 10 OVA-specific IgE responses (~15 fold) and increased day 14 OVA-specific IgG_{2c} production (~14 fold) in C57Bl/6 animals following treatment with OA-POL (Figure 25). Treatment of OVA (alum) immunized IL-12 deficient mice with OA-POL resulted in similar, dramatic decreases of OVA-specific IgE production, whereas the increases in OVA-specific IgG_{2c} levels were dampened by the absence of IL-12. In contrast, both the increased IgG_{2c} and diminished IgE responses were somewhat blunted in IFNγ -/- mice (Figure 25).

A direct comparison of the magnitude of change in OVA-specific and total Ig production is displayed in Table 5. This confirms that the magnitude of IgE inhibition (both total and OVA-specific) is slightly higher in IL-12 deficient mice as compared to C57Bl/6 mice (~15 fold inhibition versus >21 fold inhibition respectively) (Table 5). In contrast, the abrogation of existing IgE responses in IFNγ -/- mice was less efficient - only an 8-fold and 4-fold decrease in OVA-specific and total IgE synthesis respectively was noted following OA-POL treatment (Table 5). All knock out strains had greatly diminished increases in OVA-specific IgG_{2c} production, ranging from only

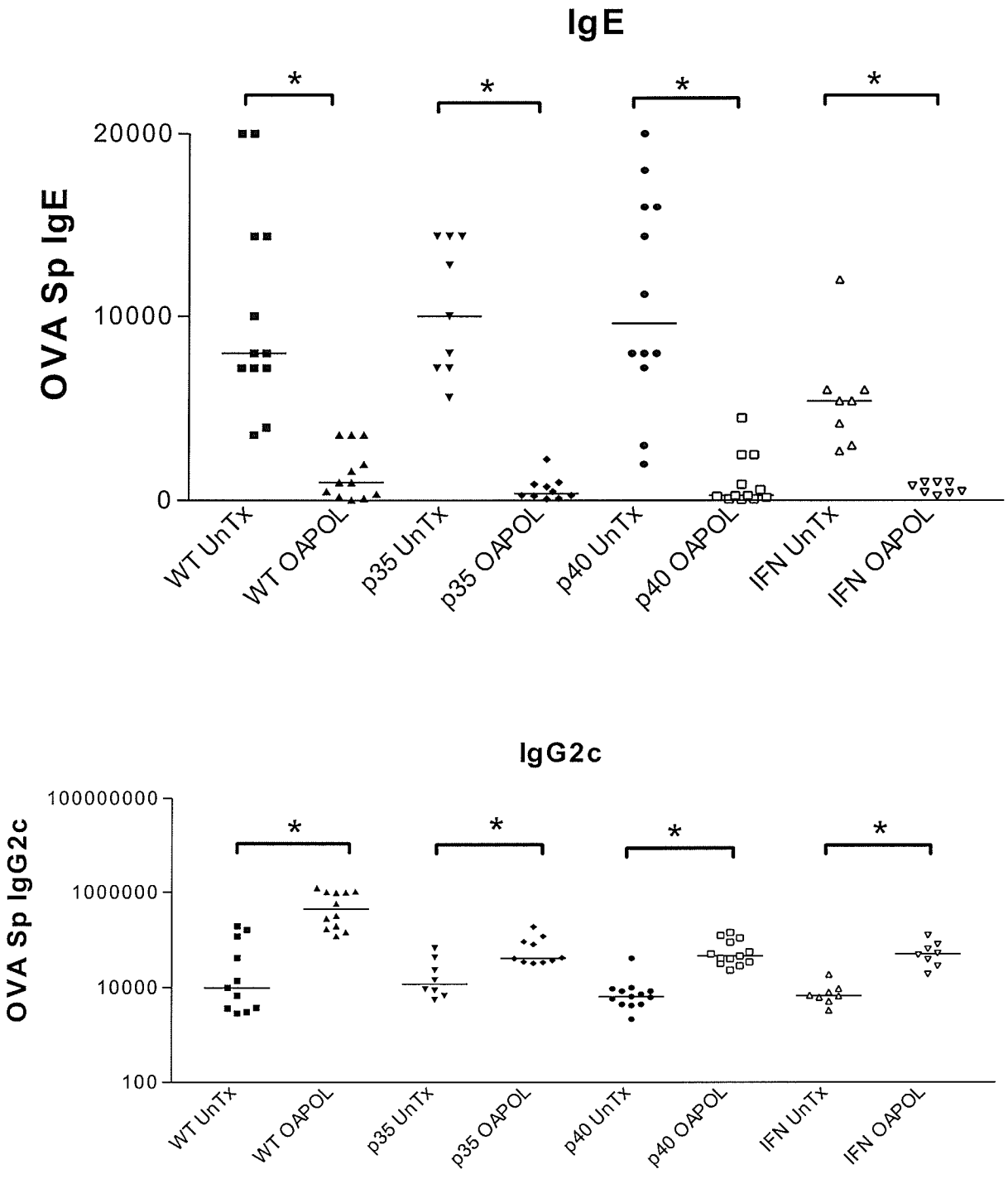

Figure 25: Inhibition of IgE synthesis in OA-POL treated, OVA (alum) immunized IL-12 -/- and IFNγ -/- mice. C57Bl/6, IL-12 p35 -/-, IL-12 p40 -/- and IFNγ -/- mice were immunized with 0.2 μg OVA (alum) and treated with nothing or OA-POL. Mice were boosted with 0.2 μg OVA (alum) on day 0 and bled on days 10 and 14 for analysis of OVA specific IgE and IgG_{2c} respectively. Median indicated by line. Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p < 0.0001$).

Table 5: Fold changes in Ab levels following redirection of established IgE responses in C57Bl/6, IFNγ -/- and IL-12 -/- mice

Strain[c]	n/x[d]	Fold Decrease in IgE levels[a]		Fold Increase in IgG2a levels[b]	
		OVA specific	Total	OVA specific	Total
C57Bl/6	16/3	14.86	6.22	13.45	1
IL-12 p35 -/-	10/3	23.71	8.6	3.11	0.73
IL-12 p40 -/-	16/3	21.69	6.02	6.93	0.8
IFNγ -/-	8/2	8.7	4.06	7.05	1.34

a - Decrease in mean IgE responses in OA-POL treated animals as measured at day 10

b - Increase in mean IgG_{2a} responses in OA-POL treated animals as measured at day 14

c - All deficient strains on a C57Bl/6 background

d - "n" mice used in "x" independent experiments

a 3 to 7-fold increase compared to the nearly 14-fold increase seen in control mice (Table 5). Total IgG_{2c} was largely unaffected by OA-POL treatment in any strain (Table 5). These data provide further support to the conclusion that IFNγ -/-, but not IL-12, is a critical requirement for optimal inhibition of IgE responses following treatment with OA-POL, one model of therapeutic intervention similar to that used in clinical allergen immunotherapy.

7.3 - Summary

OA-POL successfully abrogated both *de novo* and established IgE production in all strains of knockout mice examined. However, the magnitude of IgE inhibition differed between strains: IL-12 -/- mice were consistently able to match, or surpass the level of IgE inhibition seen in C57Bl/6 controls. IgE inhibition was consistently less efficient in IFNγ -/- mice. In contrast, all knockout mice had substantially reduced capacity to up-regulate the production of type 1 associated antibodies. These data again suggest that IL-12 is critical only in the optimal induction of type 1 immune responses, but IFNγ is important for both inhibiting type 2 immunity *and* promoting type 1 immune responses.

8.0 - IL-12 and IL-18 responsiveness in OA-POL treated mice

IFNγ, but not IL-12, is directly involved in inhibition of type 2 immune responses and is required for optimal inhibition of IgE synthesis following treatment with OA-POL. As both IL-12 and IL-18 are known for their potent IFNγ inducing properties, we next explored the responsiveness of mice to rIL-

12 and rIL-18 following OA-POL treatment. We hypothesized that OA-POL treatment causes increased responsiveness to endogenous levels of IL-12 and IL-18. As a result of increased responsiveness to IL-12 and/or IL-18, we predict there would be a greater signal driving Th1 immunity, which prevents the development of a substantial type 2 immune response.

8.1 - Increased IL-12 responsiveness in OA-POL treated mice

To test the hypothesis that OA-POL induces increased responsiveness to IL-12 and/or IL-18, we immunized mice with 100 μg of OVA or OA-POL on days 0, 2 and 4. To focus directly on the capacity of chemically modified allergen versus native allergen to modulate IL-12/IL-18 responsiveness, all immunizations were carried out in the absence of adjuvants. Following sacrifice on day 8, spleen cells were cultured in the presence of exogenous IL-12 or IL-18 ± OVA. We examined IFNγ production at both 18 hours (principally NK cell derived) and 48 hours (both NK and T cell derived). Spleen cells from OA-POL treated mice did not produce significantly more IFNγ in response to IL-12 at 18 hours, regardless of the presence of OVA (Figure 26). However, in response to IL-12 + OVA at 48 hours, spleen cells from OA-POL (saline) immunized mice produced ~2 fold more IFNγ than spleen cells from OVA (saline) immunized mice. IFNγ production in response to rIL-18 was not significantly different at any time point or culture condition examined (Figure 26). Thus, OA-POL treatment does result in significantly increased responsiveness to IL-12.

8.2 - Increased IL-12 responsiveness in OA-POL treated mice is not a result of enhanced IL-12Rβ2 expression

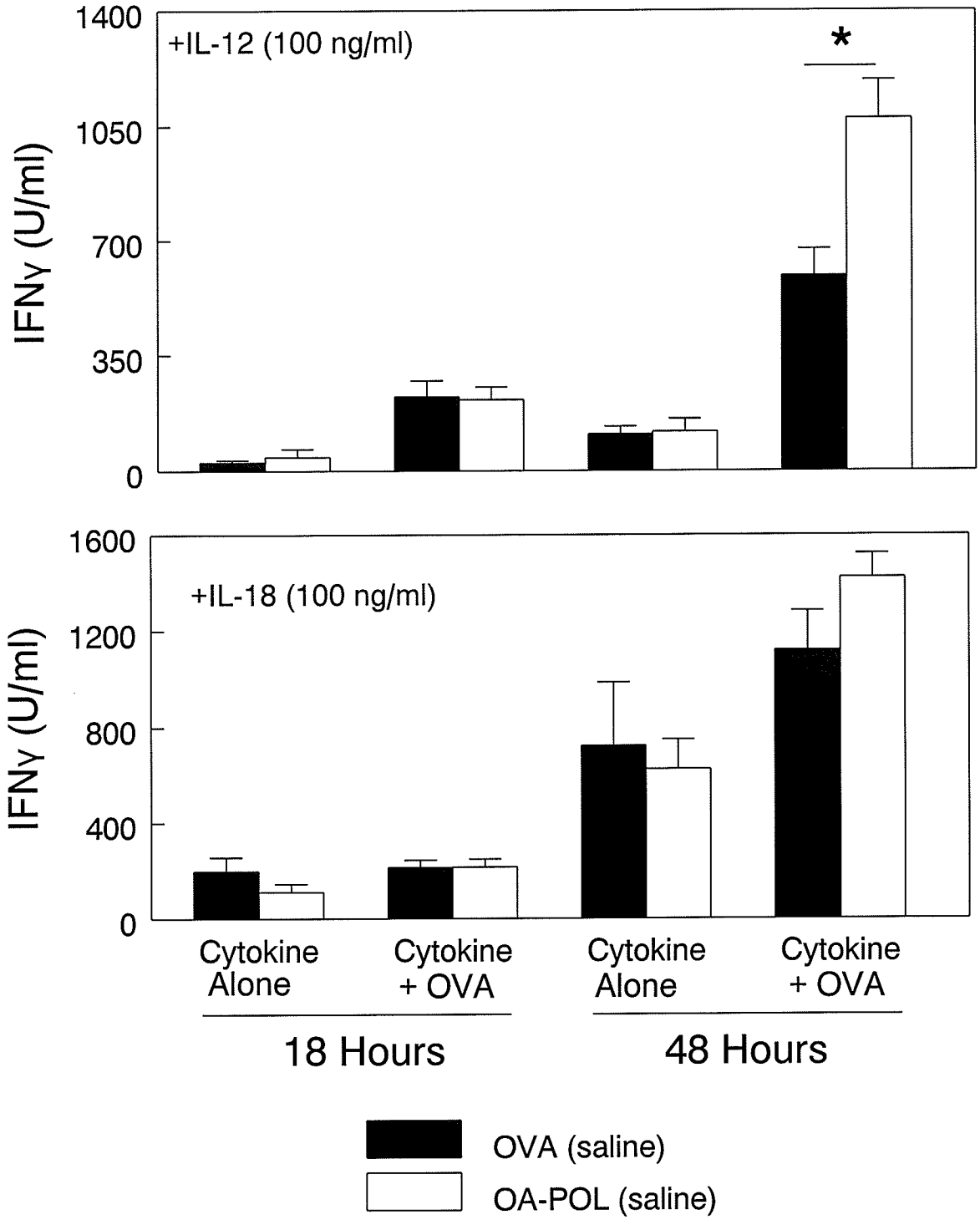

Figure 26: Increased IL-12 responsiveness in OA-POL treated mice. Mice were immunized with 100 μg OVA (saline) or OA-POL (saline) on days 0, 2 and 4, sacrificed on day 8 and spleen cells cultured as indicated. Tissue culture supernatants were harvested at 18 and 48 hours for analysis of IFNγ production. Mean + SEM shown (n = 12 mice from 3 experiments). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p < 0.05$).

To determine if increased IL-12 responsiveness seen following OA-POL (saline) immunization is a result of enhanced IL-12Rβ2 expression, we quantitated the levels of receptor mRNA in spleen cell lysates taken from OVA (saline) or OA-POL (saline) immunized mice using quantitative RT-PCR. We examined receptor mRNA levels both at the time of sacrifice (day 8) to gauge the level of endogenous receptor expression in the spleen, and after 48 hours of *in vitro* stimulation with OVA, conditions that resulted in significantly elevated IL-12 responsiveness. However, as shown in Figure 27, regardless of the condition, OA-POL (saline) immunized mice do not produce detectably greater quantities of IL-12Rβ2 mRNA as compared to OVA (saline) immunized mice. Consistent with the observation of identical biological responsiveness to rIL-18 in OA-POL (saline) immunized mice, there is no increase in mRNA levels for IL-18Rα or IL-18Rβ (Figure 27).

Thus, despite significant increases in overall responsiveness to IL-12 and IL-18 stimulation, spleen cells from OA-POL treated mice do *not* display increased receptor expression for these cytokines. However, both NK cells and T cells are present in spleen cell preparations, and both of these cells respond to IL-12 and IL-18 through IFNγ production. Since the capacity of OA-POL to inhibit developing and existing type 2 immune responses has previously been demonstrated to be T cell dependent, we hypothesized that OA-POL would act chiefly on T cells. As NK cells constitutively express IL-12 and IL-18 receptors (Hyodo, Matsui et al. 1999; Chakir, Camilucci et al. 2000) NK cell expression of these receptors may possibly mask any subtle increases in receptor expression on OVA-specific CD4+ T cells, and thus prevent us from observing significant changes in receptor mRNA expression in bulk spleen cell populations. In subsequent experiments we directly focussed on the effects of OA-POL treatment on the T cell population.

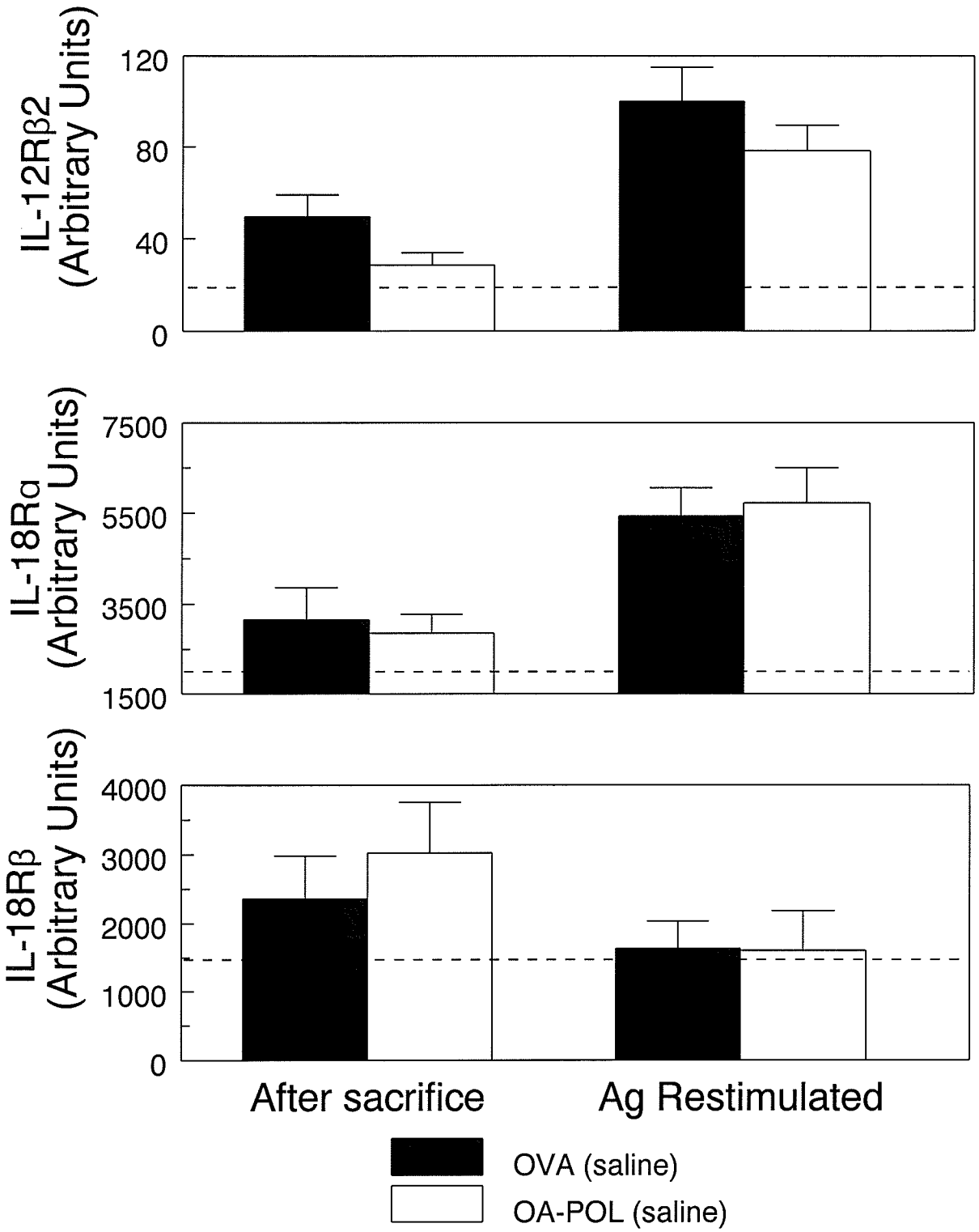

Figure 27: IL-12 and IL-18 receptor mRNA expression is not elevated in OA-POL treated mice. Mice were immunized with 100 μg OVA (saline) or OA-POL (saline) sacrificed on day 8. Receptor mRNA was detected immediately after sacrifice or after 48 hours of stimulation with OVA. Dotted line indicates receptor expression in naive mice. Mean + SEM shown (n = 8 mice from 2 experiments).

8.3 - Increased *T cell* responsiveness to IL-12 and IL-18 following OA-POL treatment

To determine whether OA-POL acts to increase IL-12 responsiveness of T cells or NK cells, we treated OVA (saline) and OA-POL (saline) immunized mice with rat anti-mouse NK1.1 on day 6 and 7 to deplete NK cells. Depletion efficiency ranged from 60-90% as assessed by both NK1.1 and CD49b staining (data not shown). OA-POL (saline) immunized, anti-NK1.1 treated mice still produced significantly more IFNγ in response to OVA + IL-12 at 48 hours (Figure 28). Interestingly, depletion of NK cells also revealed increased OA-POL induced responsiveness to IL-18. OVA + IL-18 stimulated cultures from OA-POL (saline) immunized mice produced 3-4 fold more IFNγ than similarly stimulated cells from anti-NK1.1 treated, OVA (saline) immunized mice at both 18 and 48 hours (Figure 28). These data suggest that OA-POL globally influences IL-12 and IL-18 responsiveness of T cells.

8.4 - Increased IL-18Rα and IL-18Rβ mRNA levels in NK cell depleted, OA-POL treated mice

IL-12 and IL-18 receptor mRNA levels were also compared in anti-NK1.1 treated OVA (saline) and OA-POL (saline) immunized mice (Figure 29). Despite a marked increase in IFNγ production following stimulation with OVA + IL-12, there was no detectable increase in IL-12Rβ2 mRNA levels following OA-POL (saline) immunization in anti-NK1.1 treated mice (Figure 29). However, consistent with the increased responsiveness to IL-18 + OVA, significantly more mRNA for both IL-18Rα and IL-18Rβ was found in OA-POL (saline) immunized compared to OVA (saline)

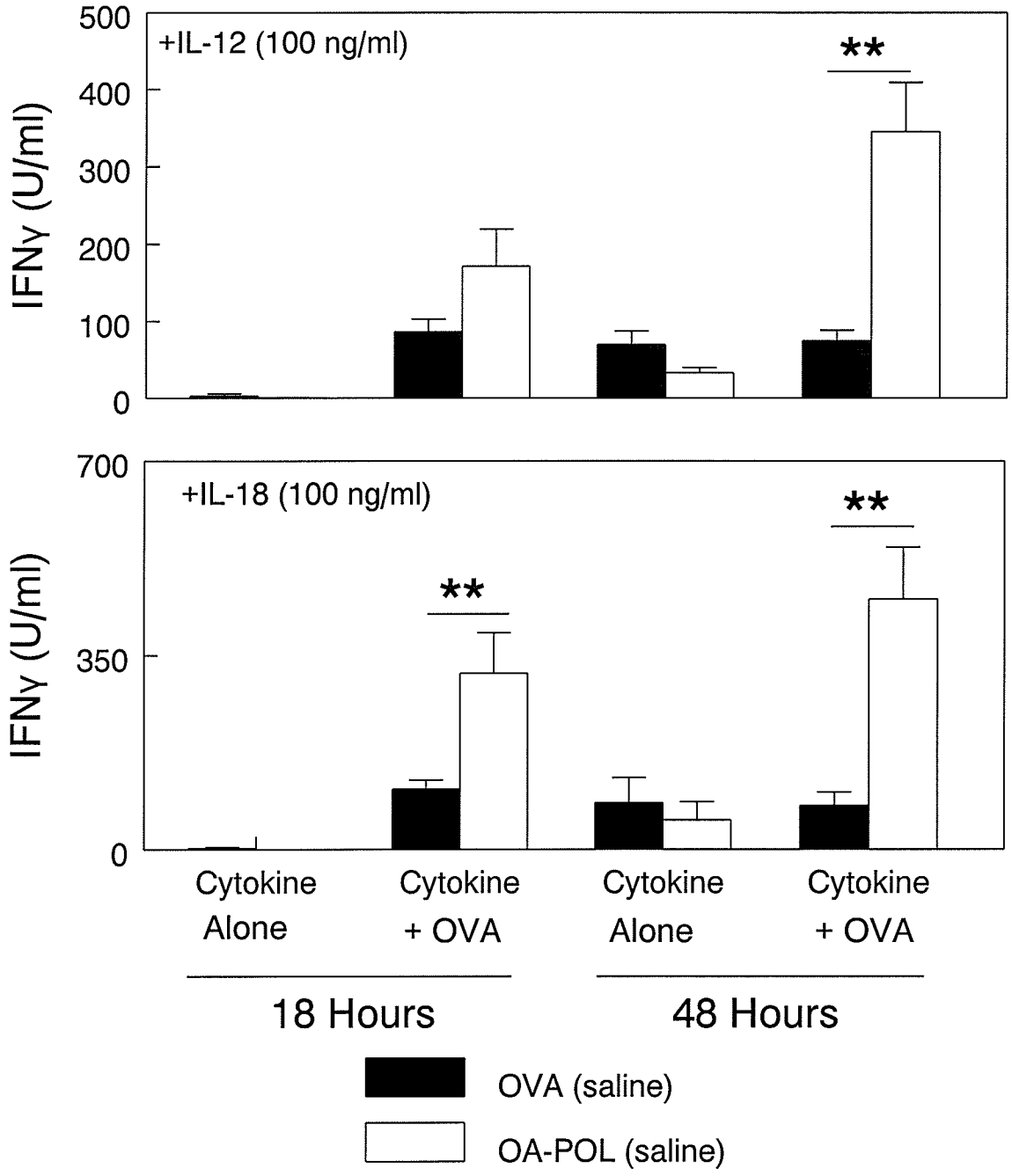

Figure 28: Increased IL-12 and IL-18 responsiveness in NK cell depleted, OA-POL treated mice. Mice were immunized with 100 μg OVA (saline) or OA-POL (saline), treated with anti-NK1.1 on days 6 and 7 and sacrificed on day 8. Spleen cells were cultured as indicated. IFNγ production was assessed. Mean + SEM shown (n = 10 mice from 2 experiments). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p < 0.05$; ** $p < 0.005$).

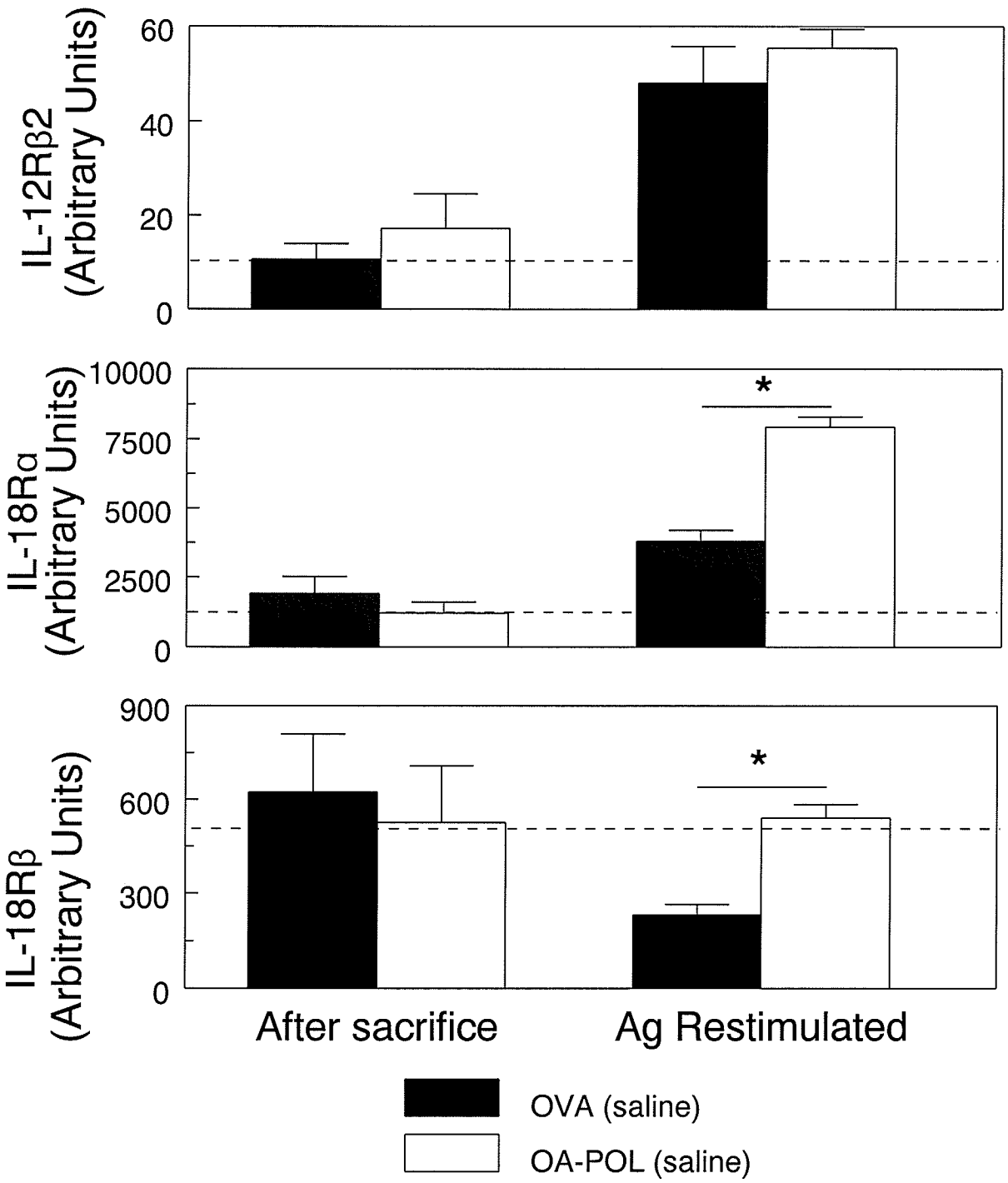

Figure 29: Increased IL-18 receptor mRNA expression in NK cell depleted, OA-POL treated mice. Mice were immunized with 100 μg OVA (saline) or OA-POL (saline), treated with anti-NK1.1, and sacrificed on day 8. Receptor mRNA was detected immediately after sacrifice or after 48 hours of stimulation with OVA. Dotted line indicates receptor expression in naive, anti-NK1.1 treated mice. Mean + SEM shown (n = 10 mice from 2 experiment). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p < 0.001$).

immunized, anti-NK1.1 treated mice (Figure 29). Since within the spleen, NK cells and T cells represent a significant majority capable of responding to IL-12 and IL-18 (Otani, Nakamura et al. 1999), and we have depleted NK cells from these animals, it seems likely that the increases in mRNA expression levels are a result of increased *T cell* expression of IL-18 receptor subunits. However, OA-POL immunization has no observable effect on IL-12Rβ2 expression.

8.5 - Increased IL-12Rβ2 expression on OVA-specific T cells

As the effects of OA-POL are OVA-specific (HayGlass and Stefura 1991), we hypothesized that the principal effect of OA-POL treatment would be on OVA-specific T cells. As these comprise <<1% of the total T cell population in whole spleen cell cultures, any subtle changes in IL-12Rβ2 mRNA expression on OVA-specific T cell populations would likely be masked by the stable expression on the majority of T cells. To examine an OVA-specific T cell enriched population, we cultured spleen cells from OVA (saline) or OA-POL (saline) immunized mice with OVA for 48 hours. Following this culture period, we purified recently activated T cells on the basis of CD4 and CD69 positivity and examined IL-12Rβ2 expression in this population. As shown in Figure 30, OVA-activated T cells from OA-POL (saline) immunized mice contained ~3 fold more IL-12Rβ2 mRNA than similar preparations of T cells from OVA (saline) immunized mice. These data demonstrate that treatment of mice with a homogeneous, chemically polymerized allergen increases IL-12 receptor expression on T cells recently activated in the presence of OVA.

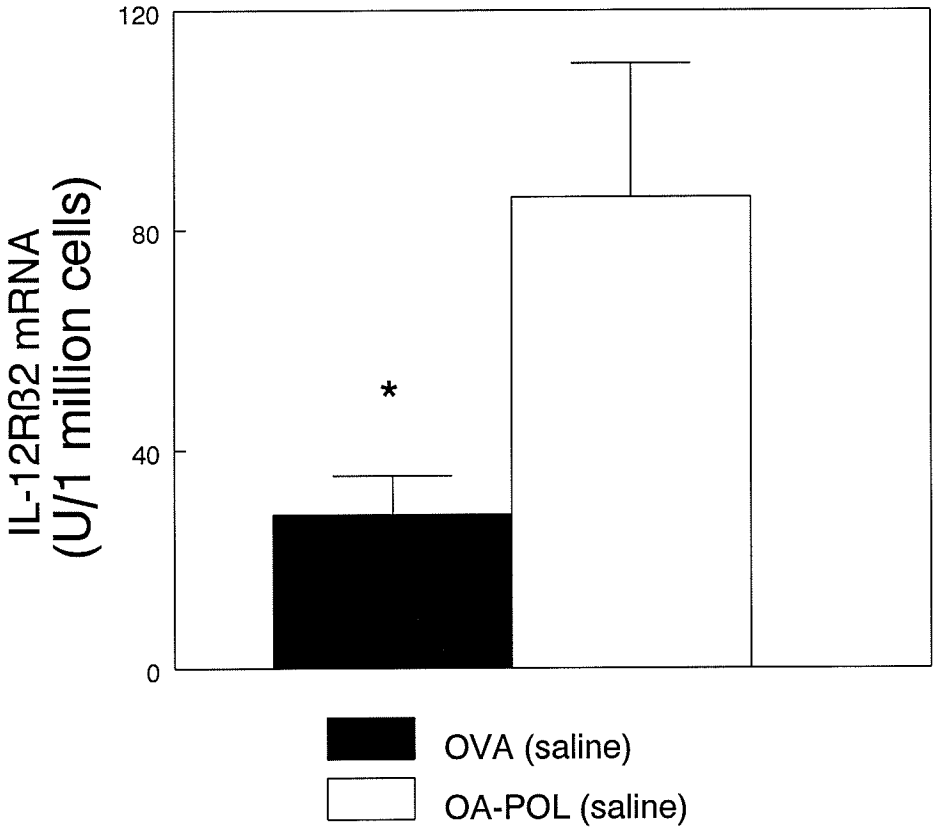

Figure 30: Increased IL-12Rβ2 mRNA expression in OVA-specific T cells. OA-POL treated mice. Mice were immunized with 100 μg OVA (saline) or OA-POL (saline) and sacrificed on day 8. $CD4^+CD69^+$ cells were sorted from 48 hr OVA-stimulated spleen cell cultures using flow cytometry. IL-12Rβ2 expression was determined as described. Mean + SEM shown (n = 8 mice from 3 experiments). Significant differences between untreated and OA-POL treated mice are indicated (Student's t test; * $p < 0.05$).

8.6 - Summary

We demonstrate here that OA-POL treatment, in the absence of adjuvant, results in markedly increased T cell responsiveness to IL-12 and IL-18, as measured by increased IFNγ production. This functional observation was confirmed by direct examination of IL-12 and IL-18 receptor mRNA expression which revealed an increase in IL-12Rβ2 expression specifically on OVA-specific T cells, and a generalized increase in T cell expression of IL-18R mRNA. These data suggest that OA-POL administration mediates increased responsiveness to endogenously produced regulators of type 1 immune responses. While we have demonstrated that the production of IL-12 p40 in response to antigen stimulation following OA-POL treatment is not elevated, we cannot completely rule out the possibility that there is an increase in the production of IL-12 p70 or IL-18.

V - Discussion

1.0 - Introduction

Allergic disease is an increasingly common immune disorder, particularly in developed nations. The underlying cause of disease is increasingly well understood (a type 2 dominated immune response to normally innocuous environmental allergens), yet the endogenous controls responsible for preventing induction of aberrant type 2 dominated immune responses or redirecting existing allergic responses, are not well understood. In this thesis, we examine the role of endogenously produced IL-12 and IFNγ in both preventing and redirecting excessive type 2 immune responses. We also explore the capacity of IL-18, a cytokine with controversial effects in allergic disease, to inhibit type 2 immunity. Lastly, we explore the mechanism of successful immune redirection observed following treatment with OA-POL. To thoroughly assess the nature of the immune response, we examine multiple measures of the balance of type 1 versus type 2 immunity including *in vivo* Ab synthesis, *in vitro* Ag-stimulated cytokine production, and *in vitro* Ag-driven type 1 and type 2 chemokine production following induction of Th1 or Th2 immune responses to exogenous protein

2.0 - Endogenous IL-12 is a potent promoter of type 1 immunity, but is not critical for inhibition of type 2 immune responses

IL-12 is a potent promoter of IFNγ synthesis and plays a critical role in facilitating naive T cell differentiation into IFNγ-producing, Th1-like effector cells. In animal models, the administration of

recombinant IL-12 as an adjuvant results in powerful, yet transient, inhibition of IgE synthesis and marked increases in type 1 Ab levels. Although administration of IL-12 in allergic models after initial sensitization profoundly reduces airway reactivity, it is consistently unable to abrogate existing IgE responses and actually increases the synthesis of type 2 cytokines. Recently, the benefit of administration of rIL-12 to individuals with mild asthma was assessed in clinical trials. While IL-12 administration decreases serum and sputum eosinophilia, there was no change in airway function, as determined by unaltered duration of late phase response and identical sensitivity to histamine challenge (Bryan, O'Connor et al. 2000). Unfortunately, the direct effects of rIL-12 administration on the nature or intensity of the immune response to allergen was not examined (i.e. cytokine production or serum IgE levels). While the effects of recombinant IL-12 are well studied, the role that endogenous IL-12 plays in limiting the severity of the allergic response has not been thoroughly examined. To address this question, we make use of both IL-12 p35 -/- and IL-12 p40 -/- mice immunized with exogenous protein antigen in adjuvant, or *Trichinella spiralis* extract and compare their immune response to those elicited in similarly treated C57Bl/6 controls.

2.1 - Impact of endogenous IL-12 on *in vitro* type 2 cytokine production

Lack of endogenous IL-12 has profound effects on Ag restimulated IFNγ production *in vitro* in mice immunized with OVA (alum) (Figure 1), OVA (CFA) (Figure 2) and *T. spiralis* extract (Figure 3). Although IFNγ production is dramatically reduced in IL-12 deficient strains of mice, IFNγ production is still readily detectable, albeit 5-10% of that seen in control animals. Decreased IFNγ production to a variety of stimuli is widely reported in IL-12 deficient mice to a variety of *in vivo* and *in vitro*

stimuli (Magram, Connaughton et al. 1996; McIntyre, Shuster et al. 1996; Ehrhardt, Ludviksson et al. 1997; Su and Stevenson 2002), supporting the role of IL-12 as a potent, perhaps the *most* potent, promoter of type 1 immunity.

In contrast to the striking effect on IFNγ production, IL-12 deficiency has no demonstrable effect on type 2 cytokine production. Levels of all type 2 cytokines examined are indistinguishable between IL-12 -/- and C57Bl/6 controls both under resting conditions and following immunization under conditions designed to elicit a variety of immune responses (Figures 1-3). Similarly, identical spleen cell IL-4 production is observed in anti IL-12 treated *Borrelia burgdorferi* or *Leishmania donovani* infected mice (Anguita, Persing et al. 1996; Engwerda, Murphy et al. 1998). IL-12 -/- mice also display no increase in IL-4 production following infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis* (Cooper, Magram et al. 1997; Wakeham, Wang et al. 1998). Furthermore, Piccotti *et al.* report no significant elevation of IL-4 or IL-10 production after *in vitro* ConA stimulation of spleen cells from IL-12 -/- mice on both C57Bl/6 and BALB/c genetic backgrounds (Piccotti, Li et al. 1998). In contrast to our findings of unaltered Th2 cytokine synthesis in IL-12 -/- mice, Magram *et al.* report increased IL-4 production in KLH stimulated, IL-12 -/- lymph node cells (Magram, Connaughton et al. 1996). Additionally, spleen cells from *Toxoplasma gondii*, anti IL-12 treated mice produce more IL-4 than those from *T. gondii* infected control mice. It must be mentioned, however, that the levels of IL-4 in control animals were undetectable and IL-4 levels in anti-IL-12 treated mice were minuscule (1.9 ± 0.5 U/ml) making comparison of the true effect of anti-IL-12 treatment difficult (Gazzinelli, Wysocka et al. 1994). In the majority of these studies however (both those supporting and contradicting our findings), the conclusions made about IL-12 as

a negative regulator of type 2 immunity are based on a single measurement of a notoriously difficult to detect cytokine, IL-4, and occasionally IL-10.

Other groups' previous findings consistently support the hypothesis that endogenous IL-12 synthesis is critical to support type 1 cytokine synthesis. However, because the existing data on the role of endogenous IL-12 production in regulation of type 2 cytokine production was incomplete, or contradictory, we examined a *panel* of type 2 cytokines under a variety of different *in vivo* and *in vitro* stimulation conditions. From these studies, we conclude that endogenous IL-12 is a redundant negative regulator of type 2 cytokine production to exogenous protein antigens. In contrast, IL-12 is required for optimal maintenance of IFNγ production in response to exogenous antigens.

2.2 - Impact of IL-12 deficiency on Ab synthesis

In order to assess the impact of IL-12 deficiency on Ab synthesis in a type 2 dominated immune response, here we immunize mice with OVA (alum) (Figure 5) and *Trichinella spiralis* extract (Figure 6) in alum. Contrary to the hypothesis put forward by us, and others (Magram, Connaughton et al. 1996), IL-12 -/- mice do not display increased IgE levels in comparison to C57Bl/6 controls under these conditions. Likewise, IgE responses are equivalent (or slightly depressed) in both strains of IL-12 -/- and control mice undergoing a type 1 dominated immune response following immunization with OVA (CFA) (Figure 4). Collectively these data argue that IL-12 is not a critical negative regulator of the induction or maintenance of IgE production.

While contrary to our initial hypothesis, these data is supported by other studies from the literature. Anguita *et al.* report no elevation of IgE production in anti-IL-12 treated mice infected with *Borellia burgdorferi* (Anguita, Persing et al. 1996). Keane-Myers *et al.* report unchanged IgE synthesis in anti-IL-12 treated, OVA sensitized and challenged A/J mice, but a significant increase in IgE levels in similarly treated C3H mice (Keane-Myers, Wysocka et al. 1998). Schijns *et al.* report no significant change in the balance of type 1 and type 2 antibody production in IL-12 -/- mice infected with murine hepatitis virus, but find elevated IgE levels in KLH (alum) immunized IL-12 -/- mice (Schijns, Haagmans et al. 1998). Thus, the data from the literature on the role of endogenous IL-12 is conflicting and suggests that differences between studies may be due to genotypic and/or differences in immunization status and the infectious organisms used.

In contrast to the lack of consistent effect of endogenous IL-12 deficiency on IgE synthesis endogenous IL-12 production possesses a potent stimulatory activity for type 1 antibody production. In mice undergoing OVA (CFA) driven, type 1 dominated immune responses, we consistently find a profound (50-80%) reduction of both total and OVA-specific IgG_{2c} production (Figure 4). Similarly, total IgG_{2c} levels are also significantly reduced in OVA (alum) and *T. spiralis* (alum) immunized mice (Figure 5 and 6). The lack of a significant decrease in OVA-specific IgG_{2c} levels in OVA (alum) immunized IL-12 -/- mice (Figure 5) likely represents weak induction of OVA-specific IgG_{2c} in type 2 responses (100-1000 fold lower than those seen in OVA (CFA) immunized mice) rather then any differences in IgG_{2c} regulation between type 1 and type 2 immune responses.

These findings agree with studies reporting decreased type 1 Ab production in IL-12 deficient mice in models of collagen induced arthritis (McIntyre, Shuster et al. 1996), *Plasmodium chabaudi* infection

(Su and Stevenson 2002), immunization with irradiated *Schistosoma mansoni* (Anderson, Shires et al. 1998) and following immunization with KLH (Schijns, Haagmans et al. 1998). Interestingly, IL-12 deficiency does not result in decreased type 1 Ab production in models of viral infection (Schijns, Haagmans et al. 1998; Oxenius, Karrer et al. 1999) suggesting alternative mechanisms of inducing type 1 Ab production in viral models. It is possible that IL-18 may play a more critical role in mediating type 1 Ab responses in viral infections, as $CD4^-CD8^+$ T cells produce more IFNγ in response to IL-18 than $CD4^+CD8^-$ T cells (Tomura, Maruo et al. 1998), and IL-12 -/- mice produce more IL-18 than control mice (Figure 15).

Collectively these data demonstrate that the absence of IL-12 has little effect on IgE production, but results in a profound reduction in IgG_{2c} synthesis. This suggests that IL-12 is critical for promoting the optimal induction of type 1 immune responses, but is not required for preventing the development of type 2 Ab responses.

2.3 - Undiminished IL-12 responsiveness in IL-12 -/- mice suggests no global shift towards hyperexpression of type 2 immunity.

IL-4 is a critical negative regulator of IL-12Rβ2 expression, and maintenance of functional IL-12 receptor expression is a key checkpoint in the differentiation of Th1 and Th2 effector cells (Szabo, Jacobson et al. 1995; Szabo, Dighe et al. 1997). Thus, the demonstration that IL-12 responsiveness (as measured by IL-12 driven IFNγ production) is not diminished in IL-12 -/- mice (Figure 7) suggests that there is no default generation of IL-12 unresponsive, Th2-like effector cells following

immunization with OVA (alum). This observation is supported by the finding of equivalent induction of IFNγ in cultures from control and IL-12 -/- spleen cell cultures stimulated with alloantigen + IL-12 (Piccotti, Li et al. 1998).

These data is particularly interesting in light of the observations that PBMCs from allergic individuals demonstrate a reduced responsiveness to IL-12 (HayGlass, Li et al. 1997; Matsui, Kaneko et al. 2000; Shikano, Kato et al. 2001) and increased prevalence of mutations in the IL-12Rβ2 (Matsui, Kaneko et al. 1999). Thus, in a situation where there *is* a demonstrated elevation of type 2 immunity (human allergic disease), there is an associated *decrease* in responsiveness to IL-12. This, provides further support for our conclusions that unaltered responsiveness to IL-12 is indicative that the immune responses of IL-12 deficient mice are not globally shifted towards a default hyperexpression of type 2 immunity.

2.4 - p35 -/- versus p40 -/- mice: possible contributions of IL-23 and IL-27

The rationale for using both p35 -/- and p40 -/- strains was driven by the understanding of IL-12 biology in the mid-to-late 1990s. Thus, to address the possibility that p40 subunits (either p40 or $(p40)_2$) possess antagonistic activities, IL-12 p35 -/- mice, are included in this study, along with IL-12 p40 -/- mice. However, with the subsequent identification of the additional IL-12 family members, the differential roles of IL-12 family members in controlling the balance of type 1 versus type 2 immunity can be explored. This is possible by examining differences in the immune response

between control mice (capable of producing IL-12, IL-23 and IL-27), p35 -/- mice (able to produce IL-23 and IL-27) and p40 -/- mice (capable of producing IL-27 alone).

Direct comparison of p35 -/- and p40 -/- mice demonstrated negligible differences in the Ab or cytokine responses to any stimulus tested, but revealed significant differences from the immune responses seen in C57Bl/6 controls. This suggests that the principal defect in IL-12 p35 -/- and IL-12 p40 -/- mice leading to diminished IFNγ synthesis is IL-12 deficiency. Neither IL-23 nor IL-27, in the absence of IL-12, is sufficient to induce *maximal* IFNγ production (although we speculate that either may be important in maintaining the low levels of IFNγ production seen in the absence of IL-12). Furthermore, all mice demonstrate no exacerbation of type 2 immunity in the absence of p35 (IL-12 -/-) or p40 (IL-12 -/- and IL-23 -/-). This suggests that neither IL-12 nor IL-23 is required for optimal inhibition of type 2 immune responses. Thus, control of type 2 immune responses may be mediated via basal IFNγ levels, (which may in turn be controlled by endogenous IL-27 or IL-18 production) or directly by IL-18 and/or IL-27. Support for a potential role for IL-18 comes from within this thesis: enhanced IL-18 responses were noted in IL-12 -/- mice (Figure 15), coupled with the observation of IFNγ independent, IL-18 inhibition of IL-4 production (Figure 16). Further experimentation, specifically designed to directly explore the relationship of IL-12, IL-23 and IL-27 in control of type 1 and type 2 immunity is required to allow more precise understanding of the complex interplay between these cytokines. This may be accomplished by directly comparing the immune responses in IL-12 p35 -/- mice, IL-23 p19 -/- mice and IL-27 p28 -/- mice. Attempts were made to undertake such studies as a component of this thesis, but reagents for the study of IL-23 and

IL-27 (knockout mice, recombinant protein and monoclonal antibodies), were controlled by Schering-Plough, and were not made available to us.

2.5 - Summary of findings in IL-12 -/- mice

Contrary to previous studies that draw mixed conclusions on the basis of a single marker of type 2 immunity, we examine production of a wide range of type 2 associated factors, under numerous stimulation conditions. IL-12 deficient mice consistently display a decreased intensity of type 1 immunity, confirming the identity of IL-12 as an important promoter of type 1 immune responses. However, the absence of any detectable increases in total or specific IgE production, and type 2 cytokine synthesis indicates that IL-12 is not required for preventing the hyperexpression of type 2 immune responses. This hypothesis is further supported by the observation of unaltered IL-12 responsiveness in IL-12 -/- mice which suggests a failure to default to the generation of IL-12 unresponsive, Th2-like effector cells in IL-12 deficient conditions.

3.0 - Endogenous IFNγ as a negative regulator of type 2 immunity

In the absence of endogenous IL-12 production, IFNγ levels are severely reduced, but IFNγ production in IL-12 -/- mice is consistently ~10% of the levels seen in normal C57Bl/6 controls (Figures 1 and 2). IFNγ itself is strongly associated with type 1 immune responses and limits development of type 2 immunity, inhibiting proliferation of Th2 clones (Mosmann and Coffman 1989) and IgE production *in vitro* (Maggi, Parronchi et al. 1992). Indeed, local administration of

either recombinant IFNγ (Lack, Bradley et al. 1996) or IFNγ encoding DNA (Dow, Schwarze et al. 1999) to the airways of allergic animals results in decreased IgE production. This suggests that exogenous IFNγ can act as a potent modulator of type 2 immunity in murine models of immediate hypersensitivity. We hypothesize that basal levels of IFNγ present in IL-12 -/- are sufficient to prevent the hyperexpression of type 2 immune responses.

Previous studies examining the role of endogenously produced IFNγ using anti-IFNγ treated mice (Hessel, Van Oosterhout et al. 1997), IFNγ -/- mice (Hofstra, Van Ark et al. 1998), or IFNγR -/- mice (Coyle, Tsuyuki et al. 1996; Bruselle, Kips et al. 1997) yield conflicting results. Some suggest IFNγ limits allergic disease (as IFNγ deficient mice demonstrate increased serum IgE levels and prolonged lung eosinphilia (Coyle, Tsuyuki et al. 1996; Bruselle, Kips et al. 1997) while others argue IFNγ promotes it, particularly airway hyperresponsiveness (Hessel, Van Oosterhout et al. 1997; Hofstra, Van Ark et al. 1998). Type 2 cytokine production is arguably one of the most important factors in development of allergic disease. However, a major limitation of the above studies is that none examined production of type 2 cytokines and chemokines from Ag stimulated, IFNγ deficient cells.

Here, we examine the role of endogenous IFNγ in controlling the development of type 2 immune responses. We make use of IFNγ deficient mice, immunized under both type 1 and type 2 inducing conditions to compare type 1 and type 2 antibody and cytokine/chemokine production in IFNγ -/- mice with that seen in control animals.

3.1 - Endogenous IFNγ as a negative regulator of type 2 cytokine synthesis

To assess the balance of type 1 and type 2 immunity during a type 2 dominated immune response in the absence of endogenous IFNγ, IFNγ -/- and control mice are immunized with OVA (alum) and spleen cell cytokine production is measured after *in vitro* OVA restimulation. IFNγ deficiency results in a consistent 200% increase in all type 2 cytokines examined (IL-4, IL-5, IL-10 and IL-13) (Figure 8). Furthermore, there is also a 50-fold decrease in the ratio of IP-10/TARC production (chemokines associated with type 1 and type 2 immune responses respectively) (Figure 9). Immunization with OVA (HKL) results in substantially lower type 2 cytokine production. Of the type 2 cytokines whose production was robust enough to be accurately quantitated (IL-5 and IL-13) similarly elevated levels were found in tissue culture supernatants from IFNγ -/- mice (Figure 10). This is in direct contrast to IL-12 deficient mice which do not display enhanced type 2 cytokine levels compared to control mice (Figures 1, 2, and 3). The generalized lack of hyperexpression of type 2 cytokines in IL-12 deficient mice and the increased expression of type 2 cytokine and chemokine expression in IFNγ deficient mice suggest that endogenous IFNγ, but not IL-12, is required for optimal negative regulation of type 2 cytokine production.

Surprisingly, most previous studies examining the role of endogenous IFNγ in allergic disease using IFNγ -/- and anti-IFNγ treated animals did not examine the production of type 2 cytokines (Bruselle, Kips et al. 1997; Hessel, Van Oosterhout et al. 1997; Hofstra, Van Ark et al. 1998). However, in IFNγR -/- mice, Coyle *et al.* report there is no increase in anti-CD3 driven IL-4 and IL-5 production by lung T cells shortly after aerosol OVA challenge (Coyle, Tsuyuki et al. 1996). They do report however that anti-CD3 driven IL-4 and IL-5 production in IFNγR -/- mice was detectable for up to two months after aerosol challenge, long after similar responses disappear in control animals (Coyle,

Tsuyuki et al. 1996). Antigen specific cytokine responses were not examined however. These data suggest IFNγ signalling is important for preventing *sustained* type 2 immunity once initiated. The significant increases in the production of all type 2 cytokines/chemokines examined in the absence of endogenous IFNγ (Figures 8 and 9) extend the previous data and highlight the importance of IFNγ in preventing *initial* induction of type 2 immunity to an exogenous protein antigen.

3.2 - Endogenous IFNγ as a regulator of immunoglobulin synthesis

Immunization of both C57Bl/6 and IFNγ deficient mice under protocols designed to elicit strong type 1 or type 2 dominated antibody responses yielded surprising results. As expected, type 1 Ab production (both total and OVA-specific) is reduced by the same magnitude (~50%) in both OVA (alum) and OVA (HKL) immunized IFNγ knockout mice (Figure 11 and 12). However, IFNγ deficient mice display a global deficiency in Ig production as levels of total and OVA-specific IgG_1 (a general marker of immune responsiveness) and IgE (a type 2 associated antibody) are also significantly diminished compared to control mice (Figure 11 and 12). Thus, rather than displaying a selective inhibition of type 1 Ab production and enhancement of type 2 Ab production, the global inhibition of Ig synthesis found in IFNγ deficient mice suggest a broader role for IFNγ in regulation of Ig production. In light of enhanced type 2 cytokine production, decreased antibody levels in IFNγ -/- mice, are particularly surprising. Similar decreases in IgE, IgG or IgA levels in the absence of endogenous IFNγ have been previously observed (Schijns, Haagmans et al. 1994; Bruselle, Kips et al. 1997; Hofstra, Van Ark et al. 1998), although the absence of IFNγ has also been reported result in more intense IgG_1 responses (Graham, Dalton et al. 1993). In all cases, the mechanisms underlying

these alterations are not explored in the cited studies. To explore the mechanisms involved in the observed decrease in Ig production, we examine both the kinetics and intensity of B and T cell activation in IFNγ -/- mice.

Exposure of immature B cells to antigen prior to final maturation results in apoptosis. Given the previous reports of increased numbers of immature B cells in secondary lymphoid organs in IFNγ -/- mice (Flaishon, Hershkoviz et al. 2000), we hypothesize that large numbers of B cells die at the immature stage in IFNγ -/- mice. This would be reflected as a decreased percentage of mature and functional B cells. However, as there is neither a decrease in the number of mature B cells in the spleen or bone marrow (Table 2) nor a decrease in the capacity of spleen cells from IFNγ -/- mice to proliferate in response to B cell mitogens (Figure 13), this explanation seems unlikely. An alternative explanation is that decreased MHC Class I and Class II expression previously observed in IFNγ -/- mice (Goes, Sims et al. 1995) results in diminished antigen presentation capacity. However, this is inconsistent with the *increased* production of type 2 cytokines reported here following *in vitro* restimulation of spleen cells from IFNγ -/- mice (Figures 8, 9 and 10).

The observation of increased proliferation of cells from IFNγ deficient animals in mixed lymphocyte reactions (MLR) (Dalton, Pitts-Meek et al. 1993), and the fact that IFNγ is known to have potent anti-proliferative effects (Gajewski and Fitch 1988; Gajewski, Goldwasser et al. 1988) also prompted us to examine the kinetics of T cell proliferation. T cell proliferation immediately after Ag driven T cell activation was markedly higher in IFNγ -/- cultures (Figure 14b). However, by day 6, Ag driven proliferation of the IFNγ -/- cells is approximately half that seen in control cultures (Figure 14b). If the same shift towards increased proliferation of T cells at an early time point observed following *in*

vitro activation with antigen is reflective of the kinetics of *in vivo* antigen driven proliferation, then it is conceivable that fewer T cells with the appropriate co-stimulatory molecules are available to provide B cell help in the early stages of the antibody response. Such a lack of effective T cell help may provide an explanation for the decrease in overall Ig synthesis.

Thus, despite dramatic elevation in type 2 cytokine production in IFNγ -/- mice, we consistently find a decrease in the overall ability to mount an Ab response to exogenous protein antigens. This deficiency in Ab production is not related to a maturational defect in B cell ontogeny, or in an inability of B cells to proliferate. It may, however, be a result of a marked shift in the kinetics of T cell activation/proliferation which is evident in IFNγ -/- mice.

Examination of Ig production in IFNγ -/- mice also yielded some interesting observation about the role of IFNγ in the regulation of IgG_{2c} production. IFNγ is the only cytokine which has been demonstrated to efficiently induce production of IgG_{2a} (Severinson, Fernandez et al. 1990; Bossie and Vitetta 1991) which is the C57Bl/6 analog of IgG_{2c}. It is not a major focus of this thesis, but it is worthwhile noting that while depressed, there is still significant induction of OVA specific IgG_{2c} following immunization of IFNγ -/- mice (titres increase from <10 to 14,000 or ~ 400,000 for OVA (alum) and OVA (HKL) immunized mice respectively) (Figure 11 & 12). In a similar fashion, it was initially reported that IL-4 was solely responsible for driving IgE production *in vivo* (Finkelman, Katona et al. 1988). The significant levels of IgE in IL-4 deficient mice (von der Weid, Kopf et al. 1994; Morawetz, Gabriele et al. 1996) led to the discovery that other factors have the capacity to induce IgE. The significant induction of IgG_{2c} in IFNγ -/- mice argues, as it did in IL-4 deficient

mice, that additional factors possess the capacity to efficiently induce B cell class switch to IgG_{2c}.

3.3 - Summary of findings in IFNγ -/- mice

Previous studies examining the role of IFNγ in regulation of type 2 immune responses to exogenous protein antigens are consistently lacking in-depth examinations of Ag-driven type 2 cytokine production. We report here, however, that IFNγ deficient mice are consistently biased towards an increased ratio of type 2 to type 1 cytokine and chemokine production during both Th1 and Th2 dominated immune responses. This is in striking contrast to IL-12 deficient mice which do not display any increases in the intensity of type 2 immunity. Furthermore, this increased type 2 cytokine production is accompanied by a global *decrease* in Ab responses. Collectively, these data also suggest that IFNγ plays an important role in regulating the overall humoral response.

4.0 - IL-18 as a negative regulator of type 2 immunity *in vivo*

Given that deficiency in IFNγ, but not IL-12, results in enhanced type 2 cytokine/chemokine responses, we hypothesize that basal IFNγ production in IL-12 -/- is sufficient to prevent hyperexpression of type 2 immunity. As IL-18 is a potent inducer of IFNγ synthesis, we examined IL-18 production in IL-12 -/- mice, reasoning that a greater reliance on IL-18 for control of IFNγ production (and thus type 2 immune responses) might be reflected by a detectable rise in IL-18 levels. IL-18 levels in the serum of naive control and IL-12 -/- mice were readily detectable, but

indistinguishable from controls. However, upon injection of LPS, IL-12 p35 -/- deficient animals displayed markedly elevated (100% increase) serum IL-18 levels as compared to similarly treated control mice as soon as 4 hours after LPS administration (Figure 15). The increased IL-18 levels observed in IL-12 -/- mice remained elevated for 4 additional days (Figure 15). Thus, in the absence of IL-12, mice have a greater propensity to produce IL-18. We hypothesize that this increased IL-18 expression, coupled with the demonstrated ability of IL-18 to inhibit IL-4 production (Figure 16) prevents the hyperexpression of type 2 immunity in these IL-12 deficient mice.

Other studies examining IL-18 responses in mice lacking IL-12 signalling do exist. Zhang *et al.* report significantly increased IL-18 production in spleen cells from MOG immunized IL-12Rβ2 -/- mice (thus deficient in only IL-12 signalling) in a murine model of EAE (Zhang, Gran et al. 2003). Interestingly, in this model, IL-12Rβ2 deficiency also results in increased IL-23 p19 mRNA expression and ultimately, in an exacerbation of EAE (Zhang, Gran et al. 2003). In contrast, Coxsackievirus B3 infected IL-12Rβ1 deficient mice (deficient in both IL-12 and IL-23 signalling) display decreased IL-18 levels in the serum and in the heart, which is in turn correlated with decreased myocarditis (Fairweather, Yusung et al. 2003).

The p35 -/- mice used in our study functionally resemble the IL-12Rβ2 mice used by Zhang *et al.* and thus are in good agreement with their findings in an EAE model (Zhang, Gran et al. 2003). It will require further study to determine if the discrepancy in IL-18 production between IL-12 -/- (i.e. p35 -/- or IL-12Rβ2 -/- mice) and IL-12/IL-23 -/- (IL-12Rβ1 -/-) mice represents a potential role for IL-23 in the control of IL-18 or differential regulation of IL-18 in viral infection and administration of

protein antigen/LPS . Sadly, in our study, serum IL-18 levels were not determined in IL-12 p40 -/- mice (which may have potentially allowed us to comment more specifically on this discrepancy) due to lack of mice.

4.1 - IL-18 as a negative regulator of type 2 cytokine synthesis

IL-18 is a potent inducer of IFNγ production, particularly in the presence of IL-12. More recently IL-18 has been attributed a powerful pro-Th2 activity as it has been demonstrated to induce type 2 cytokine synthesis, and thereby IgE synthesis (Yoshimoto, Mizutani et al. 2000; Yoshimoto, Min et al. 2003). Thus, we directly examine the effect of IL-18 on *in vitro* type 2 cytokine production from OVA (alum) immunized mice. Stimulation of spleen cells from OVA (alum) immunized mice with OVA + IL-18 results in marked inhibition of IL-4 synthesis in a dose dependent manner (Figure 16). Production of other type 2 cytokines or chemokines examined is unaffected (Figure 17). Furthermore, the inhibitory effects of IL-18 on IL-4 production are independent of IFNγ, as a similar inhibition is seen when IL-18 is added to spleen cells cultures from OVA (alum) immunized IFNγ -/- mice (Figure 16). In contrast, while IL-12 dramatically inhibited all type 2 cytokines examined, its actions are principally dependant upon its ability to induce IFNγ production (Figure 16 and 17) as previously described (Wynn, Jankovic et al. 1995; Bruselle, Kips et al. 1997; Hogan, Foster et al. 1998; Stampfli, Scott Neigh et al. 1999; Rais, Wild et al. 2002). This clearly demonstrates a marked difference between the negative regulatory capacity of these two Th1 promoting cytokines: IL-12 relies on IFNγ to mediate inhibition of Th2 immune responses, while IL-18 has the capacity to do so even in IFNγ deficient environments. It should also be pointed out that allergic individuals very

consistently demonstrate a reduced capacity to produce IFNγ in response to a variety of different stimuli (HayGlass, Li et al. 1997; Matsui, Kaneko et al. 1999; Matsui, Kaneko et al. 2000; Shikano, Kato et al. 2001; Watanabe, Kaneko et al. 2002). In this inherently IFNγ deficient environment, endogenous IL-18 may play an important role in the control of allergic disease.

The reasons for the differences between our results and the reports of IL-18 mediated enhancement of type 2 cytokine production are unclear, but significant methodological differences between our studies and those reporting enhancement of type 2 immune responses exist. Firstly, previous studies examining the effects of *in vitro* IL-18 involve extensive purification and manipulations: *in vivo* IL-2 treatment (3.6×10^6 IU rhIL-2 per mouse) to increase numbers of NK cells, α-Gal-Cer treatment to activate NKT cells, or IL-3 treatment of basophil cultures (Hoshino, Wiltrout et al. 1999; Xu, Trajkovic et al. 2000; Yoshimoto, Mizutani et al. 2000; Leite-De-Moraes, Hameg et al. 2001). In the absence of these manipulations, the induction of type 2 cytokine production by IL-18 is not seen in NK cells, NKT cells or basophils (Hoshino, Wiltrout et al. 1999; Xu, Trajkovic et al. 2000; Yoshimoto, Mizutani et al. 2000; Leite-De-Moraes, Hameg et al. 2001). Secondly, many of these studies use anti-CD3 stimulation to ensure a cytokine response detectable in the assays used in these labs (Xu, Trajkovic et al. 2000; Yoshimoto, Mizutani et al. 2000; Leite-De-Moraes, Hameg et al. 2001), whereas we stimulate *in vitro* cytokine production with antigen alone. Polyclonal stimulation of T cells results in a qualitatively different cytokine response than antigenic stimulation demonstrating that the pattern of cytokine induction is dependent on the nature of the stimulus used (Imada, Simons et al. 1995). Thus, the use of polyclonal activators may account for increased type 2 cytokine production seen in some studies (Xu, Trajkovic et al. 2000; Yoshimoto, Mizutani et al.

2000; Leite-De-Moraes, Hameg et al. 2001). Thirdly, perhaps most importantly, the dose of IL-18 used *in vivo* to promote IgE and IL-4 synthesis ranges from 7 to 65 ***micro***grams, administered over a period of about 2 weeks (Wild, Sigounas et al. 2000; Yoshimoto, Mizutani et al. 2000; Yoshimoto, Min et al. 2003). Lower doses have no discernable effect on IL-4 and IgE synthesis (Yoshimoto, Mizutani et al. 2000). The relationship between this experimental design and *in vivo* regulation remains speculative at best.

Finally, it is reported by Xu *et al.* that significant differences in IL-18 responsiveness exist between different strains of mice (Xu, Trajkovic et al. 2000). In the presence of IL-18, *in vitro* anti-CD3 stimulation of naive BALB/c spleen cells triggers IL-4 production sufficient to support the development of IL-4 producing, Th2-like cells (Xu, Trajkovic et al. 2000). In contrast, there was no induction of IL-4 from similarly stimulated C57Bl/6 spleen cells (Xu, Trajkovic et al. 2000). It is worthwhile to note that in the studies cited above, all those which find an increase in *T cell derived* type 2 cytokine production used BALB/c mice (Wild, Sigounas et al. 2000; Xu, Trajkovic et al. 2000; Yoshimoto, Mizutani et al. 2000), whereas the mice used in our study are C57Bl/6.

Collectively, there are clearly instances where IL-18 does have the capacity to promote Th2 cytokine production. However, the systems where such activities have been reported are somewhat contrived, calling into question the *biological* relevance of such findings. Our use of comparatively lower concentrations of IL-18, antigenic restimulation and bulk spleen cell cultures represents an attempt to maintain a more relevant *in vitro* system, with potentially more biologically relevant results.

As inhibition of IL-4 production is noted when IL-18 is added to whole spleen cell cultures, it is difficult to determine which cells respond directly to IL-18 (Figure 16). However, as IL-4 production was >90% inhibited following the addition of neutralizing anti-CD4 (data not shown), it seems likely that the final effect was on IL-4 producing CD4+ T cells (i.e. Th2 cells). A seminal report by Xu *et al.* demonstrates that IL-18R mRNA and protein expression can be detected only in Th1 clones and newly differentiated lines (Xu, Chan et al. 1998). Thus only Th1, and not Th2 cells, should be responsive to IL-18 (Xu, Chan et al. 1998). However, the Th2 cells examined in the study of Xu *et al.* are generated *in vitro* through antigenic stimulation in very powerful Th2 skewing conditions (IL-4, anti-IL-12 and anti-IFNγ), resulting in very clearly differentiated Th2 clones (Xu, Chan et al. 1998). Cells taken directly *ex vivo* are much less strongly polarized than *in vitro* generated lines. Just as exposure of differentiating Th2 cells to IFNγ maintains the expression of IL-12Rβ2 and allows for continued responsiveness to IL-12 (Szabo, Jacobson et al. 1995; Szabo, Dighe et al. 1997), exposure of differentiating, IL-4 producing CD4+ T cells to other endogenously produced cytokines may allow for continued expression of IL-18R, and IL-18 responsiveness. *In vivo*, this may represent a way of preventing generation of highly polarized IL-4 producing Th2 cells, and thus, the irreversible induction of type 2 immunity. Alternatively, the possibility that IL-18 is inducing the synthesis of an unidentified factor (other than IFNγ) which is in turn inhibiting the production of IL-4, cannot be excluded.

4.2 - Summary of findings on IL-18 regulation of type 2 immunity

Early investigations into the *in vivo* role of IL-18 focused primarily on its capacity to promote type 1 immune responses, particularly in the presence of IL-12. Recently, however, studies examining the role of IL-18 demonstrate its capacity to promote IL-4, IL-13 and IgE synthesis and thus argue that IL-18 possesses type 2 inducing activities. Our results demonstrate, IFNγ independent, IL-18 mediated *inhibition* of Ag-stimulated IL-4 synthesis in allergen specific recall responses. Coupled with the recent reports of a similar IFNγ independent capacity of IL-18 to inhibit IL-13 production in murine helminth infection (Helmby, Takeda et al. 2001; Helmby and Grencis 2002), these data suggest that IL-18 acts as a redundant negative regulator of type 2 immune responses via a pathway not dependent upon IFNγ.

5.0 - Mechanism of OA-POL mediated inhibition of type 2 immunity

The previous studies focus largely on examining the importance of endogenous factors in *preventing the induction* of allergic disease. However, a more pertinent therapeutic question is what are the endogenous factors which facilitate stable redirection of *existing* type 2 dominated immune responses to a type 1 dominated immune responses normally associated with clinical tolerance. Specific allergen immunotherapy is used in allergic patients to redirect the dominant Th2 immune response, however, immunotherapy does not work in all patients, for all allergens, and most importantly, the mechanisms of immune redirection are largely unknown. Thus, as a model system to explore mechanisms involved in successful immune redirection, we make use of OA-POL, a model system with a similar redirection of type 2 immune responses seen following successful immunotherapy. We show here that pre-treatment of C57Bl/6 mice with OA-POL in saline induces a dramatic down

regulation of IgE responses (>90% inhibition) with concomitantly increased IgG_{2c} levels (>100-fold) following subsequent immunization with OVA (alum) (Figure 18). Furthermore, we also demonstrate that similar inhibition of IgE and upregulation of IgG_{2c} production is seen upon subsequent boost with OVA (alum) if OA-POL is administered following primary immunization with OVA (alum) (Figure 19). Thus, the use of OA-POL allows us to investigate the importance of various endogenous factors in redirecting existing immune responses.

5.1 - Role of B cells in immune preventing the induction of type 2 immune resposnes

We have previously reported that OVA specific Abs have a dramatically lower affinity for OA-POL compared to native OVA (HayGlass and Strejan 1983). Thus, we hypothesized that B cells are not critical antigen presenting cells involved in capture, presentation and processing of OA-POL. To directly examine the role of B cells in our model, we immunize B cell deficient (μMT) mice with 2.0 μg of OVA (alum) following treatment with nothing, or OA-POL and analyze *in vitro*, OVA driven spleen cell cytokine production. OA-POL is equally effective in both C57Bl/6 and B cell deficient mice as demonstrated by similar decreases in IL-4, IL-5 and IL-13 production following OA-POL treatment (Figure 21 & 22, Table 3)). These data indicate that B cells are not required to facilitate the effects of OA-POL. We speculate that B cells are unable to present antigenic peptides generated from OA-POL, or lack the appropriate co-stimulatory molecules to redirect induction of a type 2 dominated immune response.

As in OA-POL treated mice, following nasal administration of allergen, there is a dramatic decrease in the intensity of type 2 immune responses (diminished Ag-stimulated spleen cell production of IL-4, IL-5, IL-13 and IgE production) which is abrogated in B cell deficient JHD mice (Tsitoura, Yeung et al. 2002). However, unlike treatment with OA-POL, which *increases* type 1 immune responses, there is a concomitant *decrease* in the intensity of type 1 immune responses (spleen cell IFNγ production) and a dramatic decrease in Ag-driven T cell proliferation following intra-nasal allergen administration (Tsitoura, Blumenthal et al. 2000; Tsitoura, Yeung et al. 2002). This diminishment of T cell proliferation and both type 1 and type 2 cytokine and antibody production suggests that tolerance is being actively induced rather than the change in phenotype of the elicited immune response that is seen following OA-POL treatment (Figure 18 and 19). Furthermore, the same group also report that the induction of tolerance is highly dependent on the presence of endogenous IL-10 production (Akbari, DeKruyff et al. 2001). In contrast, in our model, we see no upregulation of IL-10 production (data not shown). As both B cells and IL-10 are required to mediate the effects of intra-nasal administration of allergen (Tsitoura, Blumenthal et al. 2000; Akbari, DeKruyff et al. 2001; Tsitoura, Yeung et al. 2002) and B cells are potent producers of IL-10 (MacNeil, Suda et al. 1990; O'Garra, Stapleton et al. 1990; O'Garra, Chang et al. 1992), it is possible that the B cells represent an important source of IL-10 required for tolerance induction following intra-nasal allergen treatment. However, the differences in B cell dependence between our study and those or Tsitoura *et al.* may also lie in the use of μMT versus JHD B cell deficient mice. μMT mice are capable of IgA production and possess IgA^+ B cells, while JHD mice do not (Macpherson, Lamarre et al. 2001). This potential explanation seems unlikely however, as IgA titers are not significantly different

between OVA (alum) and OA-POL treated, OVA (alum) immunized animals (HayGlass and Stefura 1991).

The observation that OA-POL works equally effectively in C57Bl/6 and μMT mice indicates that B cells are not required for capture, presentation and processing of OA-POL.

5.2 - Role of endogenous IL-12 and IFNγ in OA-POL mediated suppression of type 2 immunity

The use of IL-12 deficient (both p35 -/- and p40 -/- mice) or IFNγ -/- mice allows us to determine the importance of these cytokines in facilitating the inhibition of type 2 immune responses seen following OA-POL treatment. While pre-treatment with OA-POL successfully down regulates OVA-specific IgE synthesis upon subsequent OVA (alum) immunization in all knockout strains, the magnitude of IgE inhibition seen in IFNγ -/- mice was markedly diminished as compared to that seen in control animals (11-fold inhibition versus 4.5-fold inhibition) (Figure 24 and Table 4). In contrast, the degree of IgE inhibition in IL-12 deficient mice (up to 30-fold inhibition) is equivalent to, or slightly greater than that seen in C57Bl/6 controls (Figure 24 and Table 4). A similar pattern of IgE inhibition is seen following administration of OA-POL to mice with existing IgE responses: reduced IgE inhibition in IFNγ -/- mice (8.7 fold inhibition), and slightly greater IgE inhibition in IL-12 -/- mice (>21 fold inhibition) compared to IgE inhibition in control mice (14 fold inhibition) (Figure 25 and Table 5).

Optimal upregulation of type 1 immunity requires both IFNγ and IL-12 as mice deficient in either cytokine develop substantially reduced IgG_{2c} production following OA-POL treatment (Figures 24 and 25, Tables 4 and 5). It is important to stress that these findings are entirely consistent with the findings of the previous section: IFNγ, but not IL-12, is a critical negative regulator of type 2 immunity.

While counterintuitive, the demonstration that inhibition of IgE is more efficient in OA-POL treated IL-12 -/- mice suggests that IL-12 may *promote* IgE synthesis. This observation does have precedents in the literature. Studies from our lab, and those carried out by Bliss *et al.* find that administration of IL-12 as an adjuvant increases spleen cell production of both IL-4 and IFNγ in C57Bl/6 and BALB/c mice (Bliss, Van Cleave et al. 1996; Rempel, Wang et al. 2000). Germann *et al.* report increased IgE production in BALB/c and CBA/J mice following immunization with KLH or phospholipase A_2 (PLA_2) with low doses of IL-12 (0.01 to 1 μg/day for 5 days)(Germann, Guckes et al. 1995). Schmitt *et al.* demonstrate that IL-12 in the presence of IL-4 can further potentiate the development of IL-4 producing Th2 effector cells (Schmitt, Hoehn et al. 1994). Finally, Wynn *et al.* report that administration of IL-12 exacerbates the Th2 inflammation and elevates serum IgE levels after immunization of IFNγ -/- infected with *Schistoma mansoni* eggs (Wynn, Jankovic et al. 1995). Taken together, these data provide compelling evidence that IL-12 can also trigger increased IL-4 and IgE production. Work carried out by Drs. Julia Rempel and Solomon Haile suggest that the ability of IL-12 to promote IL-4 production relates to increased FcγR expression on NKT cells which allows OVA-specific activation of this population of cells (personal communication). Thus, the dichotomy

between studies arguing type 2 promoting and inhibitory activities for IL-12 may be related to differences in the proliferation/activation of NKT cells within these models.

5.3 - OA-POL treatment results in increased T cell responsiveness to IL-12 and IL-18

IFNγ is crucial for preventing the development of type 2 immunity following treatment with OA-POL (Figures 24 and 25, Tables 4 and 5). Coupled with the observation that there is no increase in IL-12 or IL-18 production in spleen cells from OA-POL treated animals (Figure 23), we hypothesize that OA-POL treatment acts to increase responsiveness to endogenously produced IL-12 and/or IL-18. As IL-12 and IL-18 are potent inducers of IFNγ production, increasing responsiveness to these cytokines provides a mechanism whereby IFNγ, critical for optimal efficacy of OA-POL treatment, could be produced in the absence of elevated IL-12 or IL-18 levels. We subsequently demonstrated that spleen cells from OA-POL treated mice produce more IFNγ in response to either IL-12 or IL-18 than spleen cells from OVA treated mice (Figure 26). This indicates that OA-POL treatment increases biological responsiveness to these cytokines. Depletion of NK cells from the bulk spleen cell population does not impact the increase in either IL-12 or IL-18 driven IFNγ production suggesting that increased IFNγ production is T cell dependent (Figure 28). Elevation of IL-18R and IL-12R mRNA levels in T cell populations provides a potential explanation for increased T cell responsiveness to IL-12 and IL-18 (Figures 27 and 29). These data provide support the hypothesis that preventing the development of type 2 immune responses in the face of strong type 2 skewing signals, is associated with an increased responsiveness to IL-12 and IL-18.

Both human and murine studies attest to the regulatory role of IL-12 and IL-18 responsiveness in determining the intensity and dominance of type 2 immunity. BALB/c mice (widely considered as an "allergic" or type 2 biased mouse strain) have lower basal IL-12 responsiveness than C57Bl/6 mice (considered a "non-allergic" strain)(Otani, Nakamura et al. 1999). NC/Nga mice, which spontaneously develop atopic dermatitis and high IgE levels, exhibit substantially impaired IFNγ production following administration of IL-12 (Matsumoto, Itakura et al. 2001). In examining IFNγ responses from PBMC of allergic and normal controls stimulated with IL-12 ± IL-2 (HayGlass, Li et al. 1997; Matsui, Kaneko et al. 2000; Shikano, Kato et al. 2001) or IL-18 (Shikano, Kato et al. 2001; Watanabe, Kaneko et al. 2002), allergic individuals display significantly lower IFNγ production compared to non-atopic controls. Furthermore, genetic studies examining IL-12 and IL-18 receptor subunits in allergic individuals reveals an increased prevalence of mutations in the IL-12Rβ2 and IL-18Rα subunits, the latter of which confers IL-18 *hypo*responsiveness (Matsui, Kaneko et al. 1999; Watanabe, Kaneko et al. 2002). Finally, the presence of high levels of IL-12 p40 homodimer, a specific antagonist of IL-12 activity, in lung biopsies from asthmatic individuals, but not biopsies from control individuals suggests that inhibition of local IL-12 activity in the lungs may exacerbate the allergic response by limiting responsiveness to endogenously produced IL-12 (Walter, Kajiwara et al. 2001). Taken together with the present findings, these studies supply ample evidence to support the contention that the control of type 2 immune responses is highly dependant on responsiveness to endogenously produced promoters of type 1 immunity.

5.4 - General mechanism of OA-POL mediated inhibition of type 2 immune responses

Many mechanisms have been proposed to explain the clinical improvement observed following specific allergen immunotherapy. These include shifting the balance of type 1 versus type 2 cytokines produced by mucosal or peripheral T cells, the induction of "blocking" IgG_4 antibodies, and the formation of IL-10 producing $CD4^+CD25^+$ regulatory T cells. We believe, however, that the data indicate that the principal mechanism by which administration of chemically modified allergen inhibits IgE synthesis is through the stable recruitment of naive, OVA-specific T cells into Th1-like effector cells which ultimately results in the observed shift from type 2 to type 1 dominance of the immune response.

We do not believe the effects of OA-POL are mediated by the induction of IL-10 and/or TGF-β as is frequently seen in animal models which report immune redirection following mucosal administration of allergen (Tsitoura, Blumenthal et al. 2000; Akbari, DeKruyff et al. 2001; Wiedermann, Herz et al. 2001; Zhang, Izikson et al. 2001; Hall, Houghton et al. 2003; Zemann, Schwaerzler et al. 2003). While we do not examine TGF-β production in our model system, IL-10 production is not enhanced following OA-POL treatment (Figure 21). Furthermore, concomitant with the decreased IgE production, OVA-specific IgG_{2c} levels are increased (Figures 24 and 25, Tables 4 and 5). This differs from the global downregulation of allergen specific type 1 and type 2 immune responses seen when allergen is administered directly to mucosal tissues (Tsitoura, Blumenthal et al. 2000; Akbari, DeKruyff et al. 2001; Zhang, Izikson et al. 2001; Hall, Houghton et al. 2003; Zemann, Schwaerzler et al. 2003). An alternative explanation for the observed effects of OA-POL on the immune response - the induction of high titers of "blocking" IgG_{2c} and IgG_1 - is not supported by the undiminished effectiveness of OA-POL treatment in B cell deficient μMT mice (Figure 22 and 23; Table 3).

The specific mechanism through which glutaraldehyde polymerized allergens increase IL-12 and IL-18 receptor expression on T cells is presently unclear, but a number of potential mechanisms are possible: 1) the larger size of OA-POL and the reduced affinity of OVA-specific antibodies for OA-POL (HayGlass and Strejan 1984) target OA-POL to a population of APCs distinct from that targeted by native OVA; 2) OA-POL triggers DC maturation in a way which facilitates increased IL-12 and IL-18 receptor expression on differentiating T cells; and 3) higher concentrations of T cell epitopes in a single OA-POL molecule results in a greater density of MHC:OVA peptide complexes on the surface of the APC which provides a qualitatively different signal to T cells facilitating increased IL-12 and IL-18 receptor expression. These mechanisms will be individually examined in future experiments.

6.0 - Overall endogenous control of allergic responses

Represented in Figure 31 is a cartoon summarizing the major findings of this thesis. It is understood that the development of IFNγ producing, allergen specific effector cells (principally via activation in the presence of APC derived IL-12) leads to clinical tolerance (Figure 31, light blue arrows). In contrast, differentiation of IL-4 producing, allergen specific Th2-like effector cells (principally due to the presence of IL-4 from as yet undetermined sources during primary T cell activation) results in the production of IgE and other mediators of type 2 immunity, leading to allergic sensitization

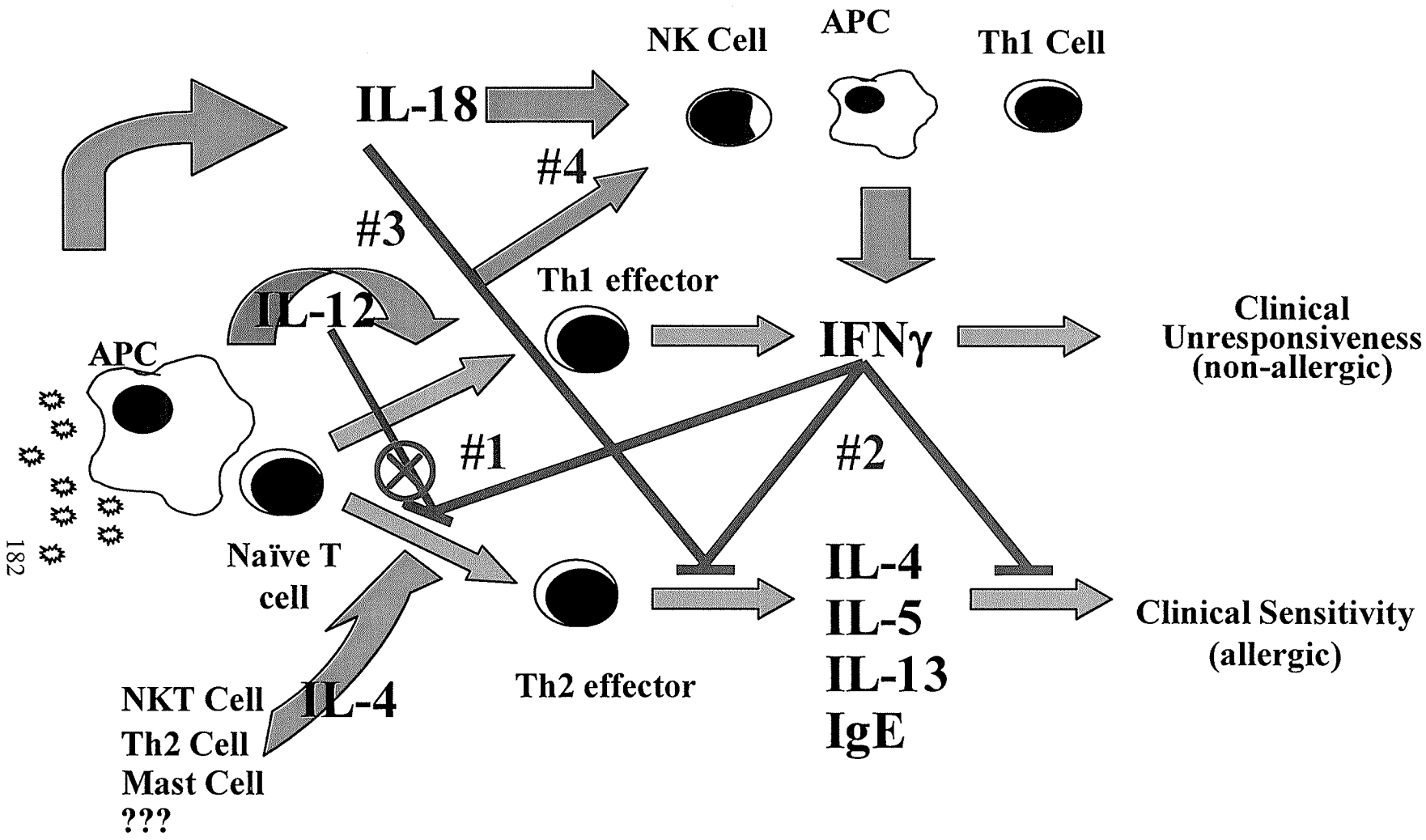

Figure 31: Endogenous control of allergic responses. Green arrows indicate activating effects, red lines indicate inhibitory effects, ⊗ indicates a non-critical inhibitory pathway .

(Figure 31, lavender arrows). Studies examining the immune response in IL-12 deficient mice demonstrate the principal role of IL-12 is as a promoter of type 1 immune responses. Unchanged type 2 cytokine or Ab production in the absence of IL-12 suggests that IL-12 is not a direct negative regulator of type 2 immunity (Figure 31, #1). In contrast, we show here that endogenous IFNγ, produced in large amounts by NK cells, T cells and APCs in response to both IL-12 and IL-18 is directly required to inhibit the synthesis of type 2 cytokines (Figure 31, #2). Indeed, immune responses in IFNγ -/- mice are characterized by significantly elevated levels of type 2 cytokine production in response to antigenic stimulation. Finally, IL-18 is known to have potent IFNγ inducing properties, and through these activities, IL-18 can contribute indirectly to the inhibition of type 2 immune responses. However, we have demonstrated for the first time that IL-18 can also act independently of IFNγ to limit the production of IL-4 from $CD4^+$ T cells. We argue that this may represent an important, complementary means of preventing induction of type 2 immunity in vivo, independent of IFNγ (Figure 31, #3).

We also demonstrate in this thesis that the OA-POL mediated prevention of type 2 immune responses has similar cytokine requirements. The use of OA-POL in IL-12 or IFNγ -/- mice demonstrates that both of these cytokines are important in promoting type 1 immune responses. However, IFNγ, but not IL-12, is critically involved in the maximal limitation of a type 2 immune response (Figure 31, #1 and #2). Consistent with the requirements for IFNγ, but not IL-12, OA-POL treatment does not result in enhanced IL-12 production. Rather, we demonstrate that the principal action of OA-POL is to enhance T cell responsiveness to IL-12 and IL-18, thereby increasing the development of Th1-like effectors and the production of IFNγ (Figure 31, #4). This argues that preventing the development of

a type 2 dominated immune response, or abrogating an existing one may be more critically dependent on the overall T cell responsiveness to endogenous Th1 inducing cytokines, rather than the sum total of Th1 promoting cytokine expression.

7.0 - Summary and concluding remarks

Studies making use of genetically manipulated knockout mice are often difficult to interpret. It is a widely acknowledged fact that the chronic absence of a cytokine can lead to the generation of compensatory mechanisms which are required to ensure "normal" development of the immune system, or ensure the viability of the organism. A lack of phenotype in a knockout mouse does not necessarily imply that the knocked out gene does not play a role in the system examined. Rather, it may reflect the redundancy inherent in many complex biological systems. The observation that IL-12 deficiency lacks any observable effect on type 2 immune responses suggests that IL-12 is, at most, redundant in this role, (possibly relying on IL-18 or IL-27 to limit type 2 immune responses in the absence of IL-12). In contrast, the finding of significantly increased intensity of type 2 cytokine production in IFNγ -/- mice strongly suggests that IFNγ does play a critical role in negative regulation of type 2 immune responses.

Recently, the use of IL-12 as a treatment for mild asthma in humans was explored. Following a course of IL-12 treatment, significant decreases are found in serum and sputum eosinophilia following antigen challenge (Bryan, O'Connor et al. 2000). Despite success in limiting serum and sputum eosinophilia, rIL-12 did not alter airway responsiveness to either histamine or antigen

challenge (as measured by the forced expiratory volume in 1 second (FEV_1))(Bryan, O'Connor et al. 2000). The effects of IL-12 administration on the overall intensity of the type 2 immune response (via measurement of Ag-driven cytokine or chemokine production) was not assessed. The overall effect of IL-12 on the underlying immune response could not be determined however as there was no examination of type 2 cytokine production, or examination of serum IgE levels. When considering the side effects of IL-12 treatment (ranging from flu-like symptoms to heart palpitations) and the lack of definitive disease modification, this study concludes that the use of IL-12 at the doses studied do not justify its use for treatment of mild asthma. The rationale for using IL-12 as a therapeutic agent for the treatment of allergic disease was based largely on murine studies, but it is important to point out that the most successful murine studies only examined the *adjuvant* effect of IL-12 administration in the short term, on developing immune responses (Germann, Guckes et al. 1995; Kips, Brusselle et al. 1996; Sur, Lam et al. 1996; Bruselle, Kips et al. 1997; Rempel, Wang et al. 1997; Lee, Fu et al. 1999). Studies from our own lab (Rempel, Wang et al. 1997; Rempel, Wang et al. 2000) and others (Germann, Guckes et al. 1995) demonstrate that the capacity of IL-12 to redirect developing immune responses is transient. The intensity of type 2 immune responses are equivalent or *higher* to those seen in untreated animals after subsequent allergen exposures in the absence of IL-12 treatment. This study clearly underlines the necessity of thorough examination of the outcome in animal models, and evaluating multiple parameters of allergic hyperresponsiveness in assessing new therapeutic approaches.

The most exciting finding in this thesis, that IL-18 can specifically inhibit IL-4 production independently of its capacity to induce IFNγ production, is in stark contrast to the recent observations

that IL-18, particularly in the absence of IL-12, acts as a *promoter* of type 2 immunity. However, critical examination of these studies reveal potential differences between their and our studies which may have accounted for the dramatic differences (see Section 4.1 of this discussion). We acknowledge the evidence that support a role for IL-18 in *promoting* type 2 immunity, but the biological relevance of these activities are presently unclear.

The observation that OA-POL treatment results in significantly increased expression of IL-12 and IL-18 receptor expression on T cells, but does not result in significantly enhanced production of the cytokine themselves is also supported by studies of human atopic patients. These studies demonstrate that there exists in allergic individuals, a significantly reduced capacity to respond to IL-12 and IL-18 (HayGlass, Li et al. 1997; Matsui, Kaneko et al. 2000; Shikano, Kato et al. 2001; Watanabe, Kaneko et al. 2002). These data suggest that attempts to manipulate *in vivo* responsiveness to endogenously produced cytokines might be a useful therapeutic strategy for treatment of allergic disease. Indeed, the result of such an intervention, redirection of type 2 dominated immune responses (rather than induction of IL-10 producing regulatory cells), suggests that it might be useful for treating allergic patients which are expected, according to the hygiene hypothesis, to have a weak "regulatory network".

The principal focus of this thesis was to explore the endogenous factors which limit the induction and severity of type 2 immune responses. We demonstrate that the type 1 immune response is dramatically reduced in both IL-12 and IFNγ -/- mice, but only IFNγ -/- mice have a detectable increase in the intensity of type 2 immune responses. We also provide a potential explanation for the

lack of increased type 2 immune response in IL-12 -/- mice: increased IL-18 production in IL-12 -/- mice, coupled with the novel observation that IL-18 can specifically, and IFNγ independently inhibit IL-4 production. Using OA-POL to redirect existing immune responses we show that B cells are not required for preventing the development of a type 2 dominated immune response through the use of OA-POL, and further confirm the role of IFNγ, but not IL-12, as a negative regulator of type immunity. Consistent with the critical requirement for IFNγ to optimally inhibit IgE production, we report that OA-POL mediates its effects not through increased IL-12/IL-18 production, but by increasing T cell IL-12 and IL-18 receptor expression.

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