THE UNIVERSITY OF MANITOBA

THE ROLE OF IL-2 AND ITS BIOLOGICAL ANALOGS IL-15, IL-7, IL-9 IN MAINTENANCE OF ONGOING ALLERGIC RESPONSES

BY

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M.D.

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ABSTRACT

Introduction: Interleukin (IL)-2, IL-15, IL-7 and IL-9 are considered IL-2-like cytokines because they share common γ-chain subunit, related signalling and biological functions. We wish to determine the impact of IL-2-like cytokines in Th1/Th2 development.

Methods: Immunize C57Bl/6 mice with recombinant mite allergen Der p1. By adding exogenous cytokines or blocking endogenous cytokines and/or CD4+, CD8+ T cells in vitro, we investigate the cellular and molecular mechanism of IL-2-like cytokines in the development of Th1/Th2 responses. Also CD8-deficient mice and IL-12-deficient mice are chosen to further extend our conclusions.

Results: We established the Th2 immunity-dominated murine model of allergy against house dust mite allergen Der p1. IL-15 selectively enhances Ag-specific IFNγ and IP-10 response and both CD4+ and CD8+ cells are required. Endogenous IL-12 is required in this augmentation, IL-15 increases cellular responsiveness to IL-12. IL-2 is not required by IL-15 and IL-15 increases the consumption of IL-2 by activated T cells. In contrast to IL-15 and IL-2, we found that IL-7 and IL-9 selectively enhance Ag-specific Th2 response without impact on Th1 cytokines.

Conclusion: IL-2 and IL-15 selectively promote Ag-specific Th1 immunity while IL-7 and IL-9 support Th2 immunity. Both CD4+ and CD8+ T cells contribute to the Ag-specific Th1 differentiation augmented by IL-15. The mechanism includes that IL-15 enhances the cellular responsiveness to endogenous IL-12, hence more Th1 cytokine production. Exogenous rIL-15 can substitute for IL-2 in Ag-specific cytokine production.

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ABBREVIATIONS

Ab -Antibody

Ag -Antigen

AICD - Activation induced cell death

APC -Antigen presenting cell

BAL -Bronchial airway lavage

CFA -Complete Freund's adjuvant

CMI -Cell mediated immunity

Con A -Concanavalin A

CTL -Cytolytic T lymphocytes

Der f -Dermatophagoides farinae

Der p -Dermatophagoides pteronyssinus

DTH -Delayed type hypersensitivity

ELISA -Enzyme linked immunosorbent assay

FCS -Fetal calf serum

FITC -Fluorescein isothiocyanate

HDM -House dust mite

IFN -Interferon

Ig -Immunoglobulin

IL -Interleukin

IL-2R -Interleukin-2 receptor

i.p. -Intraperitoneal

IRS - Insulin receptor substrates

JAK - Janus kinase

KO -knockout

LPS -Lipopolysaccharide

L. monocytogenes -Listeria monocytogenes

mAb -Monoclonal antibody

MHC -Major histocompatibility complex

Mr -Relative molecular mass

NK -Natural killer

OVA -Ovalbumin

PBMC -Peripheral blood mononuclear cells

PCA -Passive cutaneous anaphylaxis

rIL -Recombinant interleukin

SE -Standard error

SEM -Standard error of the mean

STAT - Signal transducer and activator of transcription

TCR -T cell receptor

Th -T helper cell

TGF -Transforming growth factor

TNF -Tumor necrosis factor

WT -Wild type

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INTRODUCTION

1.1 Cytokine and Immune Functions

Cytokines are proteins that are secreted by cells and exert actions on either the cytokine-producing cells (autocrine actions) or on other target cells (paracrine actions). Cytokines control the ontogeny, development, differentiation, activation and inhibition of immune and non-immune cells.

When cytokines exert their function in the immune system, they exhibit the features of **cytokine pleiotropy** and **redundancy**. Cytokine pleiotropy means that one cytokine can exert many different functions, often on different cell types, whereas cytokine redundancy refers to the phenomenon that different cytokines can induce similar biological effect as a result of similar intracellular signaling pathway. The mixture of cytokines secreted by a given cell type produces many effects through what is called **a "cytokine network"**.

In immune responses, cytokine network is critical in shaping the intensity and the type of response resulting from Ag exposure: protection, hypersensitivity or clinical tolerance.

Cytokines can be divided into different categories according to their function, structure or both. In this thesis, we focused on two criteria, one is the Th1/Th2 criteria which is based on the functional pattern of cytokine in immunity, the other is "IL-2-analog" criteria which is based on their receptor structural and signaling similarity to IL-2 receptor. They are good examples of cytokine categories. They are discussed in the following paragraphs respectively.

1.2 Type 1/type 2 paradigm

Origin of the concept

CD4 T cells control activation of B cells, macrophages and CD8 cells, therefore CD4 T cell activation is considered a pivotal event in adaptive immunity. In mid-1980's, Mosmann and others investigated polarized CD4 T cell clones in long term culture derived against a number of different Ags. They found that some clones produced IL-2, IFNγ, but not IL-4, IL-5 and IL-6, whereas other clones made IL-4, IL-5 and IL-6, but not IFNγ and IL-2. On the basis of the fact that these two CD4 T cell populations exhibited differential patterns of cytokine synthesis, they are designated Th1 and Th2 cells respectively. Th1 clones produce IL-2, IFNγ, and lymphotoxin, and Th2 clones produce IL-4, IL-5, IL-6, IL-10 and IL-13.(Mosmann, Cherwinski et al. 1986; Coffman, Varkila et al. 1991)

For the freshly derived clones, they produced a mixed pattern of Th1 and Th2 cytokines, so they were termed Th0 to reflect that they are less mature or less committed T cells in terms of cytokine synthesis. But in 1995, Kelso et al found that while the majority of freshly isolated CD4 cell clones produced different combinations of Th1 and Th2-associated cytokines, a minority of such individual fresh CD4 T cells did produce highly restricted Th1 *or* Th2 associated cytokine patterns. This finding suggests that the Th0 cells observed in vitro could be representative of diverse physiologic T cell populations and that Th1 and Th2 cells represent extremes in this spectrum of activation(Kelso 1995).

Evolvement of the concept

The concept of Th1 and Th2 model evolved with increasing knowledge of the immune system. Because the production of Th1 and Th2 associated cytokines is not limited to CD4 T cell(Carter and Dutton 1996), now this concept expands to different cell populations rather than limited to small number of T cell clones. So this paradigm now is commonly expressed as type 1 response and type 2 response. The type 1 response can be seen as a package of APC-derived IL-12, IL-18, IFNγ from CD4, CD8 and NK cells plus increased IgG2a in DTH and in pathologic autoimmunity (Mosmann and Sad 1996). The type 2 response is characterized by weaker IL-12, stronger IL-4, IL-5, IL-13 and increased immediate hypersensitivity.

Because of the concerns about how reflective clones are of in vivo responses from the T cell repertoire as a whole to generate polarized murine or human CD4 T cell Th1 and Th2 cell clones, the new concept of type 1 vs. type 2 responses provides a more practical platform to study the immune response. In the murine allergic model used in this study, spleen cell cultures including different cell populations were set up and the type 1 vs. type 2 responses were determined with current concept.

The Th1/Th2 paradigm was originally described based on the patterns of cytokine production by murine T cells. In humans the Th1 and Th2 patterns are similar, but not all the cytokines are as tightly restricted.

Significance of Type 1/Type 2 model

This model provides a framework to explore interrelationships among different cytokine synthesis, effector function and clinical consequences. Examples are listed in the following table. In this study, we focused on the role of type 1 and type 2 immunity in allergy.

TABLE 1. Relationship among type 1/type 2 responses, effector function and clinical consequences

	Type 1 response	Type 2 response
Effector molecules	Cytotoxin: perforin, granzymes, Fas ligands Cytokines: IFNγ, TNFβ, and TNFα. Ab: type1 Ab IgG2a (mouse).	Cytokines: IL-4, IL-5, IL-10, IL-13 Ab: type 2 Abs IgE, IgG1 (mouse).
Effector functions	Cell-mediated immunity predominate: 1. Macrophage activated to be highly microbicidal and amplify immune responses; coordinate immune response against intracellular bacteria; formation of granulomas in chronic infection. 2. Induces B cell to produce opsonizing Ab.	Humoral immunity predominate: 1. Activates B cells to make neutralizing Ab 2. Various effects on macrophages.
Clinical consequences	1. Infectious diseases: intracellular bacteria, fungi, protozoa. Tuberculoid leprosy 2. Inflammatory diseases: arthritis, colitis. 3. Type IV hypersensitivity	1. Infectious diseases: extracellular pathogens such as helminthes. Lepromatous leprosy. 2. Type I hypersensitivity

Reference: (Wedderburn and Woo 1999)

1.3 Cytokine environment affecting Type 1/Type 2 responses generation

Induction of CD4 T cell responses to exogenous Ag are influenced by many factors including Ag dose, physical and chemical form, route of administration, expression of costimularoty molecules, and the affinity of the TCR for its ligand, because if any single factor were independently responsible for the Th1/Th2 balance, evolutionary microorganisms would have developed ways of subverting these defenses (Mosmann and Sad 1996).

Cytokine milieu at the time of initial priming of naive T cells is considered a key factor to influence the cytokine production when cells are subsequently stimulated. IL-4 (produced by mast cells, basophils, T cells and natural T cells) induces Th2 differentiation, whereas IL-12 and IL-18 (produced by APC) are the major driving force to induce Th1 differentiation(Muraille and Leo 1998).

Th1 and Th2 cells have different abilities to transcribe the genes for IL-2 and IL-4. C-maf protooncogene product, together with NF-AT and NIP-45, are found to be critical for regulating IL-4 production. IL-2 production is regulated by NF-AT, AP-1 and NF-κB. NF-AT is induced by Ag recognition by TCR. AP-1 and NF-κB are activated by ligation of CD28 by B7.

IFNγ can not induce Th1 generation, but it can inhibit Th2 development. Because IFNγR-2 expression is dramatically reduced in Th1, but not Th2, clones after exposure to IFNγ, this results in lack of IFNγ responsiveness by Th1 cells, not Th2 cells. Th1 cells produce IFNγ and can thereby selectively inhibit the proliferation of Th2 cells.

As noted above, IL-12 is currently believed to be the major inducer of Th1 cells. Many Th2 cells do not respond to IL-12 resulting from IL-4-mediated loss of expression of the IL-12Rβ2 subunit of the IL-12 receptor.(Kalinski, Hilkens et al. 1999)

1.4 Allergic asthma and type 2 responses

Asthma is a group of disorders with 3 features: (a) intermittent and reversible airway obstruction; (b) bronchohyperresponsiveness (BHR), which is defined as an increased sensitivity to bronchoconstrictors (such as histamine or cholinergic agonists); (c) bronchial inflammation. The house dust mite is perhaps the most important allergen related to asthma (discussed later). The pathophysiology of airway constriction includes eosinophilia and Th2 lymphocyte infiltration, the presence of FcεRI+ cells and IgE expressing cells (Humbert, Menz et al. 1999)

Following allergic sensitization, T cells from atopic patients tend to produce elevated levels of Th2-type cytokines, especially IL-4, IL-13, IL-5 and IL-6, which induce and regulate IgE production and eosinophil airway infiltration (Hamelmann and Gelfand 2001). Th2 cells orchestrate asthmatic inflammation through the secretion of a series of cytokines and chemokines. Th2 cells and such cytokines have been identified in bronchoalveolar lavage fluid (BAL) and airway biopsies.

The biological function of IL-4 in asthma includes (a) promoting Ig isotype switch from IgM to IgE by B cells; (b) stimulating Th2 differentiation; (c) inducing the expression of vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells for the adhesion and transmigration of T cells, eosinophils, basophils and monocytes (Moser, Fehr et al. 1992)

IL-13 is another Th2 cytokine closely related to IL-4 because IL-13 binds to IL-4Rα and is also expressed by Th2 cells from patients with asthma (McKenzie, Bancroft et al. 1998; Corry 1999). IL-13 induces inflammation, mucus hypersecretion, subepithelial fibrosis, and eotaxin production (Zhu, Homer et al. 1999). IL-13 stimulates B cells to produce IgE, stimulates epithelial cell to secret mucus and chemokines, and also affects smooth muscle contractility. Neutralizing IL-13 completely blocks airway hyperreactivity in mouse asthma models (Wills-Karp, Luyimbazi et al. 1998).

IL-5 acts on B cells and eosinophils in the mouse, and seems to be restricted to eosinophils in human. The key role of IL-5 is in the control of eosinophilia (Dent, Strath et al. 1990; Foster, Hogan et al. 1996; Kopf, Brombacher et al. 1996). There are two roles played by IL-5 in eosinophilia: (a) IL-5 plays a critical role in the expansion of eosinophil pools in both the bone marrow and blood in response to allergen challenge of the airways. (b) IL-5 and eotaxin co-operate locally in pulmonary tissues to selectively and synergistically promote eosinophilia.

IL-4 and IL-13 cooperate within the lung to control the transmigration of eosinophils across the vascular bed into pulmonary tissues. IL-5 and eotaxin levels within the lung are regulated by IL-4 and IL-13. This mechanism allows Th2 cells to accurately co-ordinate tissue and peripheral eosinophilia.

In animal models it was found that inhibition of either the IL-4/IL-13 or IL-5/eotaxin pathways resulted in the abolition of tissue eosinophils and AHR, but only depletion of IL-5 and eotaxin concurrently results in marked attenuation of pulmonary inflammation (Foster, Mould et al. 2001).

Chemokines were reported to have a role in allergic diseases (Lukacs, Oliveira et al. 1999). Eotaxin, monocyte chemoattractant protein-1 (MCP-1), MCP-3, MCP-4, RANTES, and macrophage-inflammatory protein 1 (MIP-1) are major C-C chemokines found in bronchoalveolar lavage (BAL) from asthmatics (Alam, York et al. 1996; Holgate, Bodey et al. 1997). The main function of these C-C chemokines

were recruitment of eosinophils into the airways (Lukacs, Standiford et al. 1996; Campbell, Kunkel et al. 1998; Gonzalo, Lloyd et al. 1998). Eotaxin was first discovered in the BAL fluid of guinea pigs after allergen challenge (Jose, Adcock et al. 1994). Except chemotactic activity, eotaxin can induce increased adhesion of eosinophils to vascular endothelium via β1 and β2 integrin(Kitayama, Mackay et al. 1998). RANTES, MCP-3 and MCP-4 can also recruit eosinophils through the CCR3 receptor. It is also found that blocking MCP-1 and RANTES significantly attenuates airway hyperresponsiveness (Gonzalo, Lloyd et al. 1998). Furthermore, because of the redundancy of C-C chemokines and receptors (for instance, C-C chemokine receptor 1 (CCR1) binds MIP-1, RANTES, and MCP-3 and is expressed on a number of leukocytes including neutrophils, monocytes, lymphocytes, and eosinophils) other chemokines could have a role in the diseases.

The biological functions of chemokines are not limited to chemotaxis and regulation of transmigration. MIP-1 contributes in the pulmonary fibrotic responses after mononuclear phagocyte accumulation(Smith, Strieter et al. 1995; Smith 1996). CC and CXC chemokine members might have the capacity to modulate T cell differentiation into Th2 or Th1 type. For example, MCP-1 can drive naive T cell population towards IL-4-producing Th2 cells, whereas MIP-1α and IP-10 promotes IFNγ production by Th1 cells (Karpus, Lukacs et al. 1997). Chemokines can also regulate IgE isotype switch in B cells (Kimata, Yoshida et al. 1996). This makes chemokine receptors potential targets in the treatment of asthma and allergic disease (Wells, Power et al. 1996).Our lab first discovered a role for human IP-10 in

selectively promoting marked enhancement (20-30 fold) of type 1 responses (Gangur, Simons et al. 1998; Gangur, Simons et al. 1999).

Because of the importance of such cytokines and chemokines in the etiology and development of allergic diseases, in this thesis, the role of type 1 and type 2 cytokines and chemokines respectively were examined. The cytokines studied were: IFNγ, IL-2 (type 1), IL-4, IL-5, IL-13 and IL-10 (type 2). The chemokines include: murine IP-10 (type 1) and C10 (type 2).

1.5 Dominant approaches to study type 1/ type 2 responses

Three dominant approaches have been applied in the study of cytokine responses and each of them has limitations.

(a) Derivation and characterisation of T cell clones: Because many extrinsic factors can influence the type of clones obtained, and Ag derived clones only represent an extremely small proportion of the total allergen-specific T cell response, there is advantage in evaluation of cytokine synthesis by fresh, unselected cell populations.

(b) Polyclonally stimulated primary culture: Because of the limitations of examining derived T cells clone as listed above, many investigators have tried to examine fresh PBMC directly *ex vivo*. However, the low cytokine concentrations following

Ag-specific restimulation, particularly the low levels of IL-4 expression, result in reliance on polyclonal stimuli. Use of polyclonal activators as surrogate Ag has potential problems because such stimulation (i) triggers virtually all T cell clones rather than the small Ag-specific population, and (ii) activates different patterns of intracellular signalling(Imada, Simons et al. 1995). Direct comparison of mitogen and Ag-driven cytokine gene expression can yield very different conclusions(Monteyne, Renauld et al. 1997), emphasising the need for Ag specific approaches.

(c) Evaluation of cytokine mRNA synthesis directly ex vivo: Quantification of cytokine mRNA levels is considered a semi-quantitative method and mRNA synthesis is not always parallel with cytokine production, particularly for key cytokines such as IL-15 and IL-18 (Tagaya, Bamford et al. 1996). We will discuss the regulation of IL-15 gene expression in detail in the next section.

Defined patterns of cytokine gene expression by fresh unselected cells are strongly associated with ongoing allergic diseases. Analysis of cytokine expression *ex vivo* is an objective approach to investigate mechanisms of allergic immune responses. Because of the limitations of each approaches above, we first developed sensitive assays for cytokines and chemokines, then we decided to set up an allergen immunized murine model to allow us to quantify allergen-stimulated cytokine and chemokine production in short term, primary cultures. We developed new systems to allow detailed investigation of cytokine and chemokine production by fresh,

unselected spleen cell population in response to short term Ag, rather than polyclonal activators.

2. IL-2-like cytokines

The receptors for IL-2, IL-15, IL-4, IL-7 and IL-9 share common γ-chain (γc) and related signaling pathway. These cytokines are all T cell growth factors, so they are nominated "IL-2-like cytokines". In this section, we discussed the similarity and difference among these cytokines.

2.1 Similarities among IL-2-like cytokines

2.1.1 Structural similarities among IL-2-like cytokines

There are extremely limited amino acid sequence similarities among different IL-2-like cytokines but there are all short-chain four helical-bundle cytokines in three-dimensional structures. The first two and last two α-helices are each connected by long-overhand loops (Rozwarski, Gronenborn et al. 1994; Davies and Wlodawer 1995). The length of the helices is approximately 15 amino acids for IL-2-like cytokines. There are β-sheet structures in the AB and CD loops. Each cytokine has evolved different disulfide bonds to stabilize its structure, but helices A and D are more conserved because they are involved in the interaction with cytokine receptors which share common subunits.

2.1.2 Structural similarities among the receptors of IL-2-like cytokines

IL-2-like cytokine receptors are hetero-oligomers of subunits. They are characterized by a few conserved regions: (a) Four conserved cysteine residues and WSXWS (trp-ser-X-trp-ser) in the extracellular domain responsible for intrachain or interchain binding and (b) Box 1/Box 2 regions in cytoplasmic domain for signal transduction. Most but not all IL-2-like cytokine receptor subunits has the above conserved region.

2.1.3 Cytokines that share the common receptor chains

IL-2, IL-4, IL-7, IL-9 and IL-15 receptors share a common γ-chain (γc), IL-2 and IL-15 receptors even share the β-chain.

There are three subunits for IL-2R: IL2Rα, IL2Rβ and a common γ chains (γc). β-chain and γc are expressed on resting lymphocytes, particularly NK cells of mouse and human, they compose an intermediate affinity receptor (Kd=10^{-9} M); IL-2Rα is expressed on activated lymphocytes where it serves as a low affinity receptor (Kd=10^{-8} M), but when IL-2Rα joins β and γc, they form the high affinity receptor (Kd=10^{-11} M) allowing strong binding and internalization of IL-2 into activated lymphocytes. Thus low and high-affinity receptors are expressed on activated lymphocytes, whereas intermediate-affinity receptors are found on resting lymphocytes and NK cells(Leonard, Shores et al. 1995; Sugamura, Asao et al. 1995; Taniguchi 1995; Leonard 1996; Sugamura, Asao et al. 1996). Multiple affinities of

IL-2 binding have biological significance. First, the IL-2Rβ and γc has signal transduction function, but the IL-2Rα only has short intracellular tail and does not play a role in signaling. The importance of IL-2Rα is in the kinetics of IL-2 association with its receptor: the α-chain provides fast binding rate and more efficient formation of IL-2/IL-2Rβ/γc binding complex and thus results in high-affinity binding to the receptor complex, which is important for responding to the physiologically low concentration of IL-2 (Smith 1989). IL-2Rα is also important for internalization of IL-2. The second biological significance is that both IL-2R and IL-15R have IL-2Rβ and γc, so IL-2Rα becomes the only structural difference explaining the functional discrepancy between these two cytokines.

Because of the unique role of IL-2Rα for IL-2 but not IL-15 internalization and function, we selectively blocked IL-2Rα in this study and investigated the exclusive role of IL-2.

The functional IL-7 receptor contains the 75-kDa IL-7Rα and γc (Noguchi, Nakamura et al. 1993; Kondo, Takeshita et al. 1994). IL-7 and thymic stromal-derived lymphopoietin (TSLP) share IL-7Rα (Peschon, Morrissey et al. 1994).

Functional IL-9 receptor contains IL-9Rα and γc. IL-9Rα is a binding protein without signaling function but it increases the affinity of binding and, more importantly, determining the cellular responsiveness to IL-9.

IL-15 receptor on T cells is composed of IL-15Rα, IL-2Rβ and γc. IL-15Rα is one subunit unique to IL-15. IL-15Rα also has no signaling function but determines the capacity of a cell to respond to IL-15. It shares a number of structural similarities with IL-2Rα and even gene loci (Anderson, Kumaki et al. 1995; Giri, Kumaki et al. 1995). The IL-15 receptor on mast cell is a distinctive form denoted IL-15RX.. Its signaling transduction is different from that of IL-2Rβ and γc.

The importance of shared γ-chain is best shown in XSCID mice. X-linked severe combined immunodeficiency disease (XSCID) results from mutation of γc. XSCID mice have more serious immunological deficiency than that has IL-2-deficient mice. The following table describes the immunological difference between these two diseases. It proves that the shared γc is required for the function of multiple cytokines.

TABLE 2. Immunological features of XSCID and IL-2-deficient mice

	XSCID	IL-2-deficiency
Mutations	γc gene on the X chromosome mutation.	IL-2 gene mutation
Immunologic Feature	(a) Greatly diminished T cell development; (b) Greatly diminished T cell number; (c) Normal B cell number, but defective B cell responses.	(a) Normal T cell development; (b) Normal T cell number; (c) Decreased polyclonal T cell response in vitro; (d) Normal responses to pathogenic challenge *in vivo*.

Practically, the property of shared receptor and signaling provides us a possible way to study the effect of combined depletion of endogenous IL-2-like cytokine function. Briefly, after investigating the selective (one cytokine at a time) effect of each of the IL-2-like cytokines in this study, we will determine if combined (two or more cytokines at a time) depletion of endogenous "IL2-like" cytokine production influences expression of Th1 vs. Th2 (or type 1 vs. type 2) cytokine recall responses to exogenous antigens. The methodology is to block β-chain (hence abrogate IL-2 and IL-15 signaling) or γ-chain (hence abrogate all IL-2-like cytokine signaling). This approach will be discussed in detail in the discussion.

2.1.4 Significance of the sharing of receptor γc chain

The γc chain has no primary binding activity for IL-2-like cytokines, but it can increase binding affinity in the context of the primary binding protein for each cytokine – the α chain.

The γc chain couples to signal transduction molecules which eventually promote the proliferation of T cells. So IL-2, IL-4, IL-7, IL-9 and IL-15 have shared function as T cell growth factors. The capacity of a cell to respond to a given cytokine is determined by the unique binding chain (i.e., IL4α or IL15α chain etc), but signaling pathways is shared.

Cytokine receptor molecules are individually targeted to the cell surface, and the ligand determines the formation and stability of different receptor complexes. So one cytokine can modulate the signals of another cytokine whose receptors contain the common γ chain.

2.1.5 Signaling similarities of IL-2-like cytokines

IL-2-like cytokines share the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway.

There are two regions for Janus kinase: JH1 is catalytic tyrosine kinase domain and JH2 is pseudo-kinase region which protects from hyper-activation of JAK-STAT pathway (Ihle, Witthuhn et al. 1995). There are four known JAKs: JAK1, JAK2, JAK3 and Tyk2. The receptors of all IL-2-like cytokines share JAK3 which is coupled with γc. IL-2 and IL-15 signalling share JAK1 which is associated with IL-2Rβ-chain (Miyazaki, Kawahara et al. 1994; Lin, Migone et al. 1995). The JAK3 gene is located at human chromosome 19p13.1 or mouse chromosome 8. JAK1 gene is located at human 1p31.3 or mouse chromosome 4. One same cell generally expresses at least three of the JAK kinases.

STAT proteins are substrates for JAKs. STAT proteins are believed to bind to cytokine receptors via interactions between phosphorylated tyrosine residues on the receptor and the SH2 domains of the STAT. The STATs bind palindromic sequences

with the consensus TTNCNNNAA. After docking, phosphorylation and dimerization, STATs translocate into the nucleus and bind DNA to activate transcription. There are 7 STAT members, STAT1, STAT2, STAT3 , STAT4, STAT5a, STAT5b, and STAT6. IL-2-like cytokines also share STAT members. In activated T cells and NK cells, IL-2, 4, 7, 9, 15 all induce activation of STAT3 . IL-2, 7, 9 and 15 but not IL-4 induce tyrosine phosphorylation and activation of STAT5 in either activated or resting T cells or NK cells. IL-4 can activate STAT6. IL-2 and IL-4 can induce phosphorylation and DNA binding of STAT1. Many reports confirmed that it depends on the expression of JAK3 in the cells for the formation of STAT5-containing DNA binding complex induced by IL-2. Similar to JAKs, the same STAT can be induced by multiple cytokines, the degree of specificity conferred by different individual STATs is therefore unclear.

TABLE 3. STATs shared by IL-2-like cytokines

Receptors of cytokine	STATs
IL-2	STAT1, STAT3 , STAT5
IL-4	STAT3 , STAT6
IL-7	STAT1, STAT3 , STAT5
IL-9	STAT3 , STAT5
IL-15	STAT3 , STAT5

Except JAK-STAT pathway, IL-2 and IL-15 also share src-related tyrosine kinase pathway, induction of Bcl-2, stimulation of the Ras/Raf/MAPK pathway and ultimately results in fos/jun activation (Miyazaki, Liu et al. 1995).

The receptors of IL-2, 4, 7,9 and 15 also share IRS, insulin receptor substrate, which has a role in intracellular signalling. In primary human lymphocytes, IRS-1 and IRS-2 (insulin receptor substrates-1 and -2) are both tyrosine phosphorylated in response to IL-2, 4, 7 and 15 (Johnston, Wang et al. 1995). IRS molecules are substrates for JAK kinases and JAK kinases associate with IRS in a cytokine-dependent manner.

2.1.6 Functional similarities: T cell growth factors

All of these IL-2-like cytokines induce proliferation of T cells. This can be explained by the sharing of γc. JAK3 is physically associated with γc and it can be activated by IL-2, 4,7, 9 and 15. IL-2 and IL-15 also activate JAK1 which is associated with IL2Rβ. Each chain of the receptor associates with a distinct JAK molecule and cytokine binding results in juxtapositioning, oligomerization of JAKs and subsequent JAK activation. Activation of JAK kinase initiates tyrosine phosphorylation of cytokine receptor chain. This allows the docking of STAT5 via its SH2 domain. Then STAT5 itself is tyrosine phosphorylated, dimerized, and translocate to the nucleus, where it switches on gene transcription.

JAK3 -γc interactions are important for T cell development and proliferation responding to IL-2-like cytokines. The importance of γc and JAK3 in T cell growth can be shown by XSCID and autosomal SCID patients respectively. XSCID is

caused by the mutation of γc gene on the X chromosome, XSCID patients fail to develop T cells and NK cells (see above). SCID is charaterized by γc intracytoplasmic region mutation. It is a moderate disease compared with XSCID. The JAK3 mutation alone induces autosomal SCID. Autosomal SCID patients demonstrate serious defects in T cell development.

2.2 Differences among IL-2-like cytokines

2.2.1 Chromosome location and genomic organization

The IL-2 gene is mapped to human chromosome 4q26-28 and mouse chromosome 3; IL-15 gene is mapped to human chromosome 4q31 and mouse chromosome 8; IL-7 gene is on human chromosome 8q12-13 and mouse chromosome 3; IL-9 gene is located on human chromosome 5q31.1 and mouse chromosome 13. There is no significant sequence homology or evolutionary relatedness among these IL-2-like cytokine genes.

2.2.2 Cellular sources

IL-2, 4, and 9 are derived from T cells, but IL-7 is derived from thymic stromal cell, keratinocytes, macrophages and monocytes (Namen, Lupton et al. 1988; Namen, Williams et al. 1990). IL-15 is derived from nonlymphocytic cell types including bone marrow stromal cells, activated macrophages, monocytes, epithelial cells and muscle cells (Tagaya, Bamford et al. 1996). It is important to note that IL-15 protein

production is quite difficult to detect. Only very few reports about measurable IL-15 protein exist to date, i.e. in rheumatoid arthritis (RA) synovial fluid (McInnes, al-Mughales et al. 1996; Ziolkowska, Koc et al. 2000), although IL-15 mRNA is widely expressed by these nonlymphocytic cells with or without deliberate stimulation.

2.2.3 Regulation of expression

IL-2 expression is controlled predominately at the level of mRNA transcription and stabilization. In contrast, IL-15 expression is controlled post-transcriptionally at the levels of translation and intracellular trafficking(Bamford, Battiata et al. 1996; Tagaya, Bamford et al. 1996). Three types of IL-15 message modifications include elimination of the 5' UTR, switch of signal peptide coding sequence, and FLAG modification of 3' coding sequence. The nature of post-transcription IL-15 gene regulation explains why widely expressed IL-15 mRNA is accompanied by very limited or no protein detected. For this reason, IL-15 mRNA level cannot be used to reflect its biological function.

The quantity of IL-15 generated increases approximately 20 fold when the IL-15 signal peptide is replaced by that of IL-2. In parallel, the quantity of IL-2 secreted is reduced 40-50 fold when the IL-2 signal peptide is replaced by that of IL-15. Thus the unusual IL-15 signal peptide and/or its coding sequence appears to contribute to the inefficiency of IL-15 synthesis and secretion(Bamford, DeFilippis et al. 1998).

2.2.4 Receptor distribution and signaling

As described above, high-affinity IL-2R is expressed on activated lymphocytes. The interaction of IL-2 with high-affinity IL-2R is dependent on the amount of IL-2 produced, the levels of high-affinity receptors expressed, and the duration (Smith 1989). IL-2 can act in either an autocrine or paracrine fashion. JAK-STAT paradigm is required for IL-2 signaling as described above. Another signaling system is the Ras/Raf/MEK/MAP kinase pathway: phosphotyrosine mediates recruit of Shc, which then couples to the Ras/Raf/MEK/MAP kinase pathway, MAPK translocates to the nucleus and modulates transcription (Gesbert, Delespine-Carmagnat et al. 1998).

High affinity IL-15R is composed of IL15Rα/IL2Rβ/γc. Expression of IL-15Rα is observed in multiple tissues (eg, brain, intestine, liver, PBMCs) and cell lines. However, the respective expression levels of each of the 8 isoforms varied from one cell or tissue type to another (Dubois, Magrangeas et al. 1999). A novel 60-65 kD receptor named IL-15RX was found on mast cells. There are two IL-15 receptor/signalling pathways: IL-15 utilises IL-15RX and a new signal transduction pathway in mast cells that is distinct from IL-15Rα/IL-2Rβ/γc unutilised by T cells and NK cells. Mast cells do not respond to IL-2 since they lack the required β chain of the IL-2R. In mast cells IL-15RX recruits a JAK2/STAT5 signal transduction

pathway distinct from the JAK1,3 /STAT3 ,5 pathway used by the IL-2R or IL-15R system in T cells (Tagaya, Burton et al. 1996).

There are two types of IL-4R. The type I IL-4R is composed of IL-4Rα and γc, it is expressed on T cells and other hematopoietic cells. Major events of IL-4 signaling are mediated through JAK/IRS-2 and STAT6 pathways. An alternate IL-4R comprising IL-4Rα and IL-13Rα is expressed on non-T immune cells and it can transduce signal in a different way (Chomarat and Banchereau 1997).

Functional IL-7 receptors are expressed on pre-B cells, pre-T cells and mature T cells. IL-7R is composed of IL-7Rα and γc. JAK1 is associated with IL-7Rα and JAK3 with γc. IL-7-induced STAT1, STAT3 and STAT5 complexes have been found in human and murine B and T cell lines. Activated IL-7Rα also binds PI3K, which eventually leads to cell cycle entry and proliferation. In addition, phosphorylated IRS-1, IRS-2, c-myc and src family kinases have been implicated in IL-7R-associated signalling pathway although the details of their functions have been poorly identified (Porter and Malek 2000). For comparison with IL-2 signaling, studies showed that the function of IL-7 was diminished when the cytoplasmic domain of IL-7Rα was substituted by that of IL-2Rβ, indicating that IL-7 must induce unique signals than IL-2Rβ does (Pallard, Stegmann et al. 1999).

IL-9 receptors are expressed on pre-T cells, T cells, mast cells and B cells. IL-9R is composed of IL-9Rα chain and γc, they are associated respectively with JAK1 and

JAK3 , which become activated on binding of IL-9. Then this results in the activation of STAT1, STAT3 and STAT5 (Demoulin, Uyttenhove et al. 1996). In parallel, IRS-1 and PI3K are also reported to be independent IL-9 signalling pathway (Yin, Keller et al. 1995).

2.2.5 Different functions in the development and maintenance of immune and non-immune cells

Gene deficient mice studies reveal that the essential role of IL-2 *in vivo*, which is more sensitive than that of other cytokines, is the maintenance of T cell homeostasis. Specifically it is found that IL-2 is capable of providing the dominant cytokine signal for inducing sensitivity to AICD (Activation induced cell death) (Waldmann, Dubois et al. 2001).

IL-15 is also an anabolic agent that increases skeletal muscle mass (Quinn, Haugk et al. 1995). It can stimulate mast cell proliferation *in vitro* and *in vivo*(Tagaya, Burton et al. 1996). IL-15 may also complement the actions of IL-3 and stem cell factor in the differentiation of mast cells from their precursors(Waldmann, Tagaya et al. 1998; Waldmann and Tagaya 1999). IL-15 plays a pivotal role in the development, survival, and activation of NK cells(Waldmann and Tagaya 1999; Liu, Perussia et al. 2000). It is required for NK cell differentiation and, in addition, it stimulates expression of at least one of the MHC-specific inhibitory receptor families expressed by NK cells (Raulet 1999). IL-15-deficient mice lack NK cells and display marked

reductions in numbers of thymic and peripheral NKT cells, memory phenotype CD8(+) T cells, and distinct subpopulations of intestinal intraepithelial lymphocytes (IELs) (Kennedy, Glaccum et al. 2000). The reduction but not absence of these populations in IL-15 (-/-) mice likely reflects an important role for IL-15 for expansion and/or survival of these cells. The above functions of IL-15 are not shared by IL-2.

IL-7 plays a singularly dominant role, in terms of the ligands that bind to the γc receptor, for the production and development of lymphocytes. In IL-7 KO mice, early thymic and lymphocyte expansion are severely impaired, accompanied by a striking loss of Bcl-2 protein expression and an increased relative proportion of cells in the G0/G1 stage of the cell cycle. Short-term culture of immature thymocytes with rIL-7 caused up-regulation of Bcl-2 protein and cell survival. It has been shown that IL-7 signal transduction at a special T cell developmental point, prior to T cell antigen receptor rearrangement, is linked to an anti-apoptosis mechanism at and the cell cycle (von Freeden-Jeffry, Solvason et al. 1997). B cell development in mouse bone marrow depends critically upon IL-7 because IL-7 strongly promotes *in vivo* cell survival and maintains antiapoptotic Bcl-2/Bax ratios during the development of precursor B cells in mouse bone marrow. It is shown that the apoptotic rates of precursor B cells are enhanced in IL-7-deficient mice, associated with increased intracellular content of Bax and decreased Bcl-2. Conversely, IL-7 transgenic mice suppresses precursor B cell apoptosis and produces low Bax and high Bcl-2 levels (Lu, Chaudhury et al. 1999; Lu and Osmond 2000). NK1.1+ T cells from IL-7-

deficient mice have a normal distribution and selection but exhibit impaired IL-4 and IFNγ production (Vicari, Herbelin et al. 1996). These data suggest that the functional maturation of NK1.1+ T cells requires IL-7.

IL-9 transgenic mice revealed that IL-9 is a major anti-apoptotic factor for thymic lymphomas (Renauld, Vink et al. 1995). IL-9 is a mast cell growth factor and also exerts effects on erythroid progenitors, B cells and fetal thymocytes development. IL-9 has been shown to act on many cell types involved in asthma, including T cells, B cells, mast cells, eosinophils, neutrophils, and epithelial cells, and thus might be important in the pathophysiology of allergic asthma. Temann et al showed that IL-9 transgenic mice displayed lymphocytic and eosinophilic infiltration of the lung, airway epithelial cell hypertrophy with mucus production, and mast cell hyperplasia (Temann, Geba et al. 1998; Temann, Ray et al. 2002).

IL-4 is a major B-cell growth factor. It also exerts actions on macrophages, hematopoietic precursor cells, stromal cells, and fibroblasts (Boulay and Paul 1992). Interleukin-4 (IL-4) is a key cytokine in the differentiation of naive CD4+ T cells into Th2 cells. At the same time, IL-4 can limit the activation, expansion, and differentiation of CD8+ T cells. As a result, IL-4-deficient mice demonstrate earlier and strikingly enhanced cytolytic activity following *in vitro* restimulation, an effect exhibited by both primary and memory T cells. Secretion of IL-2 and IFNγ by CD8+ T cells from IL-4-deficient mice is also elevated, reflecting their enhanced activation (Villacres and Bergmann 1999).

With respect to established Th1/Th2 immunity, it has been found that IL-2 selectively promotes polyclonally-stimulated or Ag-specific IFNγ, but not IL-4, synthesis(Yang and HayGlass 1993). There is no report about the role of IL-7 or IL-9 on established Ag-specific immunity. Using polyclonal stimulator, there are controversial reports about IL-7's effect on Th1/Th2 responses. Some have found that IL-7 enhances IL-4 secretion from PBMC stimulated with PHA and PMA (Jiang and McGee 1998). Other reports have revealed that IL-7 increases IFNγ expression from T cells after short (6-12 hours) stimulation with anti-CD3 and anti-CD28 (Borger, Kauffman et al. 1996). There are also reports about the ability of IL-7 to promote both IFNγ and IL-4 production in polyclonally stimulated naive T cells (Webb, Foxwell et al. 1997). IL-9 has been found to upregulate IL-10 related T cell-derived inducible factors from lymphoma cells. Studies on IL-15 have also revealed controversial conclusions. One report using Ag-specific stimulation found that IL-15 enhanced IgE and Ag-specific IgG1 *in vivo* and stimulated IL-4 production *in vitro* (Ruckert, Herz et al. 1998). Using TCR transgenic mice, some investigations found that IL-15 augmented IFNγ production from CD4 T cells displaying naive or activated phenotypes (Seder 1996). Polyclonally stimulated PBMC were found to produce more IL-5 upon IL-15 stimulation (Mori, Suko et al. 1996). Because of the scarcity of reports about IL-2-like cytokine's effect on established Ag-specific Th1/Th2 immunity and also because of the similarity and difference among IL-2-like cytokines, in this study we wanted to investigate the role of IL-15, IL-7 and IL-9 on recall immunity and compare them with our previous findings about the role of IL-2.

2.2.6 Different functions in peripheral T cell homeostasis

On the immune system, there are always checks and balances. The stimulatory effect to start one immune response is balanced by the inhibitory effects of another allowing the immune system to respond vigorously but not uncontrollably to antigens. Such homeostasis maintains the number of peripheral memory T cells for long periods after antigen exposure. It has been found recently that the balance of IL-2 vs. IL-15 supports peripheral memory phenotype T cell homeostasis (Waldmann, Dubois et al. 2001).

IL-15 is constitutively produced in the body and it drives the survival of activated T cells and slow proliferation of memory T cells. IL-2 induces the AICD of T cells and also kill proliferating memory CD8 T cells (Chu, Chen et al. 1999; Lai, Gelfanov et al. 1999). Thus IL-15 and IL-2 have profound sometimes gzrosing effect on the fate of activated T cell and the total number of memory T cells.

IL-2- or IL-2Rα-deficient mice suffer from lymphoproliferative diseases, especially if infected, because of a lack of IL-2 checking(Simpson, Mizoguchi et al. 1995). IL-15Rα-deficient mice have significantly reduced number of CD8 memory phenotype T cells because no cytokine makes memory CD8 T cells divide (Lodolce, Boone et al. 1998).

IL-7 reportedly also has a role in AICD for Th1 cells, although not as dominant as IL-2. In Th1 cell line, IL-2 promotes a 4-5 fold increase in sensitivity to AICD, IL-7 is 3-5 fold less effective than IL-2, but IL-4 is not effective. In Th2 cell line, IL-2 promotes 2-6 fold increase in AICD in a concentration dependent manner, IL-4 and IL-7 do not increase AICD sensitivity (Wang, Ciardelli et al. 1997).

3 House dust mite and allergy

3.1 Epidemiology of house dust mite allergy

House dust mite (HDM) antigen has become a major risk factor for children to develop asthma. Approximately 30% of children are allergic to HDM, 30% of the sensitised children may subsequently develop the symptoms of asthma (Peat, Tovey et al. 1996; Custovic, Simpson et al. 1998; Marks 1998).

The most abundant HDM species are *Dermatophagoides pteronyssinus* in the more humid environments and *Dermatophagoides farinae* in drier regions.

3.2 Allergenic composition of house dust mite

With the development of molecular biology techniques, HDM allergens have been characterised into 13 groups by DNA cloning (Thomas and Smith 1998). Der p1, 3, 4, 6 have been identified as cysteine protease, trypsin, amylase and chymotrypsin

respectively. Der p8, 9, 10 are identified as glutathione-S-transferase, serine protease, and tropomyosin respectively. The biological functions of Der p 2, 5, 7 are unknown.

Der p1 is the first purified HDM allergen. As a major allergen, Der p1 can bind IgE in all sera from all the HDM allergic subject and 50-70% of the IgE to HDM extract is against Der p1 (Chapman and Platts-Mills 1980) in the subjects studied.

Der p1 usually exists at 100-10,000 ng/g of dust. Levels of 100 ng can induce sensitisation, and the risk increases with increasing doses (Platts-Mills and Chapman 1987; Wahn, Lau et al. 1997).

3.3 Importance of recombinant allergen in allergy model

Crude HDM extracts were used widely in clinical medicine for diagnosis and immuno-treatment purpose. Many unspecified components in HDM extract and the big variability between different batches make them difficult to standardize and may even cause unwanted allergic reaction when used in patients. The allergen composition in HDM extract is not necessarily parallel with their natural distribution and the sensitivity of the patient, this is called allergen imbalance (Chapman, Smith et al. 1997; Valenta, Vrtala et al. 1998). Allergen imbalance can affect the efficiency of desensitization where side effects to allergens in high dose could cover the therapeutic effects to other allergens and even induce additional, new sensitization.

Recombinant allergens synthesized with molecular biological techniques make it more effective for immunological treatment and studying the induction of sensitization of disease. Recombinant allergen has preserved immunogenicity comparable to that of the native extract, only a few recombinant allergens might be enough for diagnosis of sensitized subjects (Chapman, Smith et al. 1997; Valenta, Vrtala et al. 1998).

Commercial crude HDM extract usually contains glycerin, which can inhibit cell proliferation at high dose. This is also the reason that we choose recombinant allergen to study the sensitization.

4. Scope of this research

4.1 Background of the study

We previously reported that IL-2 is required for expression of Th1 but not Th2 recall responses. Because of the similarity and difference among IL-2-like cytokines as discussed above, here we wished to determine the role of IL-15, IL-7 and IL-9 in antigen-specific Th1/Th2 recall responses and its relationship with IL-2.

4.2 Objectives of the study

To compare the role of IL-9, IL-7 and IL-15 in allergen-specific Th1/Th2 recall responses and their relationship with IL-2.

4.3 Hypotheses of the study

First, we hypothesized that by using appropriate recombinant Der p1 and adjuvant we can setup murine model reflecting the immunological nature of human house dust mite allergic subjects. Then by using these allergic models, we hypothesized that IL-2 is required selectively for Ag-specific Th1 but not Th2 responses because previous studies at our lab revealed such effect of IL-2 in an OVA immunized murine model as described in introduction. Because of the similarities and differences among IL-2-like cytokines discussed previously, in the next step we hypothesized that IL-15, similar to IL-2, can selectively enhance Th1 immunity. It has been reported that cellular source of Th1 cytokine IFNγ includes three major categories: T cells, NK cells and NKT cells. If the effect of IL-15 to enhance IFNγ is Ag-specific, we will focus on the requirement of T cells and its subsets CD4 and CD8 T cells for the Th1-enhancing effect of IL-15. If the effect of IL-15 is not Ag-specific, we might focus on NK cells. As discussed previously, it has also widely known that IL-12 and IL-18 are among the most important endogenous cytokines to stimulate IFNγ production and their receptors have similar cellular distribution (especially on T cells, tested in previous hypothesis) to IL-15 receptors, we hypothesized that IL-12 and IL-18 are required for the effect of IL-15 to augment Th1 response in a Ag-specific manner. The hypotheses in details are listed below step by step.

(1) Recombinant Der p1 immunization with alum stimulates substantial type 2 immunity and low type 1 immunity.

(2) Recombinant Der p1 immunization with HKL/IFA stimulates substantial type 1 immunity and low type 2 immunity.

(3) Recombinant Der p1 elicits a similar immunity pattern to that obtained using crude whole body extracts when used in primary immunization *in vivo*.

(4) Recombinant Der p1 elicits a similar immunity pattern to that obtained using crude whole body extracts when used in restimulation in vitro.

(5) Endogenous IL-2 is required for expression of Der p1-specific Th1 but not Th2 recall responses.

(6) Exogenous rIL-2 selectively enhances Der p1-specific type 1 recall immunity.

(7) rIL-15 selectively enhances Ag-specific Th1 responses in murine allergic model.

(8) IL-12 and IL-18 is required in IL-15's function to augment Th1 immunity.

(9) rIL-15 can increase the expression of IL-12 and IL-18.

(10) rIL-15 can upregulate IL-12 and IL-18 cellular responsiveness.

(11) Both CD4 and CD8 T cell are required for the Ag-specific IFNγ responses augmented by IL-15.

(12) Endogenous IL-2 is not required in IL-15's function and IL-15 can replace IL-2 to support established Th1 immunity.

(13) rIL-15 decreases Ag-specific IL-2 production.

(14) rIL-15 increases Ag-specific IL-2 consumption.

(15) rIL-7 and rIL-9 promote Ag-specific Th2 responses in the allergic model.

(16) IL-2 is not required in the Th2 immunity and its augmentation by IL-7 and IL-9.

4.4 Summary of the study

The receptors of IL-2, IL-15, IL-7 and IL-9 share common γ-chain (γc) subunit and related signaling, these cytokines are all T cell growth factors, so they are considered "IL-2-like cytokines". Furthermore, IL-15 even shares β-chains of the IL-2R for signaling and thus shares more properties with IL-2. At the same time IL-15 and IL-2 differ substantially in numerous other immunological properties (e.g. protection from vs. induction of peripheral T cell apoptosis as reviewed above).

We previously reported that IL-2 is required for expression of Th1 but not Th2 recall responses. We wished to determine the role of IL-15, IL-7 and IL-9 in antigen-specific Th1/Th2 recall responses and its relationship with IL-2.

There are four sections in this study. In the first section, we set up a murine allergic model against recombinant house dust mite allergen. Then in the second section, using this murine model, we investigated the role of IL-2 and IL-15 on established Th1 vs. Th2 responses and the underlying molecular and cellular mechanism. In the third section, we studied the mutual relationship of IL-15 and IL-2 in recall allergic responses. After clarifying the role of IL-15 and IL-2 and their mutual impact in established immune responses, in the last section, we compared the role of IL-15 and

IL-2 with that of IL-7 and IL-9 in murine allergic model because IL-7 and IL-9 share only γc but not β-chain with IL-2. These four parts are summarized below respectively.

PART 1. ESTABLISHMENT OF A MURINE ALLERGIC MODEL USING RECOMBINANT Der p1

Immediate hypersensitivity is the most prevalent immunologic disorder in humans. In Canada it represents the most rapidly increasing chronic health problem among individuals over 15 years of age.

T cells play a pivotal role in the pathogenesis of such disorders with the nature of the allergen-specific CD4 T cell response induced following exposure to environmental antigen greatly influencing whether subsequent antigen exposure elicits atopic responses or those associated with clinical tolerance (HayGlass 1995; Romagnani 1996; Romagnani, Parronchi et al. 1997).

The major allergen of D. pteronssinus, house dust mite, has been cloned and expressed. As a major allergen, Der p1 is recognized by serum IgE of more than 80% of all house dust mite allergic humans. Of the antigen-specific IgE observed in these individuals, 50-70% binds with this allergen (Chapman and Platts-Mills 1980).

We reported development of murine models to allow analysis of factors relevant to sensitization and control of immediate hypersensitivity to house dust mite antigen. C57Bl/6 mice were immunized with Der p1 in different adjuvants and primary and recall immune responses were analyzed. The immunologic factors studied include type 1 and type 2 chemokines (IP-10, C10), cytokines (IFNγ, IL-2, IL-4, IL-5, IL-13, IL-10) and antibodies (IgG2a, IgE, IgG1), and the analysis is established both *in vivo* and in vitro.

Recombinant Der p1 immunization with alum stimulated substantial type 2 cytokine, chemokine and antibody synthesis, with lower capacity to generate type 1 responses (IP-10, IFNγ, IgG2a). These immune responses closely parallel those seen in human dust mite allergic subjects. Thus the murine model was termed a type 2 immunity model or model of allergy.

In contrast, Der p1 immunized with HKL/IFA stimulated substantial type 1, but low type 2 cytokine and chemokine expression. These immune responses parallel with the immunologic features of human clinical tolerance subjects. Therefore this murine model was named type 1 immunity model.

When used in either primary immunization *in vivo* or re-stimulation in vitro, recombinant Der p1 elicited a similar immunity pattern to that obtained using crude whole body extracts (widely used in clinical medicine). Therefore Der p1 can be used as model allergen to reflect sensitization against house dust mites.

PART 2. THE ROLE OF IL-2 AND IL-15 ON ONGOING TYPE 1/TYPE2 IMMUNITY IN A MURINE MODEL OF ALLERGY

The murine models of allergy were established as above. Subsequently, by adding exogenous cytokines or blocking endogenous cytokines and/or CD4, CD8 T cells in vitro, we investigated the role of IL-2 and IL-15 in the maintenance of type 1 and type 2 responses. We found that endogenous IL-2 is required for expression of Th1 but not Th2 recall responses, exogenous rIL-2 selectively enhances Ag-specific type 1 immunity. Similar to IL-2, rIL-15 selectively enhances Ag-specific Th1 (IFNγ and IP-10) responses by 3-10 fold while not altering Th2 (IL-4, 5, 13, C-10) associated responses. Endogenous IL-12 is required for this augmentation. IL-15 does not directly up-regulate IL-12 expression, rather, it increases cellular responsiveness to endogenous IL-12, hence type 1 cytokine production in the murine allergy model.

This is the first report that IL-15 enhances Ag-specific Th1, but not Th2, recall responses. In cellular mechanism, both CD4+ and CD8+ T cells contribute to the Der p1-specific Th1 cytokine responses augmented by IL-15. It is sharply different from the cellular mechanism of Th2 cytokine responses, where only CD4 but not CD8 T cells are required.

Our study raises the possibility of allergy treatment by IL-15, a cytokine in experimental use as an alternative to IL-2 in tumour immunotherapy.

PART 3. EXOGENOUS RECOMBINANT IL-15 CAN SUBSTITUTE FOR IL-2 IN Ag-STIMULATED CYTOKINE PRODUCTION

IL-2 and IL-15 share β- and γ- receptor subunits and signaling pathway, and both IL-2 and IL-15 can selectively enhance type 1 immunity. Albeit with so many similarities, IL-2 and IL-15 balance each other in peripheral T cell homeostasis: IL-2 induces Ag-specific T cell apoptosis while IL-15 makes such cells to turn into memory cell and survive. In this part, we studied the relationship between IL-15 and IL-2 in recall responses. We found that IL-2 is not required for IL-15 to enhance Th1 immunity, and IL-15 can substitute for IL-2 in this effect. Furthermore, IL-15 can increase IL-2 consumption by Ag-specific T cell, not accompanied by enhanced function. The biological significance of this finding is: IL-15 increases the consumption of IL-2 by activated T cells and gradually takes the place of IL-2 to maintain Th1 response when more memory T cells are forming. This draws a longitudinal balance of IL-2 vs. IL-15. The purpose of augmented IL-2 consumption is to shift quickly to a IL-15-dominated pattern, rather than enhanced or synergic function of IL-2 because we proved that IL-2 is not required for IL-15's activity and its function can be replaced by IL-15.

PART 4. COMPARISON OF REGULATIVE EFFECT OF γ-CHAIN BUT NOT β-CHAIN RECEPTOR CYTOKINES

IL-7 and IL-9 share receptor γ- but not β-chain with IL-2 and IL-15. In this last section, we analysed and compared the functions of IL-7 and IL-9 on recall Der p1-specific responses with same strategy as to IL-2 and IL-15. Different from IL-2 and IL-15, we found that IL-7 and IL-9 selectively enhance Ag-specific IL-5 and IL-13 (Th2) responses without impact on Th1 cytokines. Endogenous IL-2 has no role in IL-5 and IL-13 expressions and their enhancement by IL-7 and IL-9. Thus we saw the redundancy and specificity among IL-2-like cytokines.

In summary, we established the murine type 1 and type 2 immunity models against house dust mite allergen. With such model, we compared the role of IL-2, IL-15, IL-7 and IL-9 in antigen-specific recall immunity. We analysed the cellular and molecular mechanism of their effect and their relationships with IL-2.

MATERIALS AND METHODS

MATERIALS

1.1 Mice

C57Bl/6 mice (6 to 12 wk old) bred at the University of Manitoba breeding facility (Winnipeg, Manitoba, Canada) or purchased from Charles River Canada (St. Constant, Quebec, Canada) were used in accordance with guidelines issued by the Canadian Council on Animal Care. Mice were negative for antibodies to mycoplasma, Sendai virus, and rodent corona viruses including murine hepatitis virus by ELISA (Murine ImmunoComb, Charles River). IL-12-deficient mice (p40-/- ; p35-/-), homozygous on a C57Bl/6 background, were provided by Jackson Laboratories (Bar Harbour, ME).

1.2 Reagents

Hydrochloric acid, ethyl alcohol, sodium azide, sodium phosphate monobasic, sodium carbonate, sodium bicarbonate and paraformaldehyde were purchased from CanLab (Winnipeg, MB); bovine serum albumin was from Sigma Chemical Co. (St. Louis, MO); sodium chloride, Tween 20 and sodium hydroxide from Mallinckrodt Canada Inc. (Mississauga, ON); 2-mercaptoethanol from Kodak (Rochester, NY); fetal calf serum from Intergen company (New York, NY); Trypan blue from Matheson Coleman and Bell (Ohio, USA); Hank's balanced salt solution (HBSS), L-glutamine, penicillin, streptomycin and fungizone from Flow Laboratories (Mclean VI); RPMI 1640 from Gibco Laboratories Life Technologies Inc. (Grand Island,

NY). Complete Freund's adjuvant (CFA) from Gibco-BRL (Burlinto, ON); streptavidin-alkaline phosphatase from Jackson Immuno Research Laboratories (West Grove, PA); normal rat Ig from Biocan Scientific (Toronto, ON);

Recombinant house dust mite allergen Der p1 (kindly provided by Dr. Wayne Thomas at Institute for Child Health Research, Perth, Australia) was used in immunization. Recombinant murine IL-15 (PeproTech, Rocky Hill, NJ) was used in cell culture. Monoclonal antibodies used in cell culture include anti-IL-12 (Pharmingen Canada, ON), anti-IL-18 (PeproTech, NJ), anti-CD4 (YTS 191.1) and anti-CD8 (YTS 169.4.2.1). The last two hybridomas were kindly provided by Dr. H. Waldmann at Cambridge University, UK). Hybridomas XMG 1.2 (anti-IFNγ), SXC1 (anti-IL-10), SXC2 (anti-IL-10), S4B6 (anti-IL-2) and recombinant IL-10 standard were kindly provided by Dr. T. Mosmann (University of Alberta, Edmonton, Alberta). Recombinant interleukin-2 (rIL-2) standard was from Pharmingen. Recombinant interleukin-4 (rIL-4) standard was from WHO (provided by Dr. R. Tepper (Dana-Farber Cancer Institute, Boston, MA)) and Dr. W. Paul (National Institute of Health, Bethesda, MD).

1.3 Equipment

Laminar flow biological safety cabinet (model Nu-408EM-400) was purchased from Nuair Inc. (Plymouth, MN). Water-jacketed incubator (model 3158) was purchased from Forma Scientific, Division of Mallinckrodt Inc. (Marieta, OH). Elisa microtitre reader with Softmax Pro software was a product of Molecular Devices Corporation

(Sunnyvale, CA). Chemiluminescent ELISA reader was MicroLumat Plus microplate luminometer (EG&G Berthold, Gaithersburg, MD). Vortex-Genie were produced by Fisher Scientific (Ottawa, ON). A microscope was purchased from Carsen Medical Scientific Co. (Winnipeg, MB). Centriprep-30 concentrators were purchased form Amicon (Oakville, ON). 96 well Elisa plates, polystyrene flat-bottom tissue culture plates and 24, 48 well tissue culture plates were obtained from Corning Science Product (Missisauga, ON).

METHODS:

1. Preparation of antigens in adjuvants

There were two antigens (Der p1 and HDM) and two adjuvants (alum and HKL/IFA) used in this study.

1.1 rDer p1 and crude HDM antigens in alum adjuvants

To set up the type 2 immunity model, C57Bl/6 mice were immunised with different concentrations of Der p1 or HDM adsorbed onto $Al(OH)_3$ adjuvant (alum). Preparation of alum started with adding 2 drops of 0.2% phenol red to 20 ml of 10% $AlK(SO_4)_2$ and was followed by the addition of approximately 20 ml of 0.5 N NaCl until the solution began to turn pink. After set for 10 min, the solution was centrifuged for 3 minutes at 1000 rpm (170 g). The supernatant was discarded and the precipitate was resuspended in 0.15 N NaCl (40ml). This centrifugation procedure was repeated twice. The resulting pellet was resuspended in 40 ml of 0.15

N NaCl to yield $Al(OH)_3$ at 10-12 mg/ml. This final solution containing $Al(OH)_3$ at 10-12 mg/ml was stored at room temperature. For use, antigen was absorbed onto alum, vortex, and left for 10 min at room temperature. Three fifth volume of Hank's balanced salt solution was added prior to immunization to neutralise the pH of the solution. Following this procedure over 99% of antigen was absorbed to the alum crystals.

1.2 rDer p1 antigen in HKL/IFA adjuvants

To set up the type 1 immunity model, mice were immunised with Der p1 absorbed onto HKL/IFA. To prepare heat-killed Listeria monocytogenes, *L. monocytogenes* was grown in nutrient broth cultures overnight at 37°C on a rotator. Bacteria in log-phase growth were harvested, centrifuged, and washed three times in PBS. The recovered bacteria were resuspended in LPS-free PBS and incubated at 80°C for 1 h before washed twice with PBS. Effective bacterial killing was confirmed by taking a 200 μl aliquot to grow on nutrient agar plates overnight and absence of viable colonies. 2.44 X 10^9 bacteria/ml HKL was kept at 4°C for one month. When used for immunization, Der p1 was emulsified in IFA with HKL to obtain 0.3 ml total containing equal proportions of aqueous phase (containing Der p1) and IFA (containing HKL at 1 X 10^7 bacteria for each mouse). They were mixed in a small beaker. The mixture was emulsified by repeatedly drawing the contents of the beaker into the syringe and expelling until the emulsion was well formed by the criterion that a drop of the emulsion placed on water remained intact and did not spread.

2. Immunization and treatment protocols

2.1 Immunization to set up the type 2 immunity model

To set up the type 2 immunity model, C57Bl/6 mice were immunised with 2μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant (alum) given i.p. on day 0. The mice were re-immunised on day 28 to measure serum IgE, IgG2a and IgG1 titer in primary and secondary responses, or the mice were sacrificed on day 3-day 8 (optimal on day 6, data not shown) respectively to set up spleen cell culture.

To study the cytokine responses in the absence of endogenous IL-12 p40, IL-12p40 knockout mice were also immunised with 2μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant (alum) given i.p. on day 0 and sacrificed on day 6 to set up spleen cell culture.

2.2 Immunization to set up the type 1 immunity model

To set up the type 1 model, mice were immunised with 2 μg Der p1 in $1X10^7$ HKL/IFA on day 0 and sacrificed on day 6 to set up spleen cell culture.

2.3 Bleeding

Mice were bled from tail on day 0, 10 and 14 (primary immunization was on day 0), and 35 and 42 (i.e. 7 and 14 days after secondary immunization on day 28) to

measure serum Ig or chemokine levels. Mice sera were collected by centrifugation the day after bleeding and stored at –20 °C until analyzed individually.

3. Cell culture

3.1 Preparation of spleen cell suspension

Mice were sacrificed at various dates following in vivo immunization, and spleens were removed aseptically. Single cell suspensions in 5% newborn calf serum RMPI-1640 were prepared using a glass tissue homogenizer. Spleen cells were filtered through nytex filters to remove debris and centrifuged for 2 minutes at 2000 rpm (300g). Cells were resuspended in 10% FCS RPMI-1640. The number and percentage of viable cells was determined by counting in a hemocytometer after staining with trypan blue (0.4%) and lysing RBC with acetic acid (40%) (Meplean, ON).

3.2 Spleen cell culture

Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml (200 µl/well) alone or with predetermined optimal concentration of Der p1 (0.1, 1, 5, 20 µg/ml tested, optimal at 5µg/ml), or with HDM (50, 100, 200 AU/ml). Cell culture were setup in 96 well plates (Corning Science Products, Rochester, NY) at 37°C in RPMI 1640 supplemented with 10% FCS, 10mM L-glutamine, penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), Fungizone (0.25 µg/ml; Flow Laboratories, Mississauga, Ontario, Canada), and $2 X 10^{-5}$ M 2-ME. Duplicate cultures were

established from the spleen cells of individual mouse in each group. Culture supernatants were harvested for analysis of chemokines mIP-10, C10 and cytokines IL-4, IL-2, IFN-γ, IL-5, IL-13 and IL-10 production at 24, 48, 72 and 96 hour time points and stored at –20 °C.

3.3 In vitro cytokine addition

Exogenous recombinant human IL-2 (Cetus) and mouse IL-2, IL-15, IL-7, IL-9 (PeproTech, NJ) at different concentrations were applied in the cell culture to investigate the roles of IL-2, IL-15, IL-7 and IL-9, details were shown in Results.

3.4 In vitro cytokine or cell depletion

To study the requirement for endogenous IL-2, IL-12, IL-18, CD4 or CD8 T cells, they are respectively blocked by mAbs anti-IL-2 (PeproTech, 12.5, 25, 50,100 ng/ml), anti-IL2Rα (Pharmingen, 10, 50 μg/ml), anti-IL-12 (Pharmingen, 5μg/ml as previously(Rempel, Wang et al. 1997)), anti-IL-18 (PeproTech, 0.6, 1.2μg/ml), anti-CD4 (YTS 191.1, ¼ v/v as previously(Rempel, Wang et al. 1997)) and anti-CD8 (YTS 169.4.2.1, 10μg/ml as previously).

4 Cytokine determinations

IFNγ, IL-10, IL-2, IL-4, IL-5, IL-13 were determined by ELISA as previously described (Rempel, Wang et al. 1997). The detection limits were 0.5U/ml for IFNγ,

0.63U/ml for IL-10, 3.9pg/ml for IL-2, 2.8 U/ml for IL-4, 15.6pg/ml for IL-5 and 39pg/ml for IL13.

4.1 IFNγ

The capture Ab for IFNγ was mAb XMG 1.2 (a gift of Dr. T. Mosmann) coated at 0.75 μg/ml; ELISA plates were coated at 4 °C overnight. The capture buffer was bicarbonate buffer (0.05M, pH 9.6). Then plates were blocked 1 hour at 37 °C with a 1% BSA, 0.05% Tween 20 solution and washed. Plates were washed in between all subsequent incubations. Samples were added at 1:2 dilution of supernatant (various dilutions); IFNγ standard was produced by stimulating spleen cells in the presence of ConA and IL-12 and was calibrated against WHO-NIAID IFNγ reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, NIH, Bethesda, MD). Detection Ab was biotinylated mAb R4-6A2 (American Type Culture Collection, Rockville, ML), it was made in house, and used at 1/2500 as optimal dilution. Detection was by incubation with streptavidin conjugated alkaline phosphatase. A color reaction was produced by the addition of p-nitrophenyl phosphate (Sigma Chemical Co., Oakville, ON). The reaction was proceeding for 2 hours before plates were read at $O.D._{405\text{-}695}$. Detection limits was 0.5 U/ml for IFNγ. For all ELISA the reading time and Ab concentrations were optimized to give maximal sensitivity and accuracy.

4.2 IL-2

The capture Ab for IL-2 was rat-anti-mouse mAb (Pharmingen Clone JES6-1A12) coated at 1 μg/ml; samples were added at 1:2 dilution of supernatant (various dilutions); detection Ab was biotinylated rat-anti-mouse IL-2 (Pharmingen Clone JES6-5H4) at 1 μg/ml. rIL-2 standard was from Pharmingen. Detection limits was 4 pg/ml for IL-2.

4.3 IL-4

The capture Ab for IL-4 was rat-anti-mouse mAb (Pharmingen Clone 11B11) coated at 2 μg/ml; samples were added at 1:1 dilution of supernatant (various dilutions); detection Ab was biotinylated rat-anti-mouse IL-4 (Pharmingen Clone BVD6-24G2) at 0.25 μg/ml. rIL-2 standard was from W.H.O.. Detection limits was 2.8 U/ml for IL-4.

4.4 IL-5

The capture Ab for IL-5 was rat-anti-mouse mAb (Pharmingen Clone TRFK5) coated at 0.5 μg/ml; samples were added at 1:4 dilution of supernatant (various dilutions); detection Ab was biotinylated rat-anti-mouse IL-5 (Pharmingen Clone TRFK4) at 0.3 μg/ml. rIL-5 standard was from Pharmingen. Detection limits was 15.6 pg/ml for IL-5.

4.5 IL-13

The capture Ab for IL-13 was rat-anti-mouse mAb (R&D Clone 38213) coated at 1 μg/ml; samples were added at 1:4 dilution of supernatant (various dilutions); detection Ab was biotinylated goat-anti-mouse IL-13 (R&D Polyclonal goat IgG) at 30 ng/ml. rIL-13 standard was from Pharmingen. Detection limits was 39 pg/ml for IL-5.

4.6 IL-10

The capture Ab for IL-10 was mAb SXC-1 coated at 3 μg/ml; samples were added at 1:2 dilution of supernatant (various dilutions); detection Ab was biotinylated SXC-2. Both hybridomas were gifts from Dr. T. Mosmann, and the antibodies were purified and biotinylated at our lab. The optimal dilution of detection Ab was determined for independent lots. IL-10 standard was produced by culturing spleen cells from mice injected with 10% FCS in 10% FCS on day 5. Standard was calibrated against rIL-10 standard provided by Dr. T. Mosmann. Detection limits was 0.63 U/ml for IL-10.

4.7 IL-12 p40

The capture Ab for IL-12 p40 was anti-IL-12 p40/p70 mAb (Pharmingen Clone C15.6) coated at 1 μg/ml; samples were added at 1:1 dilution of supernatant (various dilution); detection Ab was biotinylated rat-anti-mouse IL-12 p40/p70 (Pharmingen Clone C17.8) at 0.5 μg/ml. rmIL-12 standard was from Pharmingen. Detection limits was 30 pg/ml.

4.8 IL-12 p70

Chemiluminescent ELISA for IL-12p70

IL-12p70 was measured by chemiluminescent ELISA as previously described (Lewkowich, Campbell et al. 2001). High binding white opaque plates (96-well) were used (Corning Science Products). The mIL-12 p70 was captured with rat/hamster anti-mouse p35 mAb cocktail (Pharmingen) at 2 μg/ml. The standard used was rmIL-12 (a gift of Dr. M. Gately, Hoffman-LaRoche, Nutley, NJ). Detection Ab (Pharmingen rat anti-mouse IL-12p40 mAb, Clone C17.8) concentration was 150ng/ml. The assay was developed using a 0.4 mM disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1]decan-4-yl)phenyl phosphate (CSPD) solution (Tropix, Bedford, MA) in substrate buffer (1mM $MgCl_2$, 0.1M DEA, pH 9.5). The chemiluminescent enhancer Sapphire II (Tropix, Bedford MA) was used at 1:10. Reactions were allowed to develop until maximal chemiluminescent emission was seen, typically after 30 min and read in a MicroLumat Plus microplate luminometer (EG&G Berthold, Gaithersburg, MD).

4.9 IL-18

The capture Ab for IL-18 was rat-anti-mouse mAb (R&D Systems) coated at 1 μg/ml; samples were added at 1:1 dilution of supernatant (various dilution); detection Ab was biotinylated rat-anti-mouse IL-18 (R&D Systems) at 100 ng/ml. rIL-18 standard was from PeproTech. Detection limits was 15 pg/ml for IL-18.

5 Chemokine determinations

5.1 Murine C-10

An assay to quantify C10 concentration was developed using a sandwich ELISA approach. Briefly, ELISA plates were coated with rabbit anti-mouse C10 Abs (PeproTech, Rocky Hill, NJ) at various concentrations (0.6 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, 50μl sample solution (1:160 dilution of supernatant or 1:100 dilution of serum, various dilute) was added and incubated for overnight at 4°C. The plates were washed and incubated with 50μl of biotinylated rabbit anti-mouse C10 Abs (PeproTech various concentrations evaluated, 0.1 μg/ml found optimal) at 4 °C overnight. The following steps were same as previously described. The detection limit for C10 was 4.7 pg/ml.

5.2 Murine IP-10

For mouse IP-10 ELISA, the coating Abs were rabbit anti-mouse Abs (PeproTech various concentrations evaluated, 200 ng/ml found optimal); samples were added at 1:4 dilution of supernatant (various dilute) or 1:2 dilution of serum; the developing Abs were biotinylated rabbit anti-mouse IP-10 Abs (PeproTech various concentrations evaluated, 0.1 μg/ml found optimal). The detection limit for mIP-10 was 15.6 pg/ml.

6 Antibody determinations

6.1 Murine total IgE

Total serum IgE levels were measured with sandwich ELISA. ELISA plates were coated with rat anti-mouse IgE Abs (Southern Biotechnology Association, Inc., Birmingham, AL) at 1 μg/ml overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of serum sample (with beginning dilutions ranging from 1/40 to 1/1000) was added and incubated for overnight at 4°C. Highly purified anti-DNP mouse IgE, prepared from B cell hybridoma 2682 (a gift from Dr. A. Froese, University of Manitoba) was used as the standard. The plates were washed and incubated with biotinylated epsilon specific monoclonal rat anti-mouse IgE heavy chain (0.125 μg/ml) (Serotech, UK) at 4 °C overnight. The following steps were same as previously described. The sensitivity of this assay for IgE was 0.88 ng/ml.

6.2 Murine Der p1-specific IgG1

An assay to quantify serum Der p1-specific IgG1 concentration was developed using an ELISA approach. Briefly, ELISA plates were coated with rDer p1 at various concentrations (3 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of serum sample (with beginning dilutions from 1/25) was added and incubated overnight at 4°C.

To set up the internal standard, eight C57/Bl6 mice were immunised with 2 μg Der p1 in alum as described above on day 0, 21, 42, and 63. They were bleed on day 28, 35, 49, 56, 70, and sacrificed by cardiac puncture on day 77. The serum was pooled. For the IgG1 standard, this internal standard were used with beginning dilution from 1/500, which was considered 1000 U/ml, and made eight 2-fold dilution.

The plates were washed and incubated with biotinylated monoclonal goat anti-mouse IgG1 (0.2 μg/ml) (Southern Biotechnology Associates Inc., Birmingham, AL) at 4 °C overnight. The following steps were the same as previously described. The sensitivity of this assay for Der p1-sepcific IgG1 was 15 U/ml.

6.3 Murine Der p1-specific IgG2a

An assay to quantify serum Der p1-specific IgG2a concentration was developed using an ELISA approach. Briefly, ELISA plates were coated with rDer p1 at various concentrations (3 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of serum sample (with beginning dilutions from 1/20) was added and incubated at 4°C overnight.

The same internal standard as for IgG1 was used. For the IgG2a standard, this internal standard was used with beginning dilution from 1/50, which is considered 1000 U/ml, and made eight 2-fold dilution.

The plates were washed and incubated with biotinylated monoclonal goat anti-mouse IgG2a (0.139 μg/ml) (Southern Biotechnology Associates Inc., Birmingham, AL) at 4 °C overnight. The following steps were the same as previously described. The sensitivity of this assay for Der p1-specific IgG2a was 15 U/ml.

7. Statistical analysis

For each ELISA assay, data were obtained from a titration of at least four dilutions against the duplicated standard curves run in every assay. Mean cytokine or antibody concentration plus/minus standard error is shown. Statistical significance was determined using unpaired 2-tailed Student's t-test.

RESULTS

1. PART 1. DEVELOPMENT OF SENSITIVE CHEMOKINE AND ANTIBODY MEASUREMENT ASSAYS

1.1 Development of a sensitive murine C10 ELISA

Chemokines are predominantly small molecular weight (8-12 kDa) cytokine molecules. The principal function of chemokines that have been studied to date are chemotaxis in inflammation responses. Emerging evidence indicates that chemokines also have regulative role in type 1 and type 2 responses and in the generation of an immunologic repertoire. C-C chemokines such as monocyte-chemoattractant protein-1 (MCP-1), RANTES, eotaxin, and macrophage-inflammatory protein-1 (MIP-1) have been reported as increased in asthmatic allergic patients. (Alam, York et al. 1996; Kurashima, Mukaida et al. 1996; Holgate, Bodey et al. 1997; Lamkhioued, Renzi et al. 1997). Mouse MIP-related protein 1 or C10 is highly homologous to human chemokines such as MIP-1, it can be chemotactic for B cells and CD4+ T cells. Importantly C10 is IL-4- but not LPS-inducible in macrophages and requires de novo protein synthesis that delays its appearance for at least 24 h after cell activation (Orlofsky, Lin et al. 1994). Hogaboam et al found that endogenous C10 recruited a diverse array of cell populations including lymphocytes, macrophages, and eosinophils and had a unique role in the progression of experimental allergic bronchopulmonary aspergillosis (Hogaboam, Gallinat et al. 1999). Thus one of our first tasks was to develop sensitive assay systems to measure these molecules.

To measure C10 responses in our murine model of allergy, an assay to quantify C10 concentration in culture supernatant or serum was developed using a sandwich

ELISA approach. Briefly, ELISA plates were coated with rabbit anti-mouse C10 Abs (PeproTech, Rocky Hill, NJ) at various concentrations (0.6 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of recombinant C10 standard (Pepro Tech) with beginning concentration of 300 pg/ml was added and incubated at 4°C overnight. The plates were washed and incubated with 50μl of biotinylated rabbit anti-mouse C10 Abs (PeproTech various concentrations evaluated, 0.1μg/ml found optimal) at 4 °C overnight. The following steps were the same as described above. As shown in **Figure 1A**, we determined the optimal concentrations of both coating (0.6 μg/ml) and detection (0.1 μg/ml) antibodies for C10 assay. Under such conditions, a standard curve with low background and high sensitivity was found. This assay yields very small standard errors (<<5%). The detection limit for C10 was 4.7 pg/ml, which is defined as >= background + 3 S.D..

1.2 Development of a sensitive murine IP-10 ELISA

Mouse IP-10, or CRG-2 as it was previously known, is a murine homologue of human IP-10, and belongs to a family of proinflammatory chemokines. The CRG-2 mRNA was induced by α-, β-, and γ-interferon (IFNs) and by lipopolysaccharide in macrophages. In response to rIFNγ, the CRG-2 mRNA level quickly reaches a peak between 3 and 6 h (Vanguri and Farber 1990). IP-10 selectively promotes the maintenance of established type 1 immune responses. It forms cytokine circuits with IFNγ in Th1 responses(Gangur, Simons et al. 1998; Gangur, Simons et al. 1999).

Here we developed an assay to quantify mouse IP-10 concentrations in culture supernatant or serum, so we could examine Ag driven IP-10 responses. Briefly, ELISA plates were coated with affinity purified rabbit anti-mouse IP-10 Abs (PeproTech, Rocky Hill, NJ) at various concentrations (200 ng/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of recombinant murine IP-10 standard (Pepro Tech) with beginning concentration of 1000 pg/ml was added and incubated at 4°C overnight. The plates were washed and incubated with 50μl of biotinylated rabbit anti-mouse IP-10 Abs (PeproTech various concentrations evaluated, 0.1 μg/ml found optimal) at 4 °C overnight. The following steps were the same as described above. As shown in **Figure 1B**, we determined the concentrations of both coating (200 ng/ml) and detection (0.1 μg/ml) antibodies for IP-10 assay. Under such optimal condition, a standard curve with low background and high sensitivity was shown. This assay yields very small standard errors (<<5%). The detection limit for IP-10 was 15 pg/ml, which is defined as >= background + 3 S.D..

1.3 Development of murine Der p1-specific IgG1 ELISA

As discussed in the literature review, antigen specific IgG1 is an important type 2 immunity associated antibody. An assay to quantify serum Der p1-specific IgG1 concentration was developed. Briefly, ELISA plates were coated with rDer p1 at various concentrations (3 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at 37°C. After washing, eight 2-fold dilution of internal standard with beginning dilution from 1/500 were added and incubated at 4°C overnight.

To set up the internal standard, eight C57/Bl6 mice were immunised with 2 μg Der p1 in alum as described above on day 0, 21, 42, and 63. They were bled on day 28, 35, 49, 56, 70, and sacrificed by cardiac puncture on day 77. The serum was pooled. For the IgG1 standard, this internal standard designated 500,000 U/ml were used with beginning dilution from 1/500.

The plates were washed and incubated with biotinylated monoclonal goat anti-mouse IgG1 (0.2 μg/ml) (Southern Biotechnology Associates Inc., Birmingham, AL) at 4 °C overnight. The following steps were the same as previously described. As shown in **Figure 2A**, we determined the concentrations of both coating antigen (3 μg/ml Der p1) and detection antibody (0.2 μg/ml anti-IgG1 mAb) for optimal sensitivity and reproducibility in this antigen-specific IgG1 assay. Under such optimal condition, a standard curve with low background and high sensitivity was shown. This assay yields very small standard errors (<<5%). The detection limit of this assay for Der p1-specific IgG1 was 15 U/ml, which is defined as >= background + 3 S.D..

1.4 Development of murine Der p1-specific IgG2a ELISA

As discussed above, IgG2a is type 1 immunity associated antibody. An assay to quantify serum or supernatant Der p1-specific IgG2a concentration was developed. Briefly, ELISA plates were coated with rDer p1 at various concentrations (3 μg/ml found optimal) overnight at 4°C. The plates were washed and blocked for 1hr at

37°C. After washing, eight 2-fold dilution of internal standard with beginning dilutions from 1/50 was added and incubated at 4°C overnight.

The same internal standard as for IgG1 was used. For the IgG2a standard, this internal standard was used with beginning dilution from 1/50, which was defined as 1000 U/ml.

The plates were washed and incubated with biotinylated monoclonal goat anti-mouse IgG2a (0.139 μg/ml) (Southern Biotechnology Associates Inc., Birmingham, AL) at 4 °C overnight. The following steps were the same as previously described. As shown in **Figure 2B**, we determined the concentrations of both coating antigen (3 μg/ml Der p1) and detection antibody (0.139 μg/ml anti-IgG2a mAb) for antigen-specific IgG2a assay. Under such optimal condition, a standard curve with low background and high sensitivity was shown. This assay yields very small standard errors (<<5%). The sensitivity of this assay for Der p1-specific IgG2a was 15 U/ml, which is defined as >= background + 3 S.D..

2. PART 2. ESTABLISHMENT OF A MURINE ALLERGIC MODEL USING RECOMBINANT Der p1

C57Bl/6 mice were immunized with recombinant or crude house dust mite Ag in adjuvant, recall type 1 and type 2 cytokine and chemokine responses were

subsequently evaluated in short term spleen cell culture and also in vivo. The immunologic features examined include ex vivo cytokines, chemokines, and in vivo Ab levels. Each of the indices can be divided into type 1 and type 2, this gives us a broader picture of type 1 vs. type 2 responses. Our criteria for defining the Type 1 vs. Type 2 model are based on all 3 features.

We first tried to determine the appropriate Ag to set up the allergic model. There are 13 groups of house dust mite allergen. Der p1 is the first purified HDM allergen, it has been identified as a cysteine protease(Scobie, Ravindran et al. 1994). As a major allergen, Der p1, according to reports, can bind IgE in all sera from all virtually HDM allergic subjects. Moreover, 50-70% of the IgE to HDM extract is against Der p1, demonstrating that it is a major clinically relevant allergen(Chapman and Platts-Mills 1980; Tovey, Chapman et al. 1981).

In this study, we compared the immunologic features seen following crude HDM or rDer p1 primary immunization and restimulation. In Part 2.1, mice were immunized with rDer p1. As comparison, in Part 2.2, mice were immunized with crude HDM.

2.1 Immunologic features following rDer p1 immunization

2.1.1 Determination of optimal conditions for induction and analysis of antibody and cytokine responses

To set up the type 2 immunity model against Der p1, we need to determine optimal Der p1 concentrations for primary immunization in vivo and restimulation in vitro, we also optimized the time frame to investigate such immune responses.

Optimal Der p1 dose for immunization in vivo

First, we determined the optimal dose of Der p1 for immunization. IgE responses are considered a typical type 2 antibody response, so the optimal Ag dose to induce IgE response can be used to set up our murine allergic model. C57/Bl 6 mice were immunized with 0.5, 2, 10, or 50 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were bled on day 0, 10, and 14 to measure serum total IgE level. As shown in **Figure 3**, 0.5 μg Der p1 can not induce significant IgE responses, while 2-10 μg was optimal Der p1 dose to induce significant primary IgE responses.

Spleen cell cultures in the presence of rDer p1 in vitro were set up on day 4 to day 9 respectively after primary immunization. We found that (1) day 6 is the optimal date to set up cell culture with significant cytokine responses, and (2) For the cytokine recall responses in vitro, there was no difference between the group immunized with 2 μg Der p1/alum and the one with 10 μg Der p1/alum (data not shown). So we concluded that the optimal immunization dose was 2 μg and the optimal date to set up spleen cell culture was day 6. These optimal conditions were used in the following experiments.

Optimal Der p1 concentration for restimulation in vitro

To optimize the Der p1 concentration for restimulating cytokine responses in vitro, C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone or with Der p1 at 0.1, 1, 5, 20 μg/ml respectively. Culture supernatant were harvested for analysis of IL-5 and IL-13 at 72hrs.As shown in **Figure 4,** 5μg/ml Der p1 in culture elicited significantly higher IL-5 and IL-13 recall responses than that did lower concentration Der p1, and there is no significant differences between the 5 μg/ml group and the 20 μg/ml group. We concluded that 5 μg/ml Der p1 is the optimal Ag concentration for restimulation in vitro. We also analyzed IFNγ, IL-4 responses and reached the same conclusion (data not shown).

Optimal time points to analyze cytokine responses in vitro

It is extremely important to analyze cytokine responses at a time point with high expression and low background. Different antigens could induce different kinetics for the cytokine responses. So we need optimized the time points to measure cytokine responses against Der p1 in our model. For this purpose, C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg Al(OH)3 adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone or with Der p1 at 5 μg/ml. Culture supernatant were harvested at 24, 48, 72, 96, 120 hours time points respectively after setting up cell culture. Supernatant cytokine levels were measured. As shown in **Figure 5,** kinetics of

different cytokine (IL-2, IL-4, IL-5, IL-13) responses in vitro were analyzed. Antigen-specific IL-2 and IL-4 expressions peaked at 24 hrs and gradually decreased later accompanied by increased background (Ag non-specific response). In contrast, antigen specific IL-5 and IL-13 productions reached summit at 72 to 96 hrs with low background. Based on these observations, we concluded that the optimal time points for cytokine responses were: 24 hr for IL-2 and IL-4; 72 hr for IL-5 and IL-13. Similarly, we determined the optimal time frame for other cytokines and chemokines: 48 hr for C10; 72 hr for IFNγ, and IP-10; and 96 hr for IL-10 (data not shown). In the following observation, these optimal time points were followed when we analyzed cytokine and chemokine responses.

2.1.2 Type 2 chemokine C10 responses

C10 is typical type 2 chemokine. We first analyzed C10 expression in primary culture. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 μg/ml, with 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant was harvested at 12, 24, 48 hr after setting up cell culture and supernatant C10 levels were measured.

As shown in **Figure 6**, Der p1-specific C10 expression significantly increased only after 24 hrs, and reached high level at 48 hrs after setting up culture. So C10 expression was significantly later than IL-4 expression, which peaked at 24 hrs. This

finding confirmed C10 as a late expression type 2 chemokine, the possible explanation is that C10 is IL-4 inducible from macrophages.

From **Figure 6**, we also found that CD4 T cells have a role in the expression of C10, but they were not indispensable because anti-CD4 treatment did not block C10 production, possibly as a result of the multiple (non-T cell) sources of C10 production, which also explained our finding from Figure 6 that expressed C10 was not entirely Ag-specific (i.e., media control also exhibited detectable levels of C10).

From **Figure 6**, we also compared the abilities of purified Der p1 vs. crude HDM to stimulate C10 responses. We found that both Der p1 and crude HDM stimulated Ag-specific C10 production with similar kinetics.

In the next step, we measured the serum C10 level in primary and secondary immune responses. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0 and boosted on day 28. Mice were bleed on day 0, 10, 14 and 35. Serum C10 levels were quantified. As shown in **Figure 7**, serum C10 levels significantly augmented following Der p1 immunization. So serum C10 could be used as one index reflecting type 2 immunity in vivo.

2.1.3 Type 1 chemokine murine IP-10 responses

In contrast to C10, IP-10 is a typical type 1 chemokine. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 μg/ml, with 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant was harvested on 72 hr after setting up cell culture. Supernatant murine IP-10 levels were measured.

As shown in **Figure 8**, Der p1 elicited stronger IP-10 expression in primary culture than did HDM. IP-10 expression peaked at 72 hrs, in consistent with IFNγ expression. IP-10 production was Ag and CD4 T cell-dependent, because it was abolished in the absence of Ag or after adding anti-CD4 mAbs.

We also analyzed the **serum IP-10**. In contrast to C10, serum IP-10 levels were consistently below the detection limits (15 pg/ml). The chemokine finding in this murine model revealed substantial type 2 chemokine synthesis in vivo, with lower type 1 chemokine responses. These immune responses closely parallel those seen in human dust mite allergic subjects.

2.1.4 Type 2 cytokine IL-5, IL-13, IL-4 and IL-10 responses

IL-4 is responsible for IgE class switch and suppressing Th1 cells cloning(Snapper, Pecanha et al. 1991; Parronchi, De Carli et al. 1992; Delphin and Stavnezer 1995). IL-5 can promote eosinophil growth and differentiation, it also contributes to IgE and

IgG1 synthesis in mice (DeKruyff, Mosmann et al. 1990; Purkerson and Isakson 1992). IL-13 promotes B cell growth and differentiation, inhibits macrophage inflammatory cytokine production and Th1 cells. IL-10 is produced by Th2 cells as well as APC, and it can inhibit the development and activation of Th1 cells by acting on the antigen-presenting cells.

To investigate the type 2 cytokine responses in our murine model, C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 μg/ml, with different concentrations of HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant was harvested at 24, 48, 72, 96 and 120 hr after setting up cell culture. Supernatant IL-5, IL-13, IL-4 and IL-10 levels were measured.

Figure 9 and **Figure 10** described the kinetics of IL-5 and IL-13 production respectively. We first looked at the kinetics of Der p1-specific IL-5 and IL-13 expression. It was noted that both IL-5 and IL-13 reached the plateau at 72 hour time points as shown by high Ag-specific expression and low background.

At this optimal time point, Der p1 and crude HDM stimulated IL-5 (or IL-13) expression with similar kinetics. We concluded that Der p1 was representative of whole body extract HDM in restimulating IL-5 and IL-13 production in vitro.

In the absence of Ag or after blocking CD4 T cells, IL-5 and IL-13 expression continuously stayed at the bottom level, reflecting the facts that IL-5 and IL-13 were dependent on Ag-specific Th2 cells.

Figure 11 depicted the production of IL-10 and IL-4 at their optimal time point, 96 hr for IL-10 and 24 hr for IL-4 respectively. Similarly, we found that both IL-10 and IL-4 responses were antigen-specific and CD4 T cells were required for their production. The difference was that Der p1 induced similar IL-4 expression than did HDM, but Der p1 elicited stronger IL-10 expression than did crude HDM.

2.1.5 Type 1 cytokine IFNγ and IL-2 responses

IFNγ is a representative type 1 cytokine. IL-2 can selectively promote established type 1 but not type 2 responses, so it is considered as a type 1 cytokine.

To investigate the type 1 cytokine expression in our murine model, C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 μg/ml, with different concentrations of HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant was harvested on 24, 48, 72, 96 and 120 hr after setting up cell culture. Supernatant IFNγ and IL-2 levels were measured.

Also in **Figure 11,** optimal IFNγ and IL-2 expressions were identified at 72 hrs for IFNγ and 24 hrs for IL-2 respectively. It is found that both IFNγ and IL-2 production were Ag-specific and CD4 T cell-dependent. Both recombinant Der p1 and crude HDM elicits IFNγ or IL-2 production. So Der p1 can represent whole body extract HDM in restimulating type 1 cytokine production in vitro.

We noted low IFNγ expression induced by either Der p1 (12 U/ml) or crude HDM (5 U/ml). We concluded that such murine model had lower capacity to generate type 1 cytokine responses (IFNγ) than type 2 cytokine responses (IL-5, IL-13, IL-4).

2.1.6 Type 2 antibody responses

IgE, IgG1 are typical type 2 antibodies. We investigated the serum total IgE and Der p1-specific IgG1 levels following primary and secondary immunization with Der p1/alum. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0 and boosted on day 28. Mice were bleed on day 0, 10, 14, 35 and 42. Serum total IgE, Der p1-specific IgG1 and IgG2a levels were quantified as described previously. As shown in **Figure 12**, serum total IgE level significantly elevated following primary Der p1 immunization and its augmentation accelerated after secondary immunization.

We also found strongly elevated Der p1-specific IgG1 after secondary immunization.

2.1.7 Type 1 antibody responses

Murine IgG2a is considered a type 1 antibody which is exclusively stimulated by IFNγ (Finkelman, Katona et al. 1988). We investigated the serum Der p1-specific IgG2a levels following primary and secondary immunization with Der p1/ alum. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0 and boosted on day 28. Mice were bleed on day 0, 10, 14, 35 and 42. Serum Der p1-specific IgG2a levels were quantified as described previously. We found that IgG2a levels were consistently at low level at all five time points, and that there was no significant difference at each time point (data not shown). This is in contrast to IgE and IgG1 responses. We concluded that the antibody kinetics revealed substantial type 2 antibodies synthesis in vivo, with lower type 1 antibody responses. These immune responses closely parallel those seen in human dust mite allergic subjects.

2.1.8 Comparison of type 2 and type 1 immunity models against the same antigen Der p1

In previous literature using OVA antigen, use of adjuvants alum and HKL/IFA were shown to elicit different types of immune responses. But there is still no comparable report regarding rDer p1 antigen. By using different adjuvants, we wanted to establish a Th1 and Th2 dominated model against Der p1 Ag. C57 Bl/6 mice were immunised with 2μg Der p1 adsorbed onto either 2 mg $Al(OH)_3$ (Alum group) or

$1X10^7$ in HKL/IFA (HKL group) given i.p. on day 0. Mice were killed at day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml (200 μl/well) alone or with predetermined optimal concentration of Der p1 (5μg/ml).

As shown in **Figure 13,** it is found that alum group had significantly stronger Ag-specific IL-4, IL-5, IL-13, IL-10 and C10 responses than had HKL group ($p<0.05$). Meanwhile, HKL group demonstrated significantly stronger IFNγ and IP-10 expression than did alum group ($p<0.05$). In conclusion, the recombinant Ag in Alum and HKL adjuvant lead to Th2 and Th1 dominated immunity against Der p1 respectively. This advantage allows us to pursue whether the "IL-2 like" cytokine's effect is unique to atopic or exhibited in both those with hypersensitivity and clinical tolerance.

Thus by using recombinant Ag and appropriate adjuvants, we describe type 2 and type1 immunity-dominated murine model against Der p1 respectively. Therefore such murine models were named *type 2 immunity model* and *type 1 immunity model* respectively.

2.2 Immunologic features following crude HDM immunization

In the above type 2 murine models, mice were immunized with Der p1 in vivo, after setting up spleen cell culture in vitro, cytokine expression against rDer p1 or crude

HDM in vitro were compared. We concluded that recombinant antigen elicited a similar immune response to that obtained using crude whole body extracts in vitro.

To compare the abilities of Der p1 vs. HDM for primary immunization in vivo, in this part, we immunized the mice with crude HDM and investigated the in vitro cytokine expression when Der p1 and HDM were used as recall antigens. C57 Bl/6 mice were immunised with 100 AU crude HDM adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed at day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml (200 μl/well) alone or with Der p1 (5μg/ml), crude HDM (100 and 200 AU/ml) respectively. Culture supernatants were harvested for analysis of chemokines mIP-10 (72hr), C10 (48hr) and cytokines IL-4 (24hr), IFN-γ (72hr), IL-5 (72hr), IL-13 (72hr).

As shown in **Figure 14**, for all the type 1 and type 2 chemokines and cytokines measured, rDer p1 elicited a substantial part (30%~90%) of the responses against whole body extract. We concluded that the response against rDer p1 reflected the majority part of the response for whole body extract.

Thus, from the results of Der p1 in primary in vivo immunization and restimulation in vitro, we found that recombinant Der p1 induced similar immune responses to that obtained using crude whole body extracts (widely used in clinical medicine). It was confirmed that Der p1 can be used as model allergen to reflect sensitization to house dust mites.

2.3 Conclusion for the murine allergic model against rDer p1

We report development of a murine model to allow analysis of factors relevant to sensitization and control of immediate hypersensitivity to house dust mite antigen. Such immunologic factors include chemokines, cytokines and antibodies, and the analysis is established both in vivo and in vitro.

Recombinant Der p1 immunization with alum stimulated substantial type 2 cytokine, chemokine and antibody synthesis, with lower capacity to generate type 1 responses (IP-10, IFNγ, IgG2a). These immune responses closely parallel those seen in human dust mite allergic subjects. Thus the murine model was termed a type 2 immunity model or model of allergy.

In contrast, Der p1 immunization with HKL/IFA stimulated substantial type 1, but low type 2 cytokine and chemokine expression. These immune responses parallel with the immunologic features of human clinical tolerance subjects. Therefore this murine model was named type 1 immunity model.

When used in either primary immunization in vivo or restimulation in vitro, recombinant Der p1 elicited a similar immune response to that obtained using crude whole body extracts (widely used in clinical medicine). Therefore Der p1 can be used as model allergen to reflect sensitization and maintenance of hypersensity to house dust mites.

3. PART 3. THE ROLE OF IL-2 AND IL-15 IN ONGOING TYPE 1/TYPE2 IMMUNITY IN A MURINE MODEL OF ALLERGY

3.1 Established type 1 vs. type 2 responses are differentially dependent on IL-2

It has been known for a long time that IL-2 is required for the priming of CD4 T cells. But the role of IL-2 in established Th1 and Th2 cell repertoire was first studied in early 1990s. Yang and others found that Th1, but not Th2, recall responses are highly IL-2 dependent(Yang and HayGlass 1993). This finding was based on the OVA system, we wanted to initially confirm this finding with the Der p1 model of allergy established here.

Spleen cells from mice immunized with Der p1 in alum to generate Th2-biased immunity were cultured with Der p1 in the presence of: neutralising anti-IL2 Ab, irrelevant control Ab, or rIL-2 to determine the impact of blocking endogenous IL2 production vs. supplementing IL2 on allergen driven Th1 vs. Th2 gene expression.

3.1.1 Role of IL-2 on type 1 responses

As shown in **Figure 15A**, we confirmed that IFNγ and IP-10 (Th1) readouts expression significantly diminished after blocking endogenous IL-2, and that their expression significantly elevated (2.5-4.5 fold) after adding exogenous IL-2.

We extended our approach by blocking IL-2Rα which is the unique receptor of IL-2 and not shared by other IL-2-like cytokines. According to literature reports, IL-2Rα is a low affinity receptor subunit expressed on activated lymphocytes, it is not required for signalling but important for IL-2 binding and internalisation into lymphocytes (details discussed later).

From **Figure 15B**, it can be seen that IFNγ expression also seriously decreased after blocking IL2Rα.

In conclusion, endogenous IL-2 is required for the expression of established type 1 immunity and exogenous IL-2 enhances type 1 immunity.

3.1.2 Role of IL-2 on type 2 responses

From **Figure 15A**, it was also found that IL-4 and C-10 (Th2) expression are not affected by either blocking endogenous IL-2 or adding exogenous IL-2.

We concluded that Th1, but not Th2, responses are highly IL-2 dependent and exogenous IL-2 selectively enhances type 1 but not type 2 responses.

3.2 Established type 1 vs. type 2 responses are differentially dependent on IL-15

Because IL-15 shares β and γ receptor chains and aspects of the activation pathway of IL-2 signaling, it is possible that it has impact similar to IL-2 on Th1/Th2 responses. Thus, we first examined the global impact of rIL-15 on Th1/Th2 recall responses.

The Th2 murine model was set up as previously and exogenous IL-15 were added to the spleen cell culture in the presence or absence of Ag. As shown in **Figure 16**, it is found that IL-15 selectively enhanced Th1 (IFNγ and IP-10) responses by 3-10 fold while not altering Th2 (IL-4, IL-5, IL-13, C-10) responses. Furthermore, IL-15's effect was Ag-dependent and dose-dependent (**Figure 17**), the optimal concentration of IL-15 to stimulate Ag-specific IFNγ expression was 60 ng/ml.

3.3 Endogenous IL-12 is required for the IFNγ responses enhanced by IL-15

In the next step, we investigated the mechanism of IL-15's effect to enhance IFNγ responses. Several factors have been reported to be able to promote IFNγ production. They include: (a) elevated IL-12 production: from dendritic cells and macrophages; (b) IFNγ: auto-stimulation from T cells, NK or NKT cells; (c) IFNα: from leukocytes, can stimulate IFNγ by CD4 T cells in the absence of IL-12; (d) TNF-α: from macrophage; (e) IL-18: from Macrophage, can promote IFNγ in synergy with IL-12; (g) TGFβ: from chondrocytes, monocytes, and T cells, a cytokine with both enhancing and inhibitory effect on IFNγ production.

IL-12 and IL-18 are reportedly the most important factors to promote IFNγ production by Th1 cells(Tomura, Maruo et al. 1998; Chang, Segal et al. 2000; Nakanishi, Yoshimoto et al. 2001). To examine the requirement of endogenous IL-12 or IL-18 for IL-15's capacity to selectively enhance type 1 immunity, Th2 murine model was established as previously in the absence or presence of rIL-15 (60ng/ml). Blocking Abs against IL-12 or IL-18 were added to the spleen cell culture respectively. Naive rat IgG (10 μg/ml) was used as control Ab (data not shown).

As shown in **Figure 18A**, IFNγ levels were significantly decreased after blocking endogenous IL-12 with anti-IL12 mAbs. In the presence of rIL-15, blocking endogenous IL-12 induced over 85% loss of IL-15-enhanced-IFNγ expressions.

In marked context from **Figure 18B**, it was revealed that blocking endogenous IL-18 did not alter the Ag-specific IFNγ production, neither did it alter the enhanced IFNγ expression by rIL-15.

In **Figure 18C**, we found that blocking both IL-12 and IL-18 did not further decrease IFNγ levels than did blocking IL-12 alone (shown in Panel A).

Thus, we concluded that endogenous IL-12, but not IL-18, is required for IFNγ responses against Der p1 and its enhancement by exogenous IL-15.

3.4 IL-15 does not directly increase IL-12 expression

IL-15's activity of IL-12-dependent augmentation of IFNγ could due to the up-regulation of IL-12 expression by IL-15, so we then measured the IL-12/IL-18 levels and compared them in the presence or absence of exogenous IL-15. The type 2 immunity model was set up, spleen cells were cultured with Der p1 without or with rIL-15. After 24 and 72 hours, culture supernatant were harvested for analysis of IL-12 p40, IL-12 p70 and IL-18.

As shown in **Figure 19**, at both 24 hour and 72 hour time-point, IL-12p40 and IL-18 levels were quantified. At either time-point, there was no Ag-specific IL-12 or IL-18 expression, neither was there significant difference between the group with exogenous IL-15 and the one without IL-15 added.

Because p40 is a widely used, but not very precise, surrogate for functional IL-12, IL-12p70 was also examined by chemiluminescent ELISA. It was continuously lower than the detection limit (1pg/ml) in each group (data not shown).

We then focused on earlier time points and analyzed the supernatant as early as 9 hours after setting up cell culture, there was consistently no antigen-specific IL-12 or IL-18 expression (data not shown).

Based on these data, we concluded that there is no evidence that rIL-15 directly increases expression of IL-12 or IL-18.

3.5 IL-15 increases IL-12 *responsiveness* to promote IFNγ production

If IL-12 is required for the capacity of IL-15 to selectively enhance type 1 immunity, but IL-15 did not directly enhance IL-12 expression, an alternative possibility is that IL-15 enhances the IL-12 responsiveness of IFNγ-producing cells (including T cells, NK cells and NKT cells).

To test this hypothesis, we compared IL-12 responsiveness (rather than production) in the absence and presence of exogenous IL-15. Using IL-12-deficient mice, we compared the cell responsiveness to constant concentration of rIL-12 in the absence and presence of rIL-15. Briefly, IL-12 knockout mice (IL-12p40-/-, C57Bl/6 background), used to eliminate potential role of endogenous IL-12, were immunized with 2μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-12 (at a constant low concentration of 3 pg/ml) and/or rIL-15 (2.5, 10, 50, and 60 ng/ml). After 72 hours, culture supernatant was harvested for analysis of IFN γ.

The findings in **Figure 20** includes that (i) in the absence of IL-12, even high concentration of IL-15 (60 ng/ml) only induced very limited IFNγ production (less than 5-10% of that produced in wildtype mice by rIL-15); (ii) the IFNγ level in the "Ag plus IL-12 plus IL-15" group was significantly higher than the arithmetic sum value of the "Ag+IL-12" group and "Ag+IL-15" group (compare "D" with "E").

In summary, in the previous experiments, it was found that both IL-12 (data not shown) and IL-15 (data shown in Figure 17) or their combination (data not shown) at the above concentration had no Ag-nonspecific effect. So we concluded that IL-15 augments the Ag-specific IL-12 responsiveness, thus promotes IFNγ expression.

3.6 Both CD4 and CD8 T cells are required for IL-15 to enhance IFNγ responses

In this section, we tried to define the cellular basis of IL-15's capacity to selectively enhance Th1 responses. IFNγ-producing cells include T cells, NK cells and NKT cells, all of which can express IL-15R(Ohteki, Ho et al. 1997; Waldmann and Tagaya 1999).

Because in previous studies (Figure 17), it was found that IL-15's effect is Ag-specific, we first investigated the role of CD4 and CD8 T cells. To examine the requirement of CD4 and CD8 T cells, type 2 immunity model was set up as previously in the absence or presence of rIL-15 in spleen cell culture. Blocking Abs

against CD4 and CD8 were added to spleen cell cultures. Purified anti-CD4 mAb from YTS 191.1 hybridoma culture supernatant were added to cultures at 1/4 dilution. Anti-CD8 mAbs were added to the culture at final concentration of 10 μg/ml. Their concentrations were optimised previously (data not shown). Naive rat IgG (10 μg/ml) was used as control Ab (data not shown).

As shown in **Figure 21**, IFNγ levels in response to Ag alone significantly decreased after blocking *either* CD4 *or* CD8, and were abolished after blocking both CD4 and CD8. In the presence of exogenous IL-15, blocking *either* CD4 *or* CD8 could significantly decrease but not eliminate IFNγ expression. IFNγ was eradicated after blocking both CD4 and CD8 T cells. At the same time control Ab naive rat IgG did not have any impact on IFNγ synthesis (data not shown).

In contrast to IFNγ, also from **Figure 21**, we found that IL-5 expression, a Th2 response, was not changed after blocking CD8, but, as expected, was manifestly reduced in the presence of anti-CD4 mAb.

We conclude that both CD4 and CD8 T cells are required for Ag-induced IFNγ responses and its enhancement by IL-15. In contrast, only CD4 but not CD8 T cells are required for Ag specific IL-5 responses.

Our study revealed that the cellular basis of IL-15's effect on Th1 recall responses requires both CD8 and CD4 T cell.

3.7 Conclusions for the role of IL-2 and IL-15 on established type 1/type 2 immunity

Endogenous IL-2 is required for expression of Th1 but not Th2 recall responses, exogenous IL-2 selectively enhances Ag-specific type 1 immunity.

Similar to IL-2, IL-15 selectively enhances Ag-specific Th1 (IFNγ and IP-10) responses by 3-10 fold while not altering Th2 (IL-4, 5, 13, C-10) associated responses.

Endogenous IL-12 is required for the molecular mechanism of this augmentation. IL-15 does not directly up-regulate IL-12 expression, rather, it increases cellular responsiveness to endogenous IL-12, hence type 1 cytokine production and steering to Th1 responses is achieved in the type 2 immunity model.

In cellular mechanism of IL-15's effect, both CD4+ and CD8+ T cells contribute to the Der p1-specific Th1 cytokine responses augmented by IL-15. It is sharply different from the cellular mechanism of Th2 cytokine responses, where only CD4 but not CD8 T cells are required.

Our study raises the possibility of allergy treatment by IL-15, a cytokine that is currently in experimental use as an alternative to IL-2 in tumour immunotherapy

(Waldmann, Tagaya et al. 1998; Waldmann and Tagaya 1999; Waldmann, Dubois et al. 2001).

4 PART 4. EXOGENOUS RECOMBINANT IL-15 CAN SUBSTITUTE FOR IL-2 IN ANTIGEN-STIMULATED CYTOKINE PRODUCTION

4.1 Exogenous IL-15 can replace IL-2 in dominant type 1 responses

Because of similarities between IL-15 and IL-2 in signaling, and having demonstrated the activity of IL-15 in selectively promoting type 1 gene expression in recall Ag responses, we wanted to determine if this activity was direct or via activation of enhanced IL-2 synthesis. Our strategy to address this question was to block endogenous IL-2 activity by (a) anti-IL-2 Ab or (b) anti-IL-2Rα Ab and examine the capacity of IL-15 to augment IFNγ responses. Because IL-2Rα is the unique receptor for IL-2, use of an Ab to block this receptor will not influence cytokine signaling via the IL-15R.

Type 2 immunity model was set up as previously. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml). Anti-IL2Rα (50 μg/ml) or anti-IL2 (100 ng/ml), both concentrations optimized previously, were added to the culture. 72 hours culture supernatants were analyzed for IFNγ expressions.

As shown in **Figure 22**, two findings include (i) in the absence of rIL-15, blocking IL-2 with anti-IL2 mAb or anti-IL2Rα mAb significantly decreased IFNγ expression ($p<0.001$) (Upper Panel). This confirmed previous findings and confirmed the effectiveness of these 2 mAbs; (ii) lower panel, in the presence of rIL-15, we found that IL-15 retains its ability to enhance IFNγ despite after blocking either IL-2 or IL-2Rα ($p>0.05$) (Lower Panel).

Thus, our conclusion was that this activity of IL-15 to selectively enhance type 1 immunity is independent of IL-2. This means IL-15 can substitute for IL-2 in dominating type 1 responses.

4.2 Exogenous IL-15 decreases supernatant IL-2 level in a dose dependent manner

In recent years, the role of IL-2 and IL-15 in governing T cell homeostasis becomes a hot point in immunology research because of its potential biological significance. Most notably it was found that IL-2 is required for AICD whereas IL-15 inhibits this process. IL-15 supports whereas IL-2 inhibits the survival of memory T cells, thus maintaining specific immune responses to foreign pathogens. The balance between IL-2 and IL-15 determines the future of the immune response(Chu, Chen et al. 1999; Waldmann, Dubois et al. 2001). But there is no report about the direct impact of each on the other.

In light of this, the direct impact of IL-15 on IL-2 expression was investigated. Type 2 immunity model was set up as previously, mice were killed at day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (2.5, 10, 50, or 60 ng/ml). 24 hours supernatant IL-2 levels in the absence vs. presence of different concentrations of exogenous IL-15 were examined.

As shown in **Figure 23**, it was found that supernatant Ag-specific IL-2 levels in short term (24hr) spleen cell culture (recall responses) were significantly *decreased* in the presence of exogenous IL-15.

To exclude the possibility of delayed IL-2 gene expression by IL-15, we also measured IL-2 levels at later time points (data not shown) and confirmed that supernatant IL-2 levels in the IL-15 group continuously and significantly lower than those in the Ag only group.

This result suggests that IL-15 decreases supernatant IL-2 levels in a dose dependent manner. The possible mechanism includes either less IL-2 is produced or more IL-2 is internalized into cells in the presence of rIL-15, or both.

4.3 Exogenous IL-15 does not inhibit IL-2 production but increases the cellular consumption of IL-2

To clarify whether this observation reflected less IL-2 production or more IL-2 consumption by the cells, type 2 immunity model was set up, spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml), IL-2Rα was blocked with mAbs (10 or 50 μg/ml). By this way, no IL-2 can be internalized into the cells, supernatant IL-2 levels were then compared in the absence and presence of exogenous IL-15.

As in **Figure 24**, it was found that IL-2 levels had no difference between "IL-15 presence group" and "IL-15 absence group" after anti-IL2Rα mAbs were added.

We reasoned that IL-15 does not directly inhibit IL-2 production, rather, it increases the uptake of IL-2, thus reduces supernatant IL-2. The possible mechanism could be through increased IL-2R expression. Our conclusion is that IL-15 can increase the consumption of Ag-driven IL-2.

4.4 Conclusions for the impact of exogenous IL-15 on IL-2

IL-2 and IL-15 share β- and γ- receptor subunit and signaling pathway. Both IL-2 and IL-15 can selectively enhance type 1 immunity. IL-2 is not required for IL-15 to exert this function, and IL-15 can substitute for IL-2 in this effect.

Albeit with so many similarity, IL-2 and IL-15 balance each other in peripheral T cell homeostasis: IL-2 induces Ag-specific T cells apoptosis while IL-15 makes such

cells to turn into memory cell and survive. IL-15 can increase IL-2 consumption by Ag-specific T cell, not accompanied by stronger function, and more likely aims at promoting the balance toward its own part.

5. PART 5. COMPARISON OF REGULATIVE EFFECT OF γ-CHAIN BUT NOT β-CHAIN RECEPTOR CYTOKINES

To further investigate the potential role of other γc associated cytokines in controlling the balance of Th1/Th2 in immunity, we began an assessment of the roles of IL-7 and IL-9 in this process.

5.1 Role of exogenous IL-7 on established type 1/type 2 responses

IL-7 shares receptor γ-chain and signaling with IL-2 and IL-15. Extensive studies revealed its central role in regulating development of B and T cells. It is the only non-redundant cytokine identified to date in lymphopoiesis. But its role in regulating mature T cells and recall immune responses is ill defined, beyond its capacity to promote T cell proliferation. IL-7 was reported to preferentially promote IL-4 or to enhance IFNγ over IL-4 (Dokter, Sierdsema et al. 1994; Gombert, Tancrede-Bohin et al. 1996) (Borger, Kauffman et al. 1996). These contradictory results were obtained using different polyclonal activators (Con A, PHA, PMA, anti- CD3/CD28), indicating to us the need for Ag-dependent systems.

With our murine model of allergy, we will determine (i) does IL-7 preferentially

promote type 1 or 2 gene expression in recall Ag responses, (ii) is this activity direct or is it via activation of enhanced IL-2 synthesis by examining Ag plus rIL-7 +/- neutralizing anti-IL2 mAb.

First, we investigated the global effect of rIL-7 (at similar concentration as rIL-15 or rIL-2 used in above experiments) on type 1 and type 2 recall responses. C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the presence of rIL-7 at 2.5 or 25 ng/ml. Culture supernatants were harvested for analysis of type 1 and type 2 cytokines and chemokines expressions at optimal time points.

We found that, different from rIL-2 and rIL-15, rIL-7 had no effect on IFNγ or IP-10 synthesis (data not shown), but rIL-7 significantly enhanced Ag-specific IL-5 and IL-13 production. We focused on the IL-5 and IL-13 expression, as shown in **Figure 25**, rIL-7 (2.5 or 25 ng/ml) did not stimulate Ag non-specific (ie. spontaneous) IL-5 or IL-13 expression, but it enhanced Ag-specific IL-5 and IL-13 expressions by 3 fold.

We concluded that rIL-7 selectively enhanced Ag-specific IL-5 and IL-13 (Th2) responses without altering Th1 responses. This is markedly different from IL-2 and IL-15.

In the next step, we investigated whether IL-2 has a role in IL-7's function of

enhancing Th2 response. Type 2 murine model was set up as previously, spleen cell culture was set up in the presence of Der p1 plus rIL-7 (2.5 ng/ml and 25 ng/ml). As shown in **Panel A1** of **Figure 27**, there are six groups, the left 3 groups had no anti-IL2 added, the right 3 group had anti-IL2.

There were 2 findings from Panel A1, (a) In the "Der p1" groups, we found that IL-5 expression had no change after blocking IL-2 ($p>0.05$), this was consistent with our previous finding that IL-2 is not required for IL-5 responses; (b) In the "Der p1+ rIL-7" groups, we found that enhanced IL-5 expression had no change after blocking IL-2 ($p>0.05$).

In conclusion, endogenous IL-2 has no role in the Ag-specific IL-5 responses and its enhancement by rIL-7.

Similarly, from **PanelA2** of **Figure 27**, we concluded that endogenous IL-2 had no role in the Ag-specific IL-13 responses and its enhancement by rIL-7.

5.2 Role of exogenous IL-9 on established type 1/type 2 responses

Like IL-7, IL-9 also shares receptor γ-chain with IL-2 and IL-15. There is very few report regarding IL-9's effect on Ag-specific recall Th1 or Th2 responses. Using the same strategy as to IL-7, we first investigated the function of rIL-9 on established type 1 vs. type 2 responses. First, we investigated the global effect of rIL-9 on type 1

and type 2 recall responses, then we focused on the needs of endogenous IL-2 for this function.

C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the presence of rIL-9 at 2.5 or 25 ng/ml. Culture supernatants were harvested for analysis of type 1 and type 2 cytokines and chemokines expressions at optimal time points.

We found that, similar to IL-7 and different from rIL-2 and rIL-15, rIL-9 had no effect to change IFNγ or IP-10 synthesis (data not shown), but rIL-9 significantly enhanced Ag-specific IL-5 and IL-13 production. As shown in **Figure 26**, 2.5 ng/ml rIL-9 had Ag non-specific effect on IL-5 expression, but this non-specific effect was not as strong as its Ag-specific effect and was limited to IL-5, but not IL-13. We found that rIL-9 enhanced Ag-specific IL-5 and IL-13 expressions by 2 fold.

In conclusion, rIL-9 selectively enhanced Ag-specific IL-5 and IL-13 (Th2) responses without altering Th1 responses. This is different from IL-2 and IL-15.

In the next step, we investigated whether IL-2 has a role in IL-9's function of enhancing Th2 response. Type 2 murine model was set up as previously, spleen cell culture was set up in the presence of Der p1 plus rIL-9 (2.5 ng/ml and 25 ng/ml). As shown in **Panel B1** of **Figure 27**, there are six groups, the left 3 groups did not have

anti-IL2 added, the right 3 group had anti-IL2.

In the Der p1+ rIL-9 groups, we found that enhanced IL-5 expression had no change after blocking IL-2 ($p>0.05$).

In conclusion, endogenous IL-2 has no role in the Ag-specific IL-5 responses enhanced by rIL-9.

Similarly, from **Panel B2** of **Figure 27**, we concluded that endogenous IL-2 has no role in the Ag-specific IL-13 responses enhanced by rIL-7.

5.3 Conclusion for IL-7 and IL-9 on established type 1/type 2 responses

In contrast to IL-2 and IL-15, we found that IL-7 and IL-9 selectively enhanced Ag-specific IL-5 and IL-13 (Th2) responses without impact on Th1 cytokines. Endogenous IL-2 had no role in IL-5 and IL-13 expressions and their enhancement by IL-7 or IL-9.

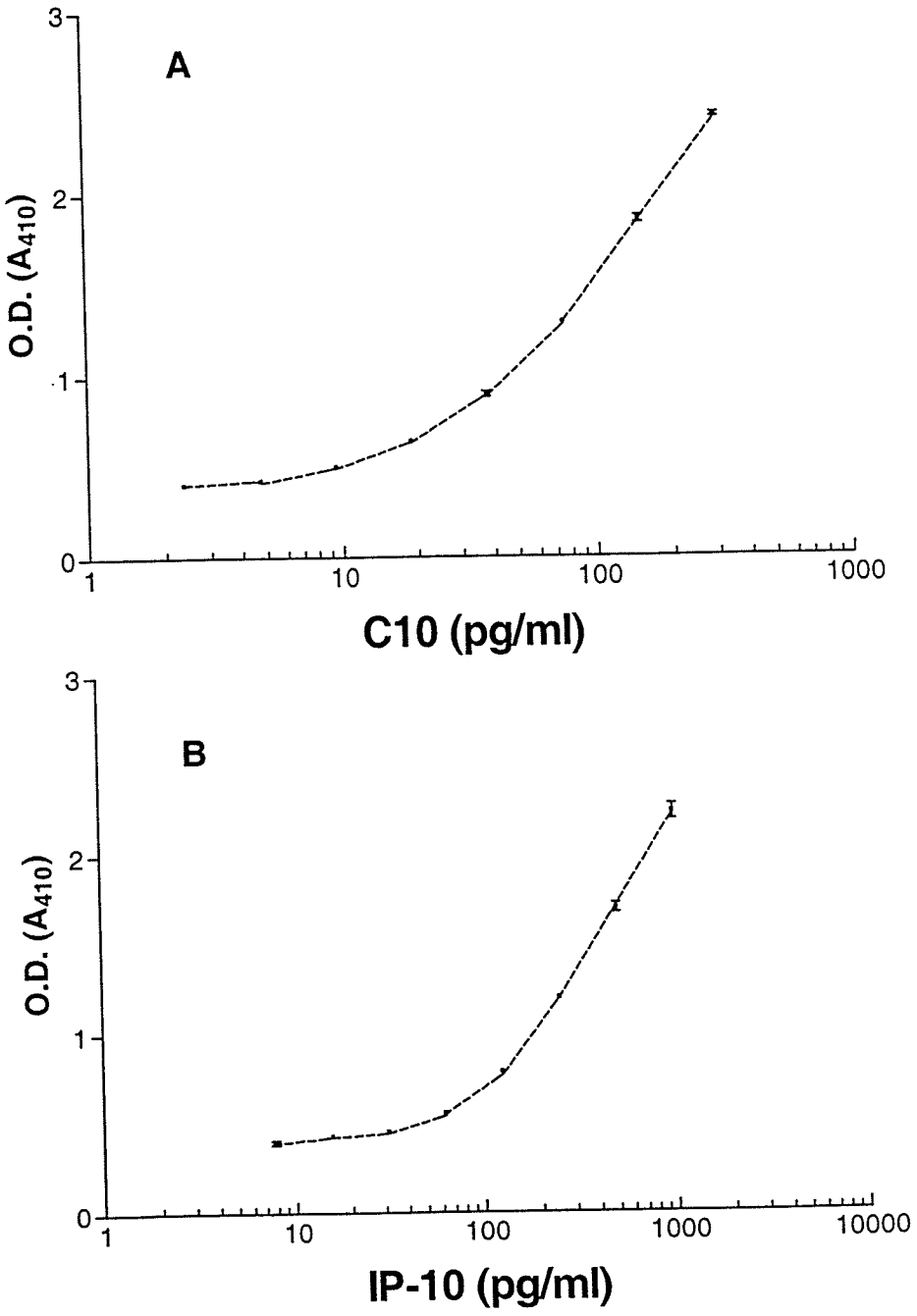

Figure 1. Sensitive murine C10 and IP-10 ELISA. **Panel A.** C10 assay, O.D. values were shown for eight 2-fold dilutions of recombinant C10 standard with concentration from 300 pg/ml (first well) to 2.34 pg/ml (last well); **Panel B.** IP-10 assay, O.D. values were shown for eight 2-fold dilution of recombinant IP-10 standard with concentration from 1000 pg/ml (first well) to 7.8 pg/ml (last well). Data shown were means O.D. +/- SEM of duplicates per chemokine concentration. Detection limits were 4.7 pg/ml for C10 and 15.6 pg/ml for IP10, read at 60 minutes.

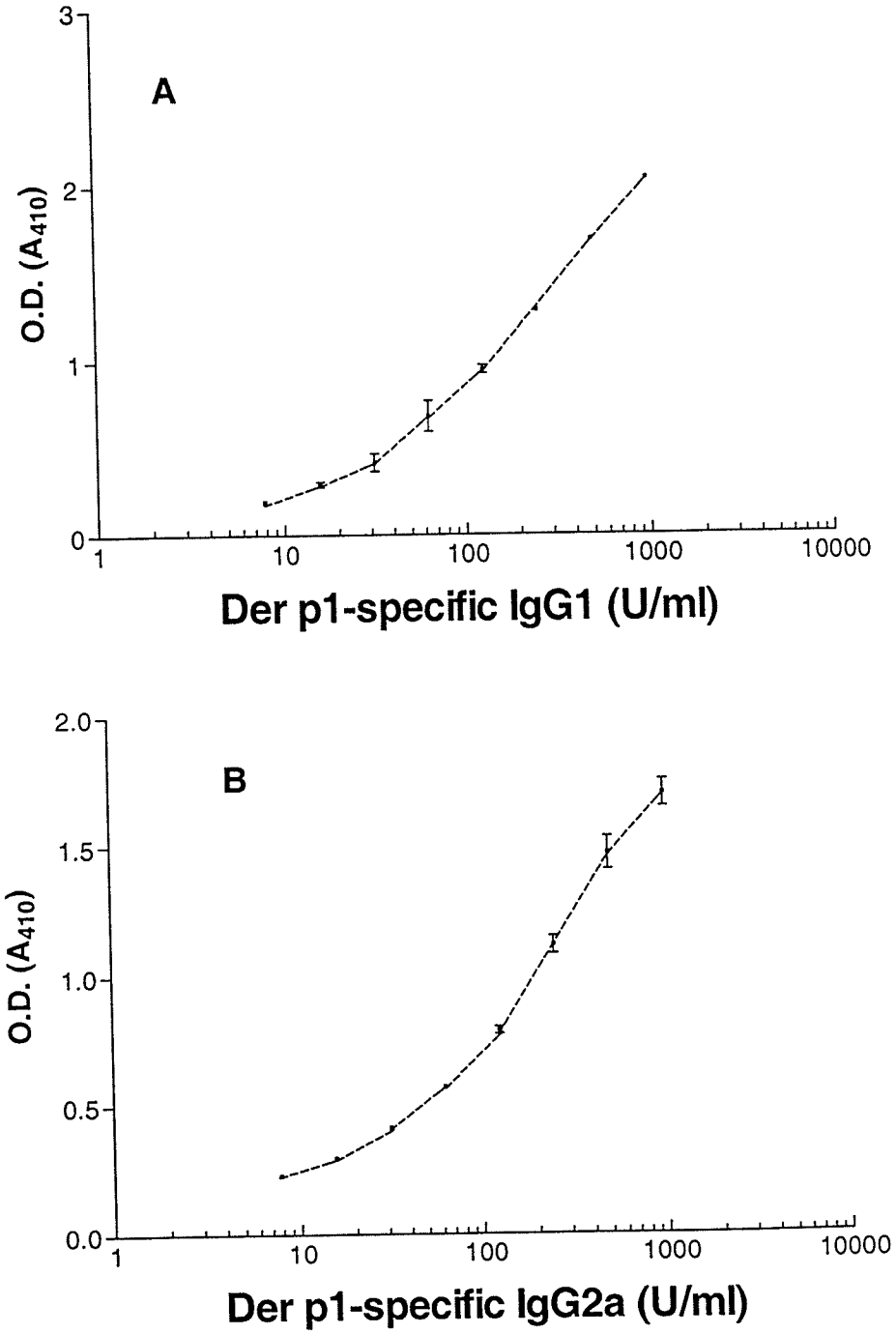

Figure 2. Sensitive murine Der p1-specific IgG1 and IgG2a ELISA. **Panel A**. Antigen-specific IgG1 assay, O.D. values were shown for eight 2-fold dilution of internal standard with dilution from 1/500 (or 1000 U/ml, first well) to 1/6400 (or 7.8 U/ml, last well); **Panel B**. Antigen-specific IgG2a assay, O.D. values were shown for eight 2-fold dilution of internal standard with dilution from 1/50 (or 1000 U/ml, first well) to 1/640 (or 7.8 U/ml, last well). Data shown were means O.D. +/- SEM of duplicates per antibody concentration. Detection limits were 15.6 U/ml for IgG1 and IgG2a. Note: The unit definitions for IgG1 and IgG2a were different, details see text.

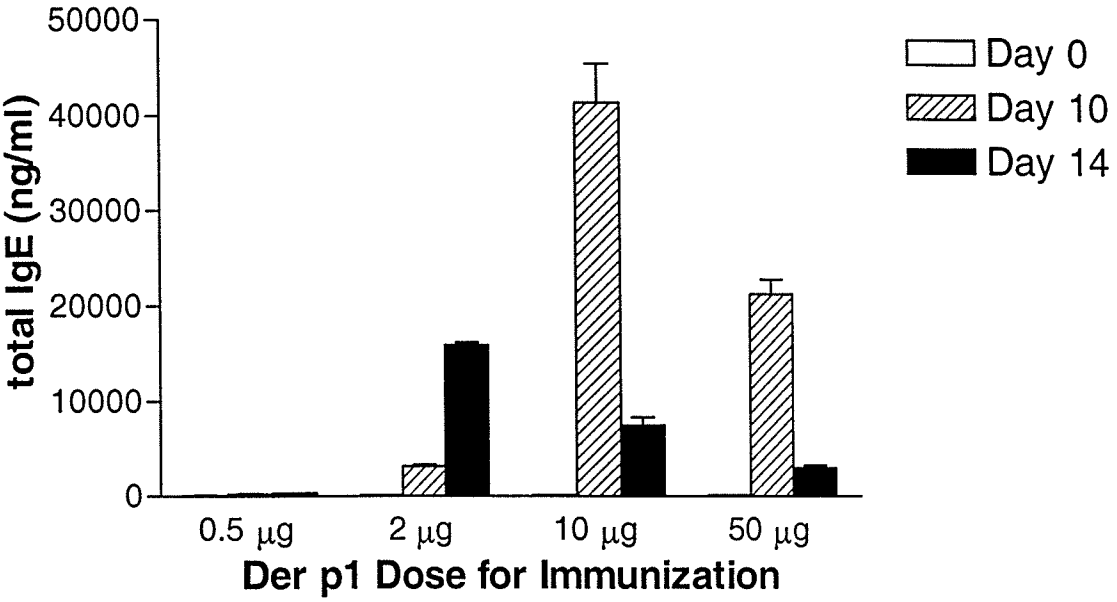

Figure 3. 2-10 µg was the optimal Der p1 dose for primary immunizations. C57/B16 mice were immunized with 0.5, 2, 10, or 50 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were bled on day 0, 10, and 14 to measure serum total IgE level. The results are pooled from 2 representative experiments, 12 mice.

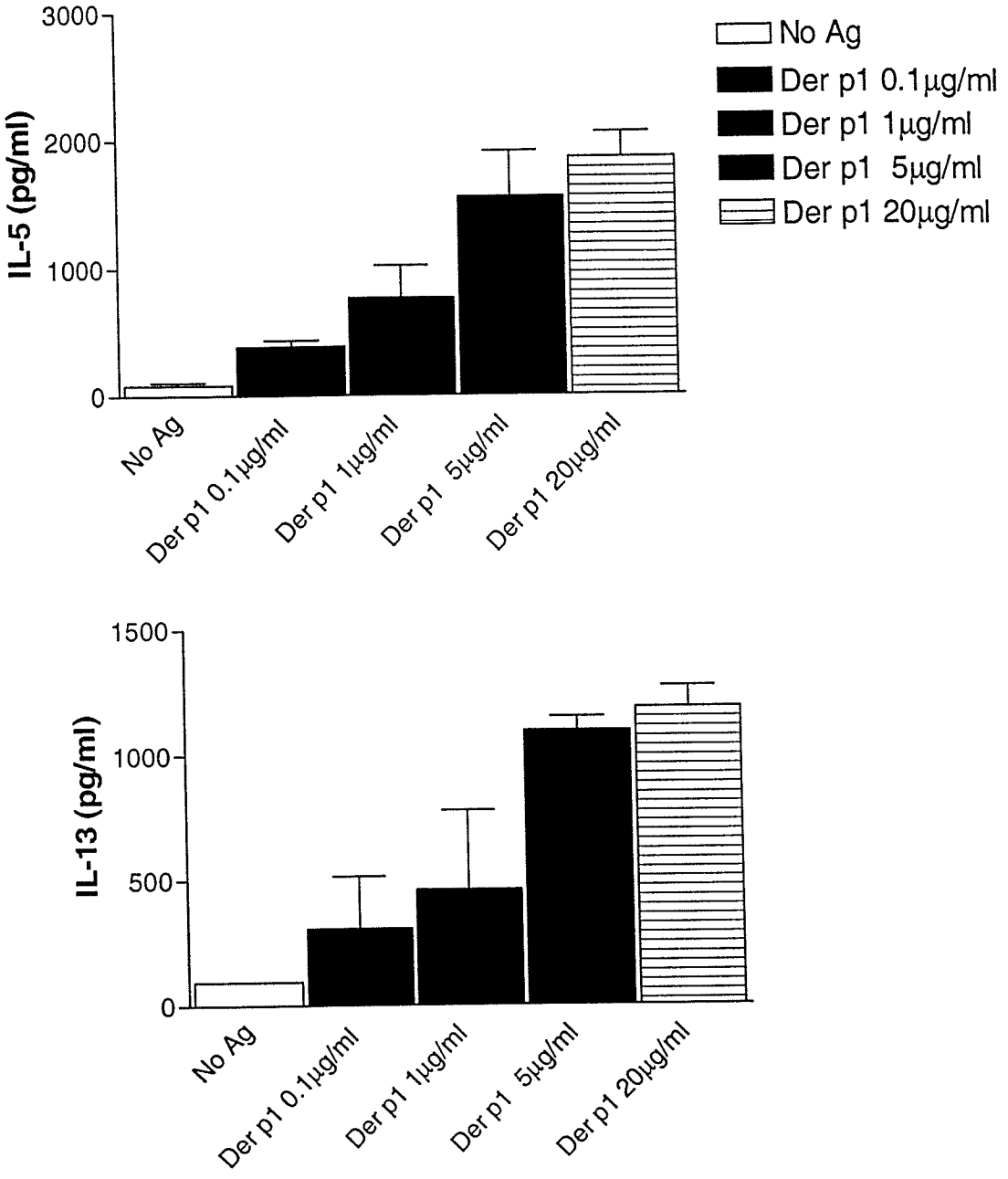

Figure 4. 5µg/ml was optimal Der p1 concentration for restimulation in vitro. C57/B16 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone or with Der p1 at 0.1, 1, 5, 20 µg/ml respectively. Culture supernatant were harvested for analysis of IL-5 and IL-13 at 72hrs. The results are 2 representative experiments, 6 mice.

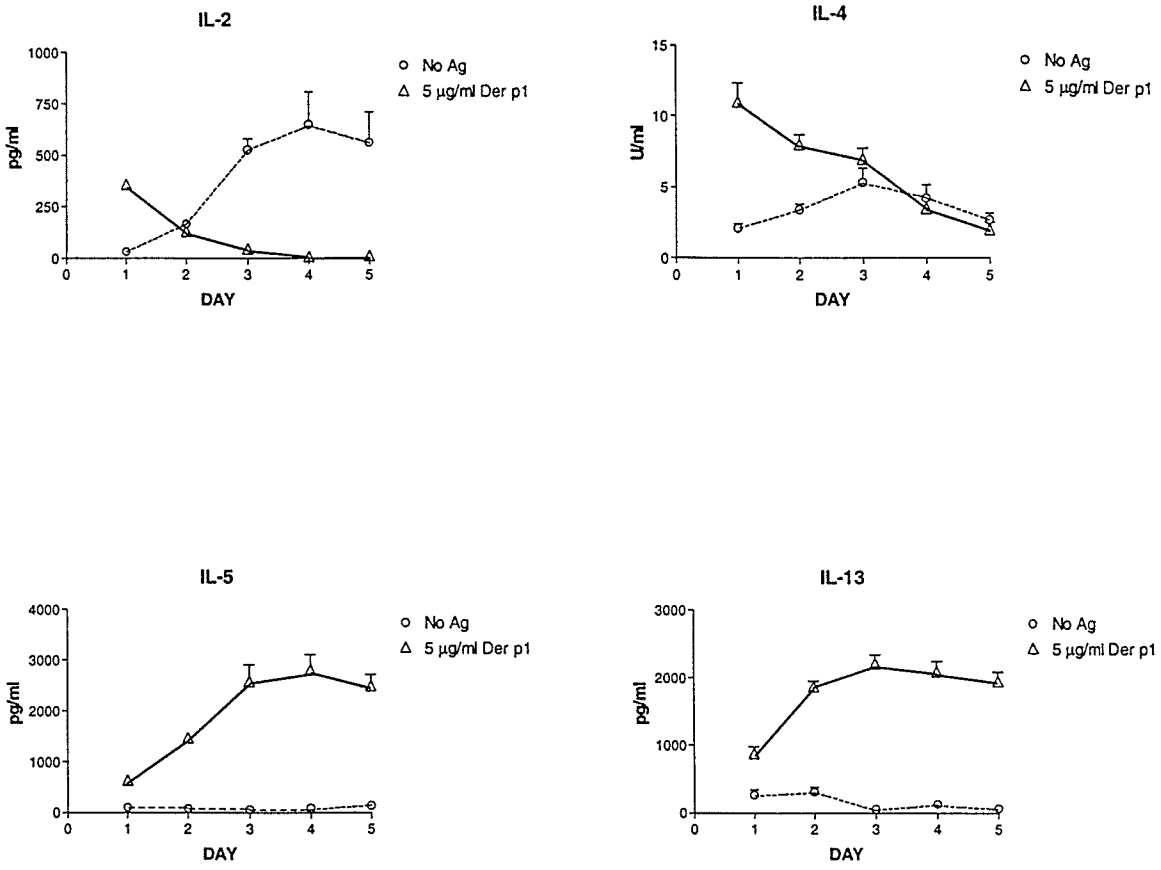

Figure 5. Kinetics of cytokine responses in vitro. C57/B16 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone or with Der p1 at 5 µg/ml. Culture supernatant were harvested on day 1, 2, 3, 4, 5 time points respectively after setting up cell culture. Supernatant cytokine (IL-2, IL-4, IL-5, IL-13) levels were measured. The results are pooled from 2 representative experiments, 8 mice.

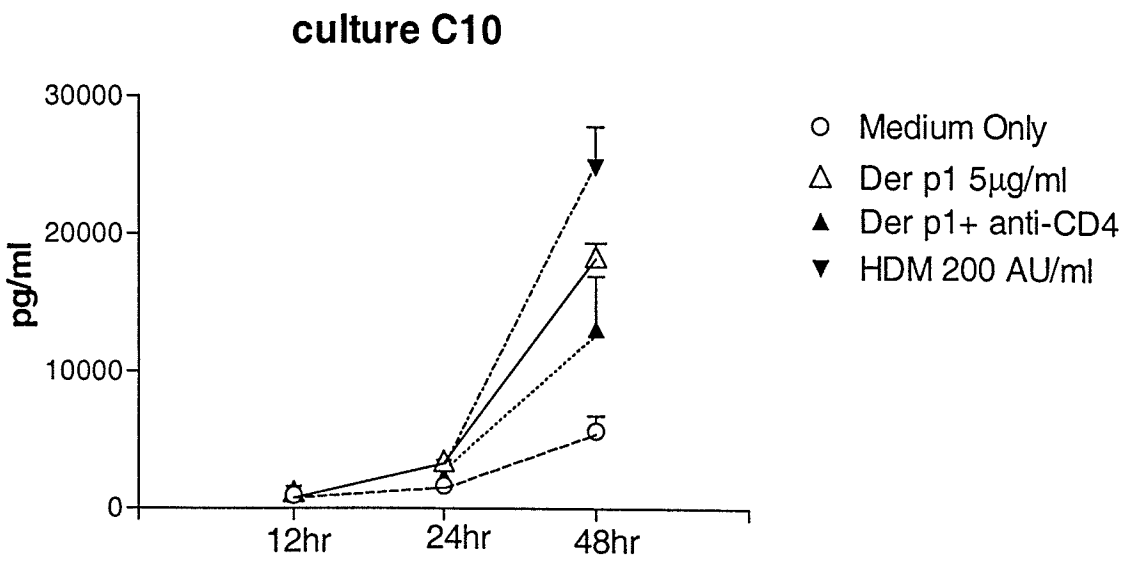

Figure 6. Both Der p1 and crude HDM stimulate Ag-specific C10 production in primary culture. C57/Bl 6 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 µg/ml, with 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant were harvested on 12, 24, 48 hr after setting up cell culture. Supernatant C10 levels were measured. The results were one representative experiment of 4 experiments did, 4 mice.

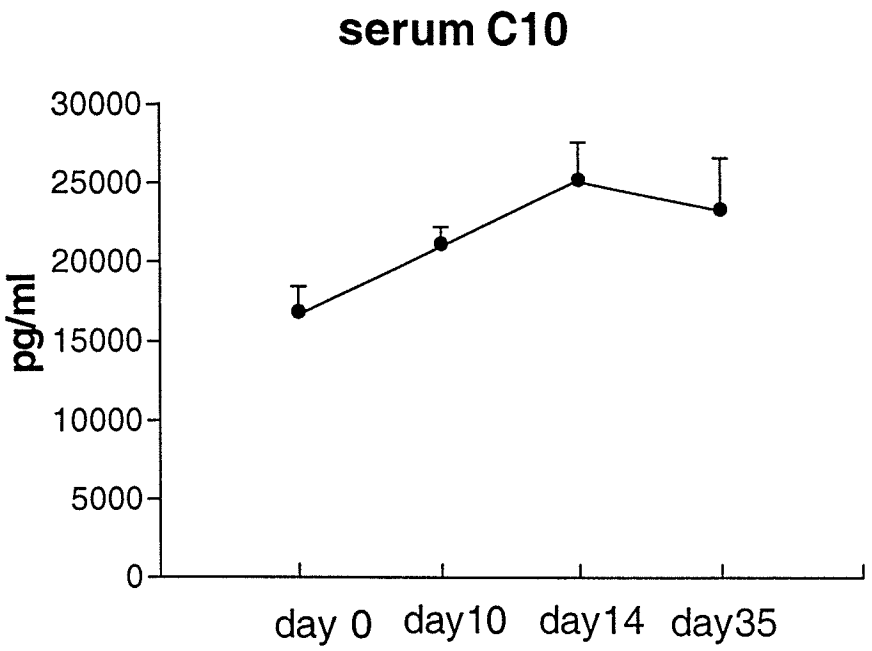

Figure 7. Serum chemokine C10 levels significantly augment following Der p1 immunization. To measure C10 expression in vivo, C57/Bl6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0 and boosted on day 28. Mice were bleed on day 0, 10, 14 and 35. Serum C10 levels were quantified. The results are pooled from four representative experiments, 13 mice.

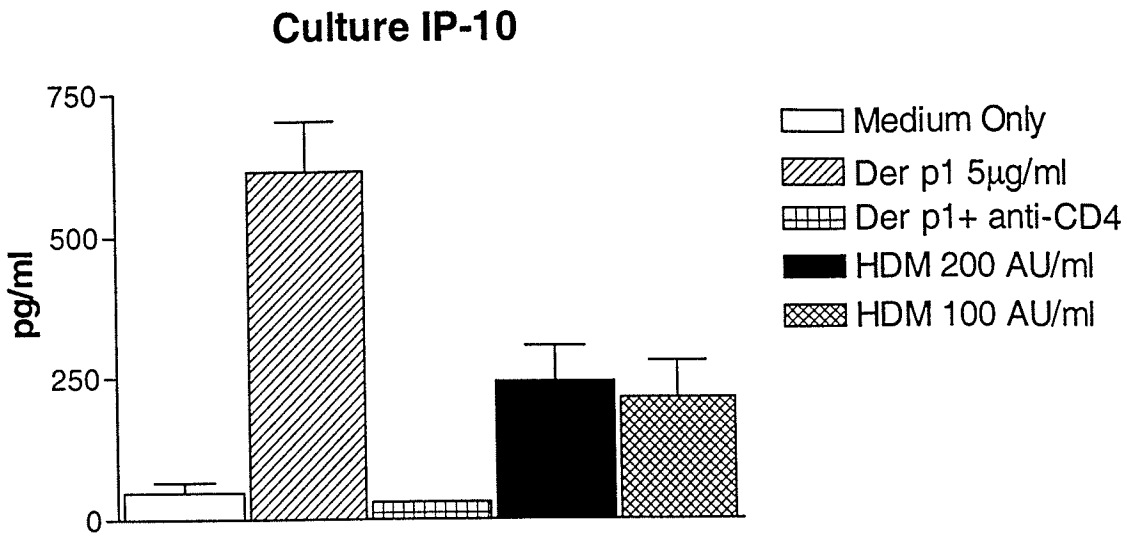

Figure 8. Der p1 elicits stronger IP-10 expression in primary culture than does HDM. C57/B16 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with Der p1 at 5 µg/ml, with 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant were harvested on 72 hr after setting up cell culture. Supernatant murine IP-10 levels were measured. The results are one representative experiment of 3 experiments did, 4 mice.

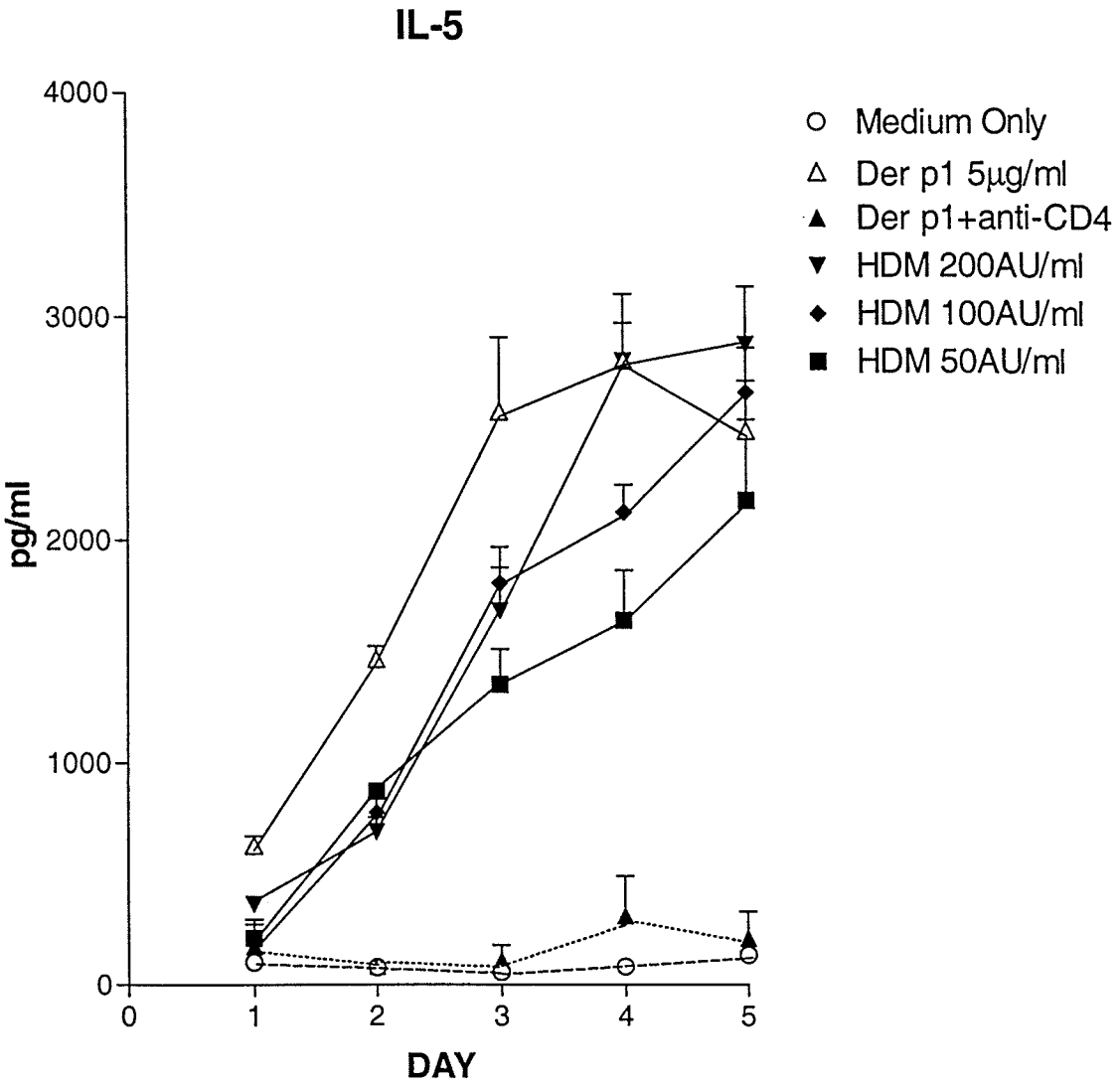

Figure 9. Der p1 and crude HDM stimulate IL-5 production with similar kinetics and intensity. C57/Bl6 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with 5 µg/ml Der p1, with 100 or 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant were harvested 24, 48, 72, 96, 120 hrs after setting up cell culture. Supernatant IL-5 levels were measured. The results were pooled from 2 representative experiments, 8 mice.

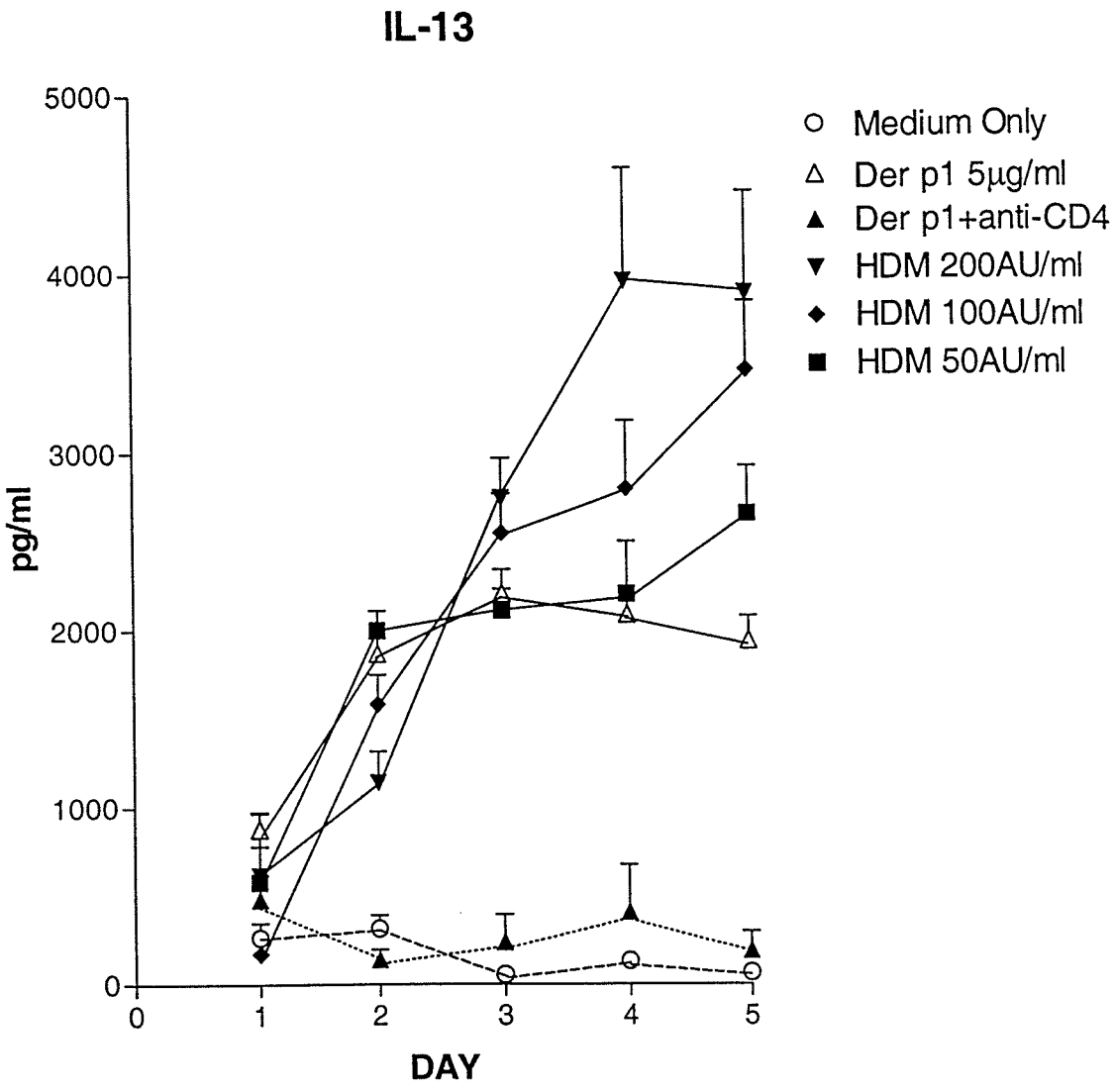

Figure 10. Der p1 and crude HDM stimulate IL-13 production with similar kinetics and intensity. C57/B16 mice were immunized with 2 µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrifaced on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with 5 µg/ml Der p1, with 100 or 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant were harvested on 24, 48, 72, 96, 120 hrs after setting up cell culture. Supernatant IL-13 levels were measured. The results were 2 representative experiments, 8 mice.

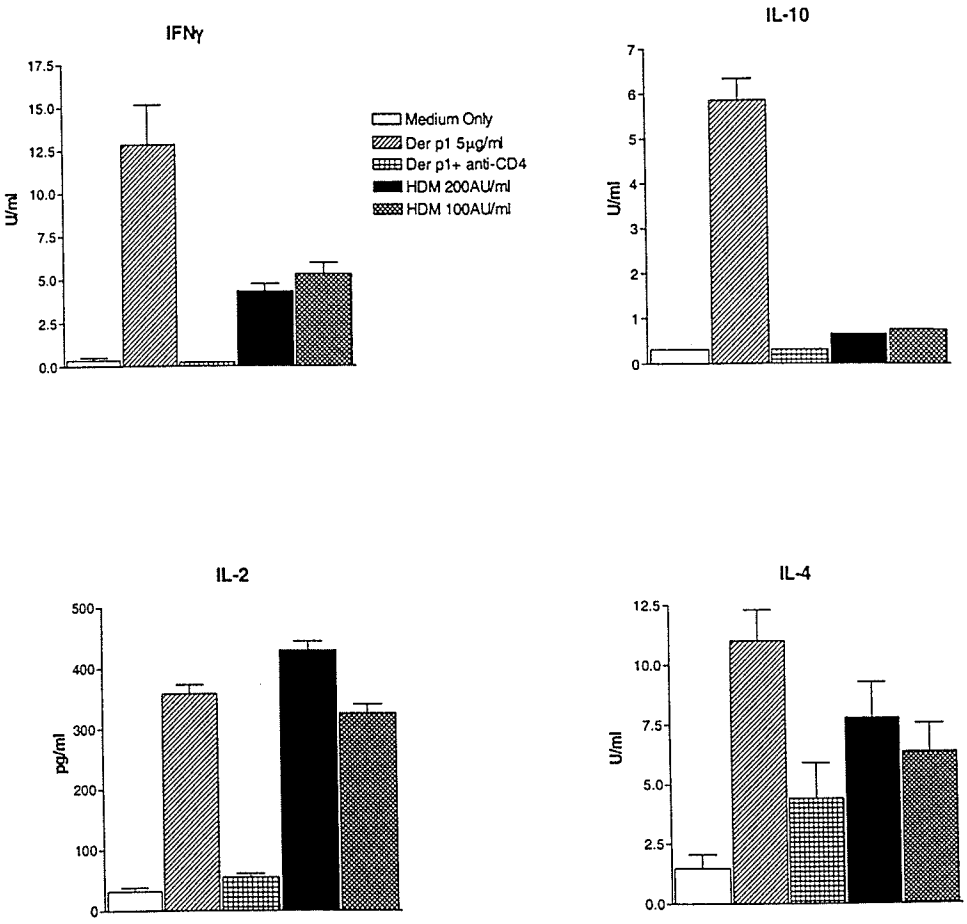

Figure 11. Der p1 elicits stronger IFNγ, IL-10 and similar IL-2, IL-4 expression than does crude HDM. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were sacrificed on day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml alone, with 5 μg/ml Der p1, with 100 or 200 AU/ml HDM or with anti-CD4 at ¼ (v/v) respectively. Culture supernatant were harvested on 24, 48, 72, 96, 120 hrs after setting up cell culture. Supernatant IFN γ, IL-10, IL-2 and IL-4 levels were measured at optimal time points, i.e. 24 hrs for IL-2 and IL-4, 72 hrs for IFNγ, and 96 hrs for IL-10. The results were pooled from 2 representative experiments, 8 mice.

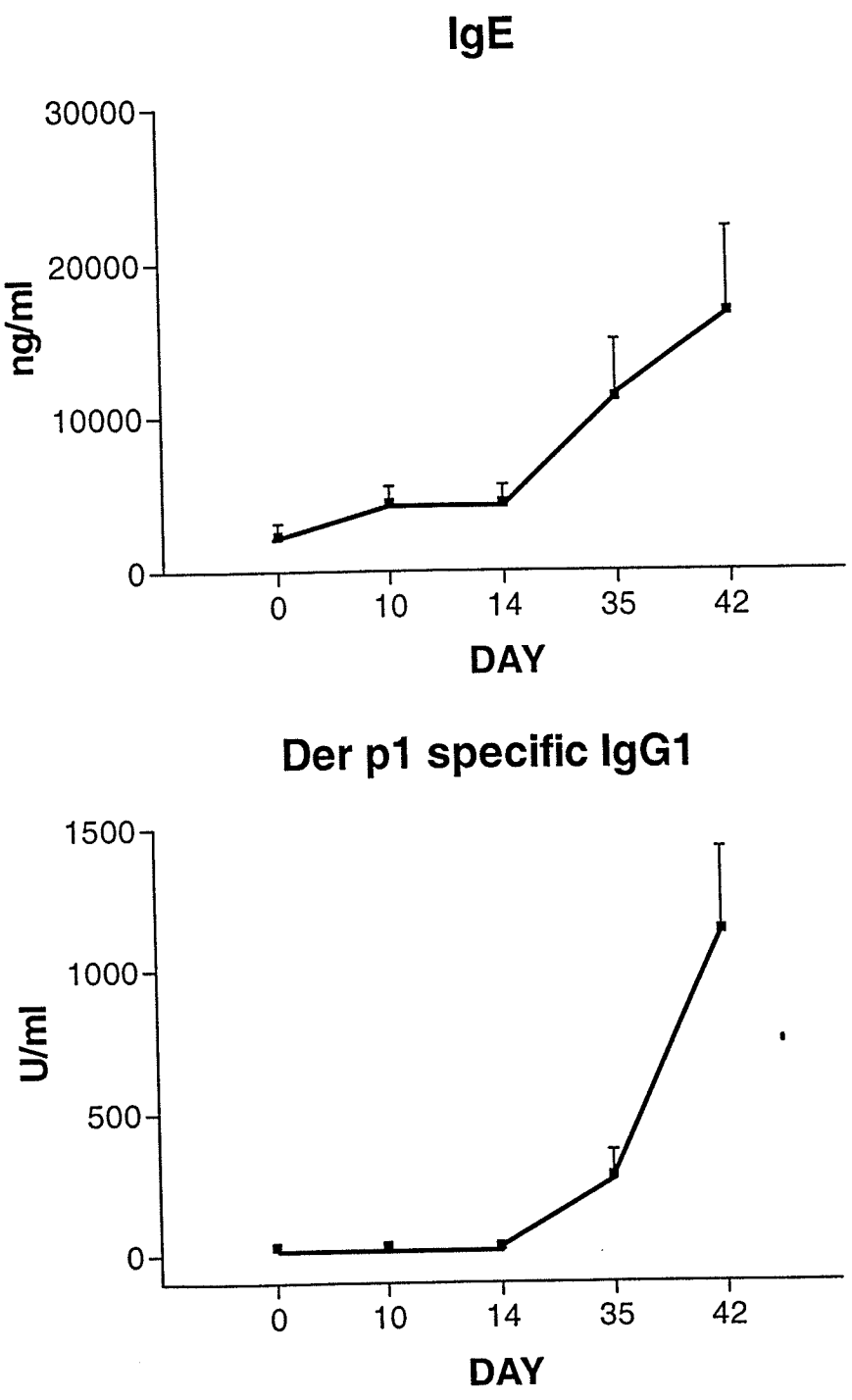

Figure 12. Der p1 induced serum IgE and IgG1 responses. C57/Bl 6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0 and boosted on day 28. Mice were bleed on day 0, 10, 14, 35 and 42. Serum total IgE and Der p1-specific IgG1 levels were quantified. The results are one representative experiments of 8 performed totally, 8 mice in this representative experiment.

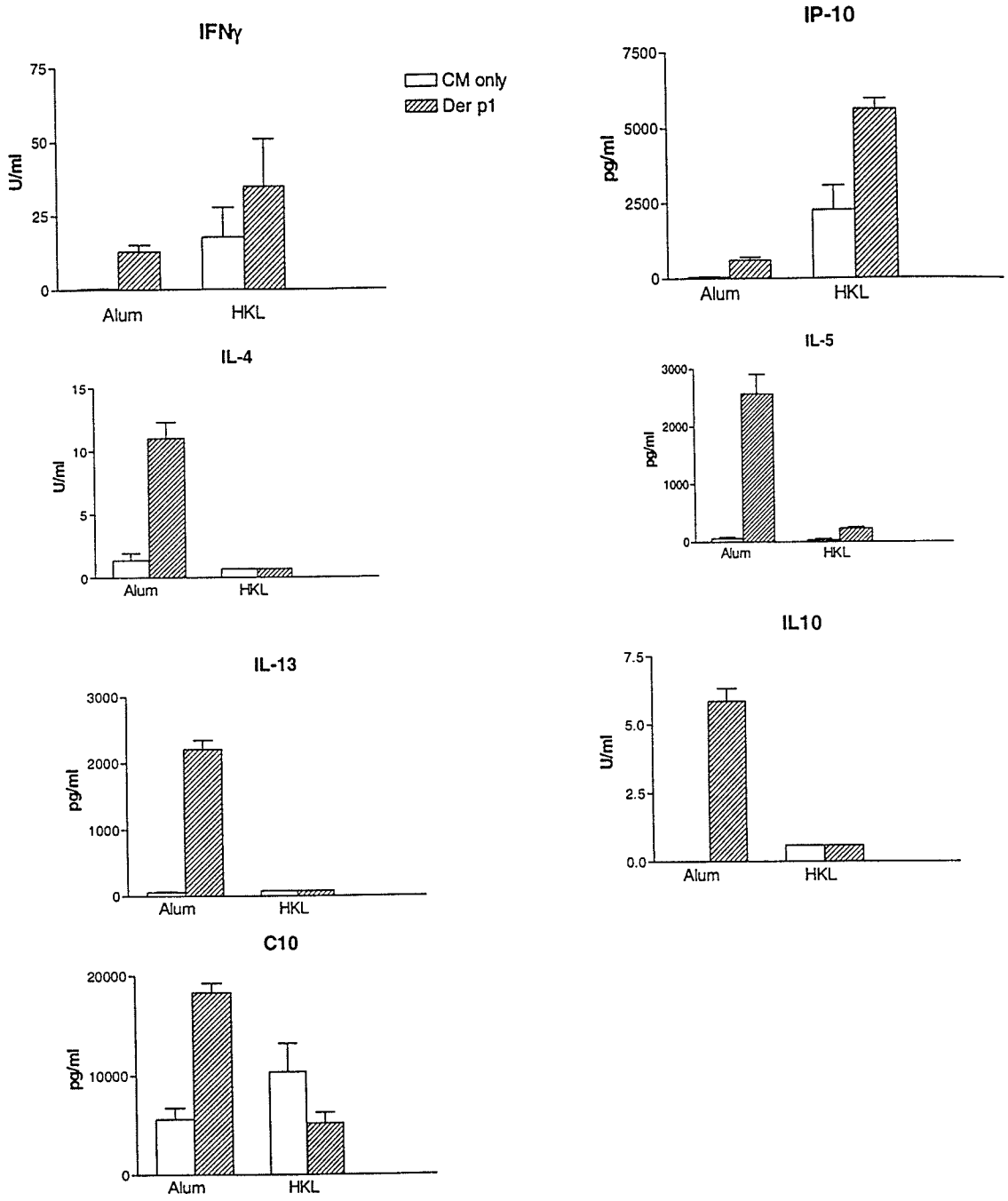

FIGURE 13. Alum and HKL adjuvant lead to Th2 and Th1 responses against Der p1 respectively. C57 Bl/6 mice were immunized with 2 μg Der p1 adsorbed onto either 2 mg $Al(OH)_3$ adjuvant (Alum group) or $1X10^7$ in HKL/IFA (HKL group) given i.p. on day 0. Mice were killed at day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml (200 μl/well) alone or with predetermined optimal concentration of Der p1 (5 μg/ml). Culture supernatants were harvested for analysis of chemokines mIP-10 (72hr), C10 (48hr) and cytokines IL-4 (24hr), IL-2 (24hr), IFN-γ (72hr), IL-5 (72hr), IL-13 (72hr) and IL-10 (96hr). The results are the means +/- SE of 2 experiments, 8 mice.

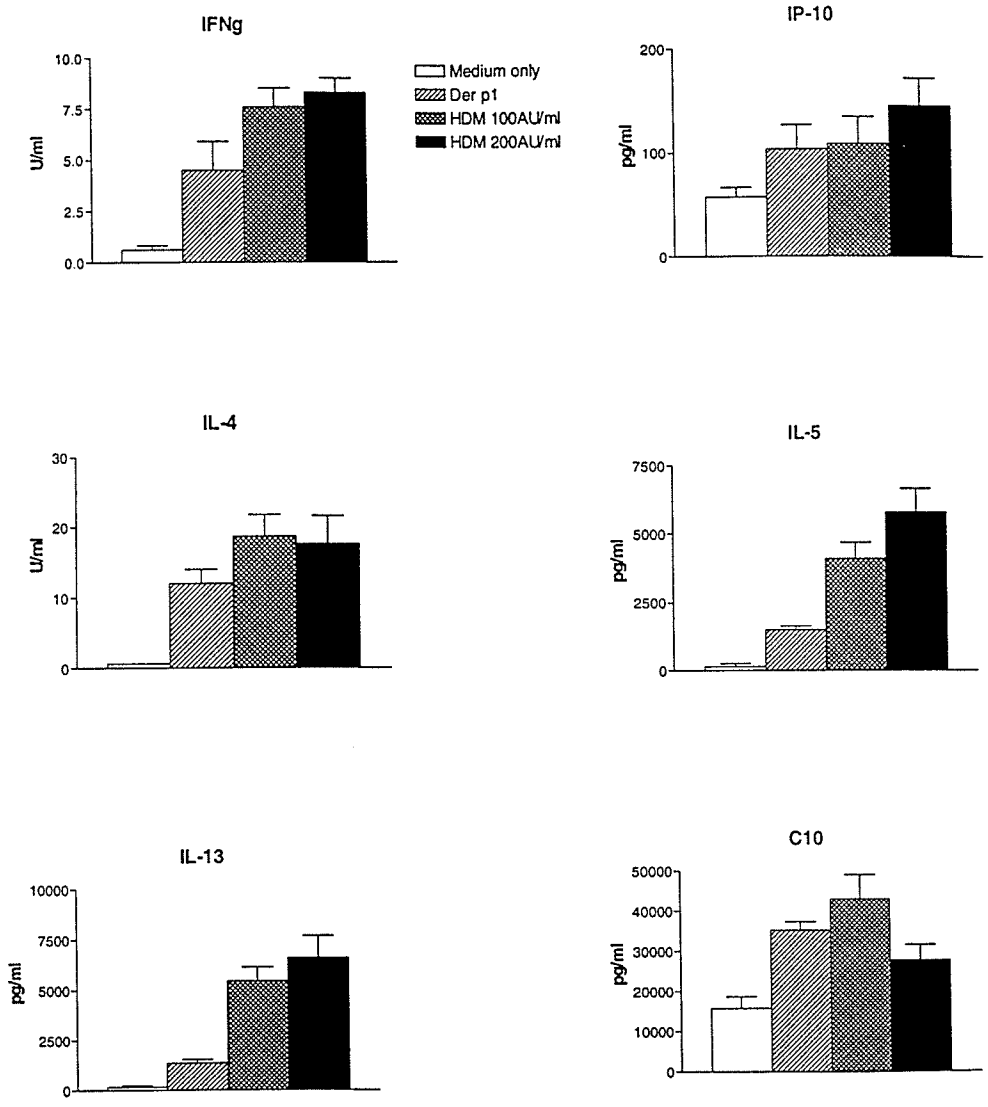

FIGURE 14. Response against rDer p1 reflects substantial part of response against whole body extract HDM following primary HDM immunization. C57 Bl/6 mice were immunized with 100 AU crude HDM adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed at day 6. Spleen cell suspensions were cultured at $7.5X10^6$ cells/ml (200 µl/well) alone or with Der p1 (5µg/ml), crude HDM (100 and 200 AU/ml) respectively. Culture supernatants were harvested for analysis of chemokines mIP-10 (72hr), C10 (48hr) and cytokines IL-4 (24hr), IFN-γ (72hr), IL-5 (72hr), IL-13 (72hr). The results is from one representative experiments, 4 mice.

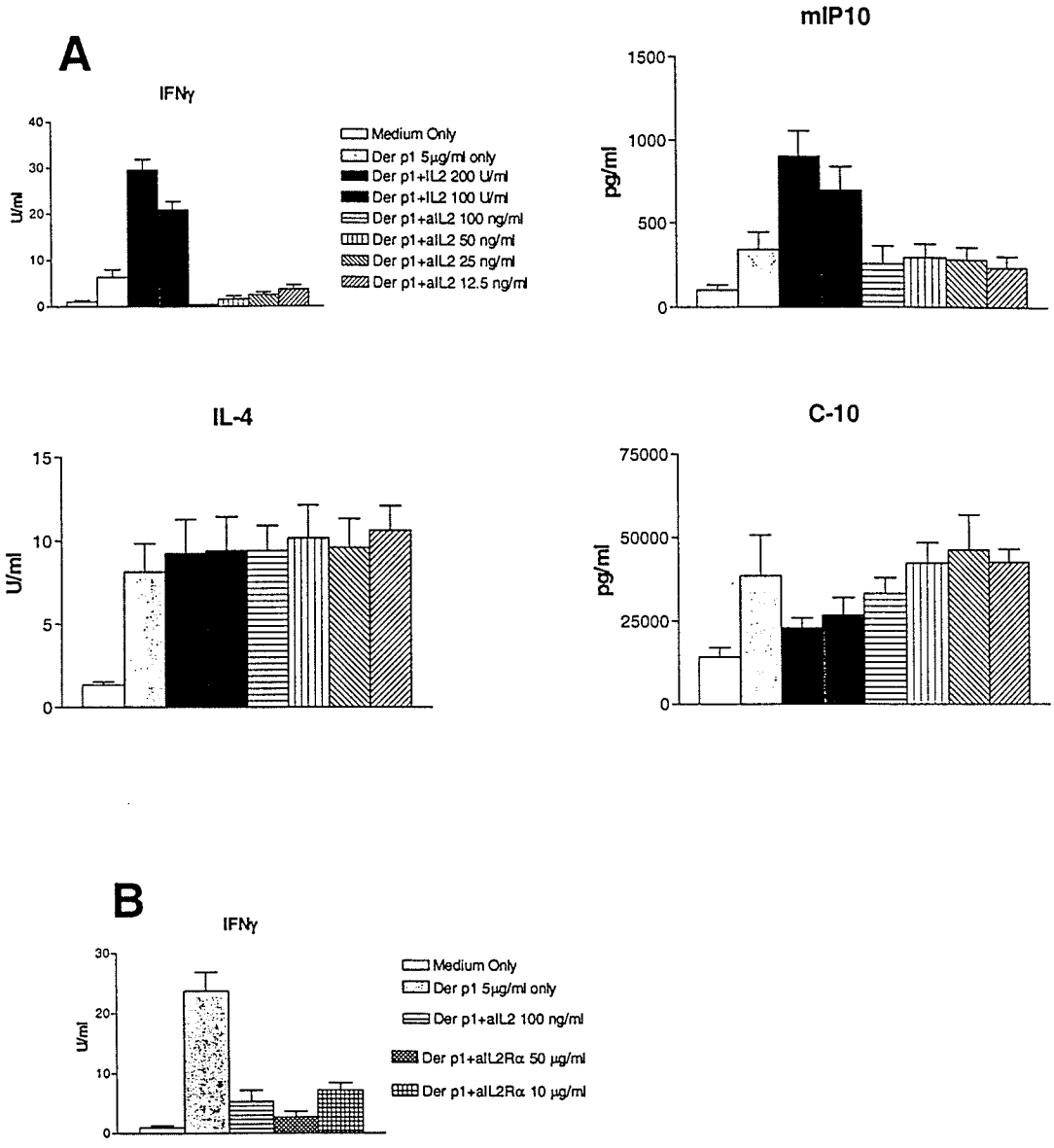

Figure 15. Th1, but not Th2, cytokine and chemokine responses are highly IL-2 dependent. C57 Bl/6 mice were immunized with 2μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed at day 6. Spleen cells were cultured with Der p1 in the presence of neutralising anti-IL2 Ab, anti-IL2Ra Ab (**Panel B**), irrelevant control Ab, or rIL2. Culture supernatants were harvested for analysis of chemokines mIP-10 (72hr), C10 (48hr) and cytokines IL-4 (24hr), IFNγ (72hr). The means +/- SE of two representative experiments were shown. 4 mice for Panel A, 3 mice for Panel B.

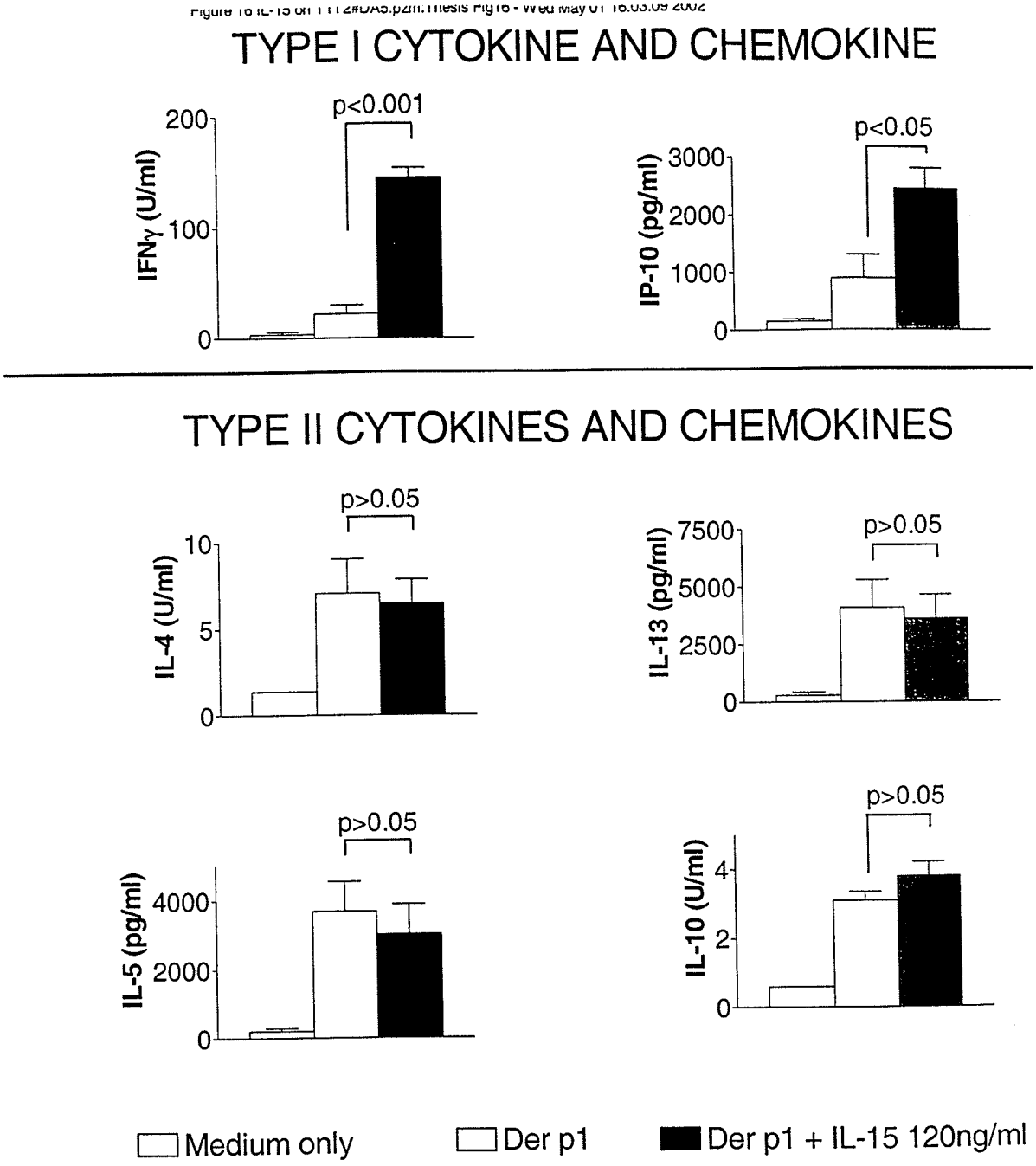

Figure 16. IL-15 selectively enhances Th1 but not Th2 responses. C57 Bl/6 mice were immunized with 2μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed at day 6. Spleen cells were cultured with Der p1 in the presence of rIL-15 (120 ng/ml). Culture supernatants were harvested for analysis of IP-10 (72hr), IL-4 (24hr), IFNγ (72hr), IL-5 (72hr), IL-13 (72hr) and IL-10 (96hr). The results are the means +/- SE of one representative experiment, 4 mice.

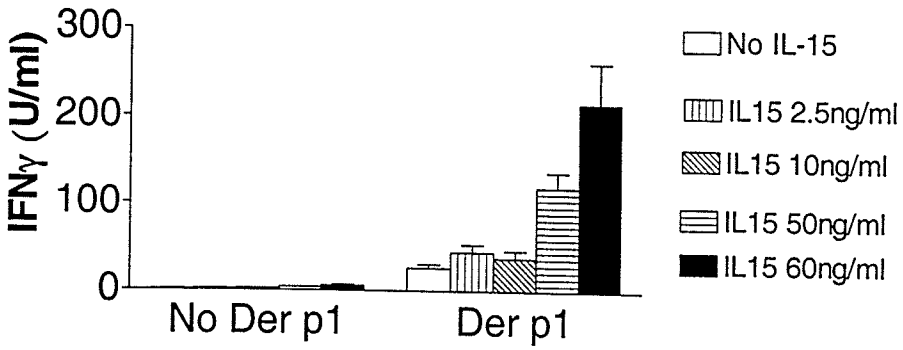

Figure 17. IL-15 augments the Ag-specific IFNγ response in a dose dependent manner. C57 Bl/6 mice were immunized with 2μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the presence of rIL-15 at 2.5, 10, 50 or 60 ng/ml. Culture supernatants were harvested for analysis of IFN-γ at 72hr. The results are the means +/- SE of two experiment pooled, 7 mice.

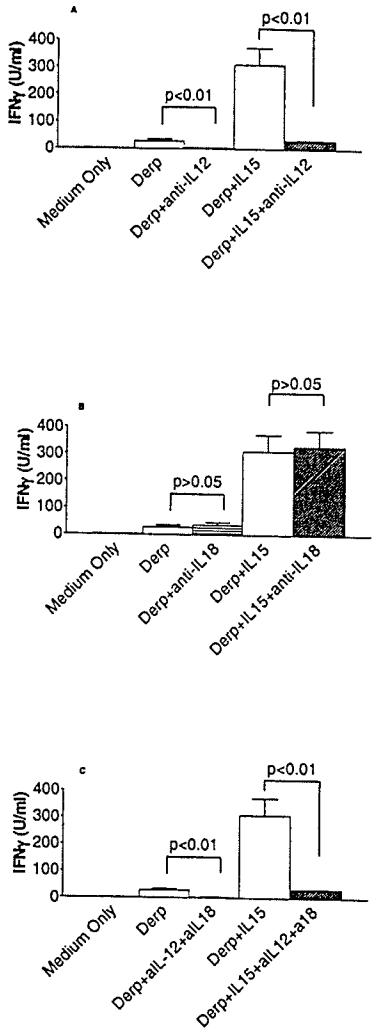

Figure 18. Endogenous IL-12, but not IL-18, is required for the IFNγ responses against Der p1 and enhanced by IL-15. C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml) with/without anti-IL12, anti-IL18, or both. Culture supernatants were harvested for analysis of IFNγ at 72 hr. The results were the means +/- SE of one representative experiment, 4 mice.

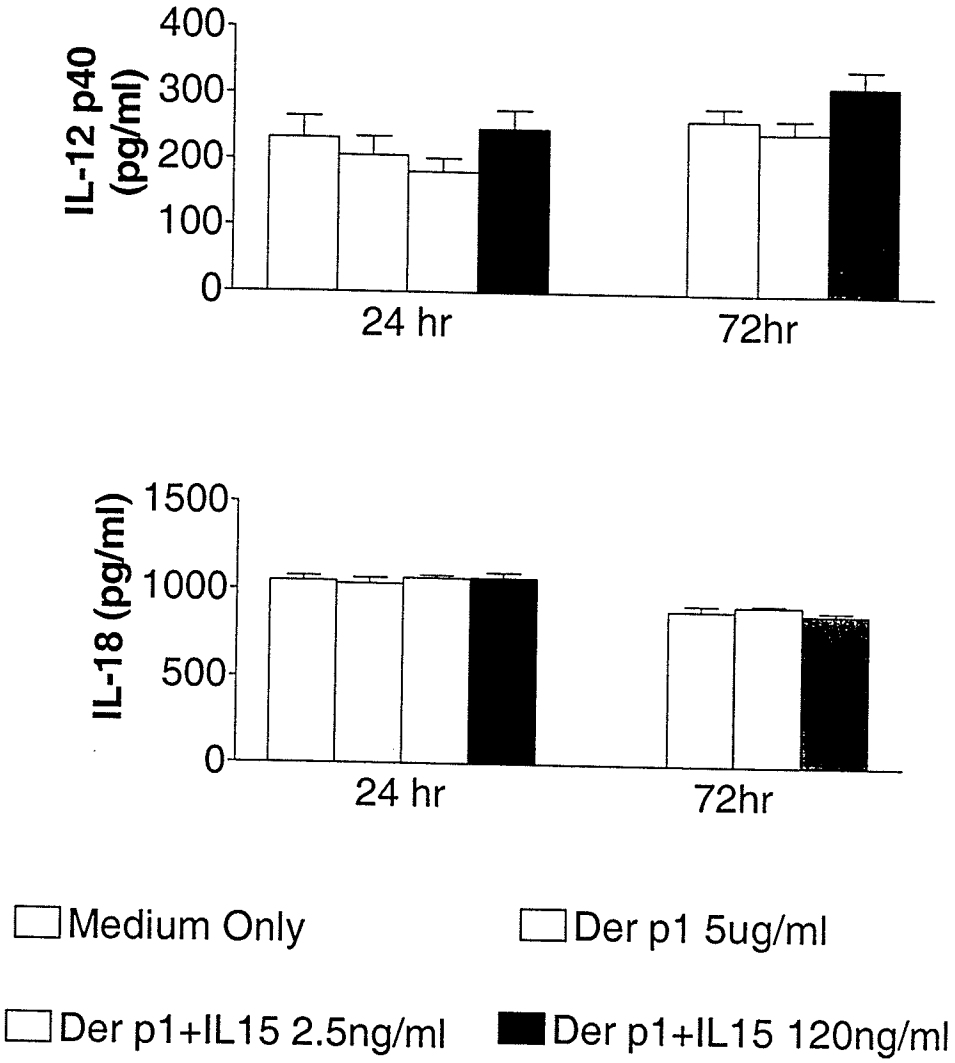

Figure 19. IL-15 does not directly increase IL-12 or IL-18 levels. C57 Bl/6 mice were immunized with 2 μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (2.5 or 120 ng/ml). After 24 and 72 hours, culture supernatants were harvested for analysis of IL-12 and IL-18. The results are the means +/- SE of one representative experiment, 4 mice.

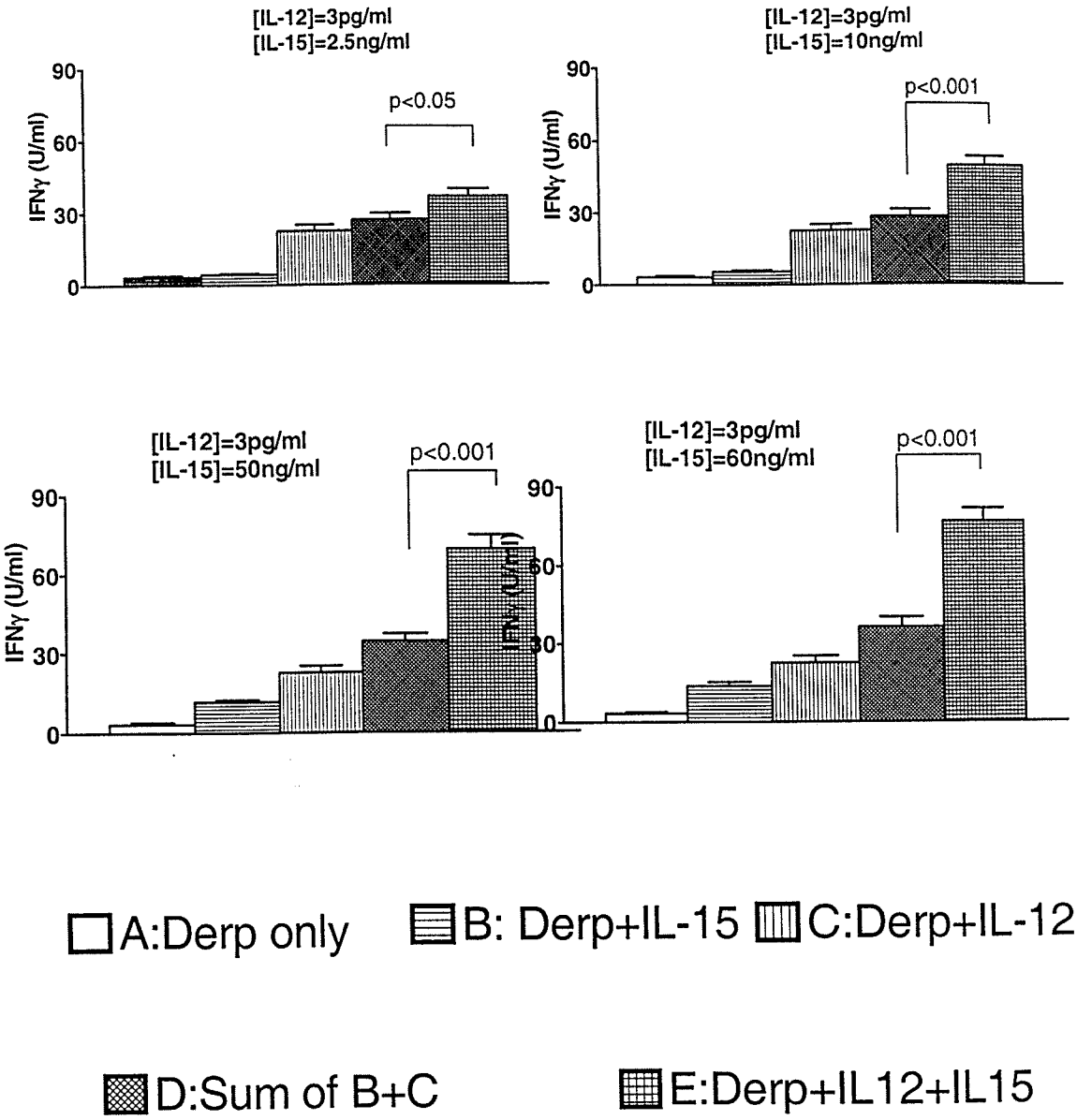

Figure 20. IL-15 increases Ag-specific IL-12 *responsiveness* in IL-12 knockout mice. IL-12 deficient mice were immunized with 2μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-12 (3pg/ml) and/or rIL-15 (2.5, 10, 50 and 60 ng/ml). After 72 hours, culture supernatants were harvested for analysis of IFNg. The results are the means +/- SE of one representative experiment, 4 mice.

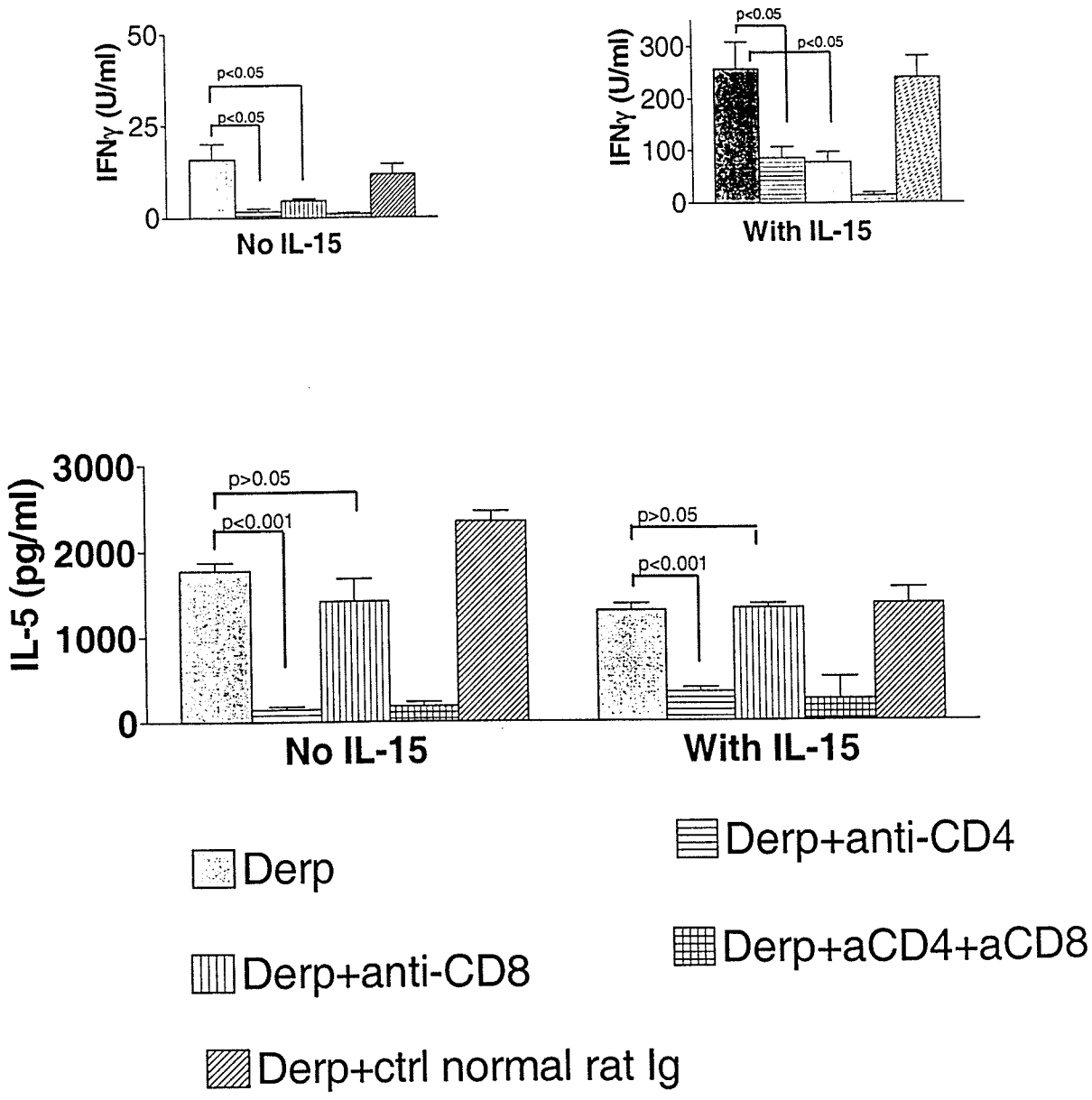

Figure 21. IFNγ response depends on both CD4 and CD8 T cells, but IL-5 response only depends on CD4 T cells. C57 Bl/6 mice were immunized with 2 μg Der p1 adsorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml). Anti-CD4 (1/4 v/v), anti-CD8 (10 μg/ml) or both were added to the groups indicated. 72 hours culture supernatants were for analysis of IFNγ and IL-5 levels. The results are the means +/- SE of one representative experiment of 4 performed, 4 mice.

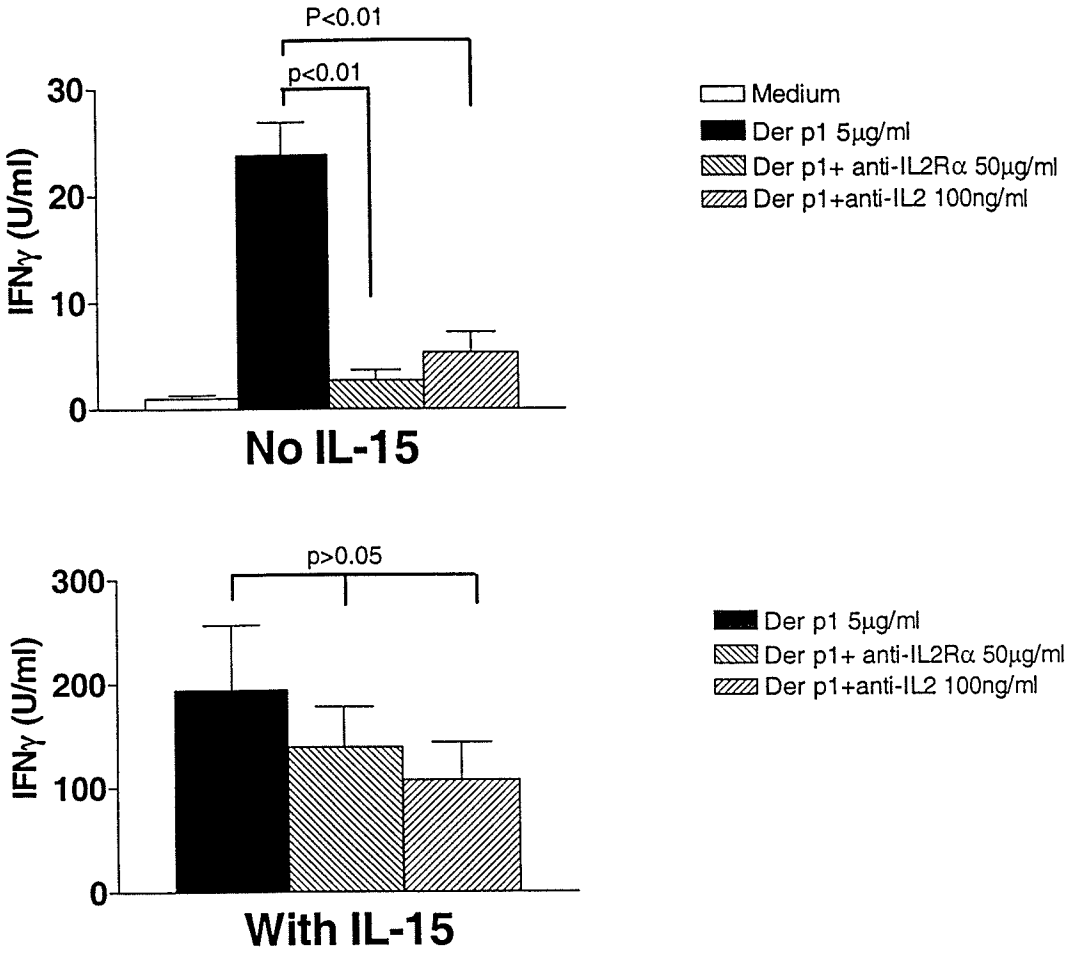

Figure 22. IL-2 is not required for IL-15 to enhance IFN γ. C57 Bl/6 mice were immunized with 2 μg Der p1 adsorbed onto 2 mg Al(OH)3 adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml). Anti-IL2Rα (50 μg/ml) or anti-IL-2 (100 ng/ml) were added to the groups indicated. 72 hours culture supernatants were for analysis of IFN γ levels. The results are the means +/- SE of one representative experiment, 3 mice.

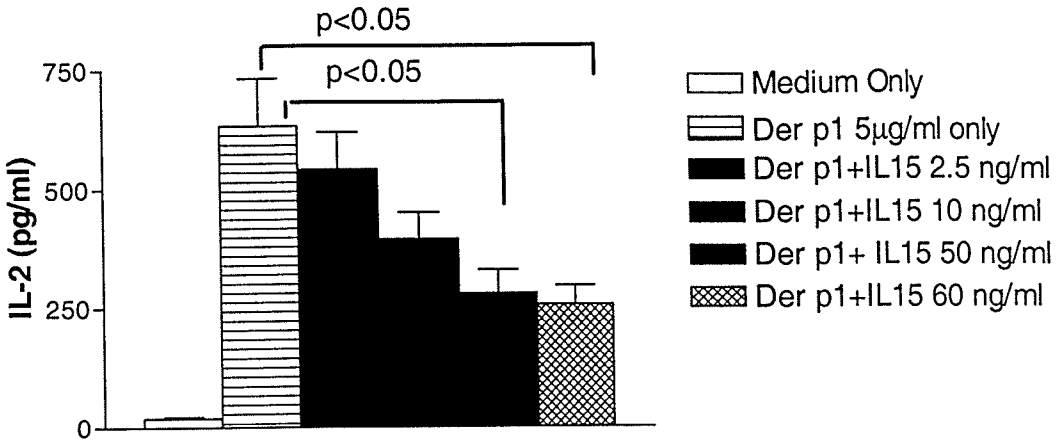

Figure 23. Exogenous IL-15 decreases supernatant IL-2 level in a dose dependent manner. C57 Bl/6 mice were immunized with 2µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (2.5, 10, 50, 60 ng/ml). 24 hours culture supernatants were harvested for analysis of IL-2 levels. The results are the means +/- SE of one representative experiment, 3 mice.

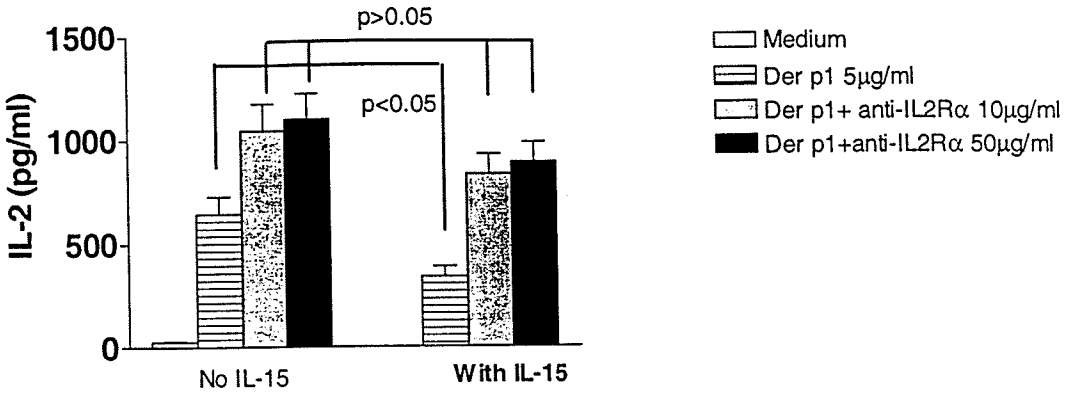

Figure 24. IL-15 does not directly inhibit IL-2 production. C57 Bl/6 mice were immunized with 2µg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-15 (60 ng/ml). Anti-IL2Rα (10 or 50 µg/ml) was added to the groups indicated. 24 hours culture supernatants were harvested for analysis of IL-2 levels. The results are the means +/- SE of one representative experiment, 3 mice.

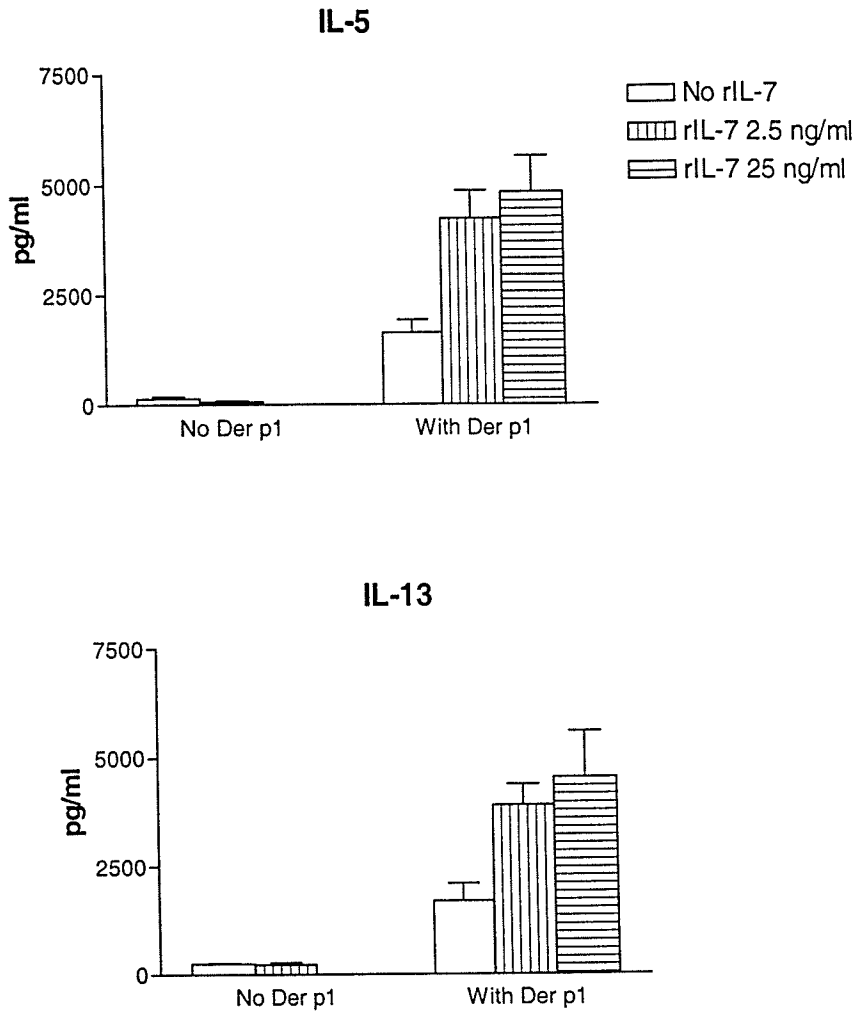

Figure 25. rIL-7 augments Ag-specific IL-5 and IL-13 response. C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the presence of rIL-7 at 2.5 or 25 ng/ml. Culture supernatants were harvested for analysis of IL-5 and IL-13 at 72 hr. The results are the means +/- SE of two representative experiments, 7 mice.

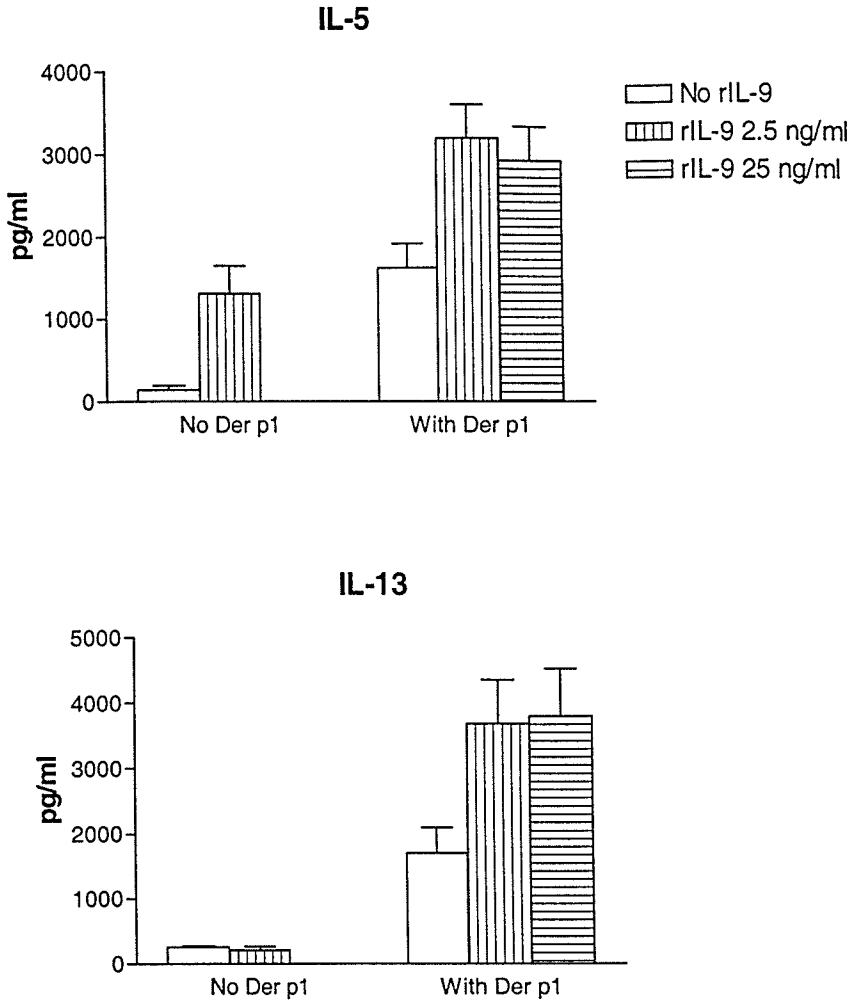

Figure 26. rIL-9 augments Ag-specific IL-5 and IL-13 response. C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the presence of rIL-9 at 2.5 or 25 ng/ml. Culture supernatants were harvested for analysis of IL-5 and IL-13 at 72 hr. The results are the means +/- SE of two representative experiments, 7 mice.

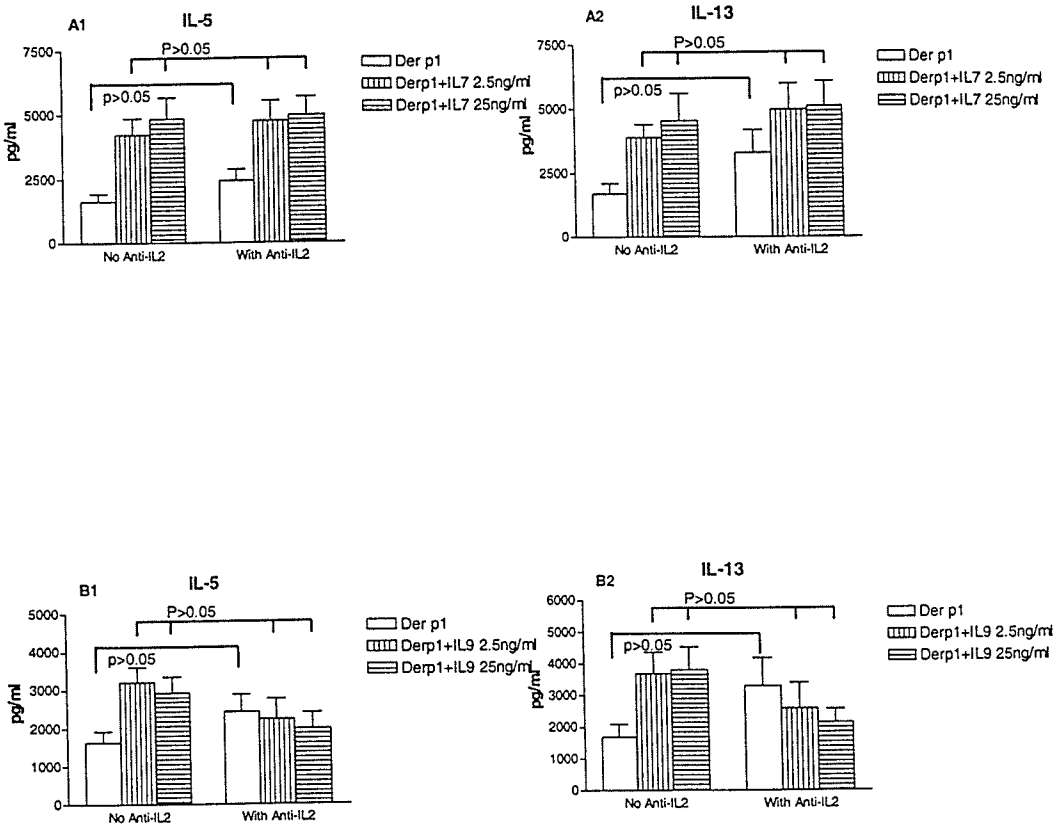

Figure 27. IL-2 has no effect on IL-5 and IL-13 expression enhanced by rIL-7 and rIL-9. C57Bl/6 mice were immunized with 2 μg Der p1 absorbed onto 2 mg $Al(OH)_3$ adjuvant given i.p. on day 0. Mice were killed on day 6. Spleen cells were cultured with Der p1 in the absence or presence of rIL-7 (2.5 or 25 ng/ml, **Panel A**) or rIL-9 (2.5 or 25 ng/ml, **Panel B**). Anti-IL2 (100 ng/ml) were added to the groups indicated. Culture supernatants were harvested for analysis of IL-5 and IL-13 at 72 hr. The results are the means +/- SE of one representative experiments, 3 mice.

DISCUSSION

1. Novelty and significance of the murine allergic model against rDer p1

1.1 Broader picture of Th1 vs. Th2 cytokine and antibody responses.

As stated in the introduction, Der p1 has been found as a major allergen of house dust mite. Since then, some studies tried to set up allergy model with Der p1. Until now, most such models are in vivo models which focus on the airway or pulmonary changes after Der p1 immunization. Clarke et al found that small amount of Der p1 (1 μg) used in both primary sensitization subcutaneously and intranasal challenge could produce an extensive pulmonary eosinophilic inflammation similar to those inflammation found in human asthma (Clarke, Thomas et al. 1999). But he found the degree of inflammation was not related to total sera IgE titer and was indeed produced in its absence. Tournoy, Kao, et al independently found Der p1 sensitization induced development of AHR, eosinophil and lymphocyte infiltration in airway, lung and BALF accompanied by allergen specific IgE (Kao, Wang et al. 2000; Tournoy, Kips et al. 2000). Especially Lee observed Der p1-specific IgG1 and IgE response, BAL eosinophilia accompanied by enhanced IL-5 production after Der p1 immunization (Lee, Fu et al. 1999). Among those in vivo models, the morphology of airway and lung changes were carefully studied, and in some studies serum allergen specific IgE and IgG1 were also observed, so the concept of "allergy" model are defined by local asthma-like inflammation, systemic IgE elevation and perhaps clinical symptoms. Because it is widely believed that asthma or allergy is related to Th2-biased immunity against allergen, we need investigate allergen-specific Th1 vs. Th2 cytokine responses to confirm that the immunological nature of Der p1

hypersensitivity. For this purpose, in vitro model is required to observe the T cell induced allergen-specific cytokine responses.

Thomas group first developed in vitro Der p1 model of allergy. After intranasal inoculation of Der p1 every 3-4 days from day 0 to day 29, they set up in vitro assays of Der p1-specific cytokine production from spleen cells, draining LN and non-draining node. They found that both Th1 (represented by IL-2 response) and Th2 (represented by IL-4 response) were evident in spleen cell and draining LN culture, with the Th1 response occurring earlier (day 7) and the Th2 following (day 21) after repeated immunization (O'Brien, Ooi et al. 1996). But their studies were based on IL-2 (Th1), IL-4 (Th2), IL-3/GM-CSF (overall marker of Th1 and Th2) and did not include IFNγ, IL-5, IL-13 and other Th1/Th2 cytokines which is considered important in asthma pathogenesis (see introduction). Chiang group was another group who carried out research on in vitro Der p1-specific cytokine responses, but the cytokines they studied were limited to IFNγ and IL-5 (Lee, Fu et al. 1999).

To focus on immunologic features of Der p1 induced allergy, we quantified multiple immunologic responses including ex vivo cytokines and chemokines responses as well as in vivo Ab responses. Each of the indicators can be divided into type 1 and type 2, giving us a broader picture of type 1 vs. type 2 responses.

As in the above models, we found that very low dose of Der p1 (2 μg/mouse for immunization, 6.7 X 10^{-7}μg/ spleen cells for restimulation) is optimal to set up this

allergic model. The restimulation concentration is especially lower compared with usual OVA concentration for restimulation (Yang and HayGlass 1993). So it is concluded that Der p1 is an effective allergen to stimulate allergic responses.

We found that the 6th day after immunization is the optimal date for allergen restimulation in vitro in order to elicit optimal allergen-specific cytokine responses. 24hr after restimulation, Ag-specific Th2 cytokines IL-4 production first peaked, then IL-5, IL-13 reached plateau at 72 hrs and IL-10 peaked last at 96 hours. Th2 chemokine C10 responded later than IL-4 did and still increased significantly after 48 hours. Th1 cytokine IFNγ and IP-10 reached plateau at 72 hours, which is obviously late because IL-12 level increased as early as 9 hours and already peaked at 24 hours. We spend time to carefully observe Ag-specific IFNγ responses and we found that IFNγ was hard to detect at 24 and 48 hours but it quickly peaked at 72 hours. This finding was different from Thomas finding above. Th2 cytokine and chemokine not only come earlier, but also be stronger than Th1 cytokine and chemokine, and augmented serum C10 and IgG1 titer are found after immunizations while IP-10 and IgG2a are undetectable or unchanged. So our model revealed that Der p1 (alum) stimulated substantial type2 cytokine/chemokine/Ab responses with lower capacity to generate type 1 responses, parallel those seen in human dust mite allergic subjects.

From literature review, chemokines were reported to have a role in allergic diseases (Lukacs, Oliveira et al. 1999). According to these reports, MCP-1, RANTES, and MIP-1 are major C-C chemokines found in bronchoalveolar lavage (BAL) from

asthmatics (Alam, York et al. 1996; Holgate, Bodey et al. 1997). The main function of these C-C chemokines that were measured was recruitment of eosinophils into the airways (Lukacs, Standiford et al. 1996; Campbell, Kunkel et al. 1998; Gonzalo, Lloyd et al. 1998). It was also found that blocking MCP-1 and RANTES significantly attenuates airway hyperresponsiveness (Gonzalo, Lloyd et al. 1998). The biological functions of chemokines were not limited in chemotaxis. MIP-1 contributed in the pulmonary fibrotic responses after mononuclear phagocyte accumulation(Smith, Strieter et al. 1995; Smith 1996). Human IP-10 in selectively promotes (20-30 fold) IFNγ responses. Thus it is valuable to study allergen-specific chemokine responses in mouse allergic model.

For this purpose, we found stronger Th2 C10 responses and weaker Th1 IP-10 responses in vitro and in vivo. Especially we found elevated serum C10 levels after immunizations. This is the first serum C10 report in allergic mouse model, and it provides C10 the possibility to be used as a convenient immunologic index for dust mite allergy.

1.2 Th1 dominated immunity model vs. Th2 dominated immunity model

As discussed above, we found that Der p1 immunized with alum could stimulate strong Th2 immunity with lower capacity to generate Th1 responses. If we can set up a Th1 dominated immunity model against the same allergen, it is more clear to compare the immunologic nature of our model of allergy, and the two models will

allow us to pursue whether the IL-2 like cytokine effect is unique to atopic or exhibited in both those with hypersensitivity and clinical tolerance in the future.

There are many reports using OVA as antigen with different adjuvant to induce Th1 and Th2 dominated immunity respectively. Usually alum is the most frequently used adjuvant to stimulate Th2 immunity (Yang and HayGlass 1993) , while Freund's adjuvant containing heat-killed Listeria monocytogenes (HKL) strongly promotes the development of a Th1 immunity (Hsieh, Macatonia et al. 1993). Meanwhile Der p1 itself was reported as an immune modulator to diminish Th1 immunity. Comoy et al found that introduction of Der p1 in the complete Freund's adjuvant (CFA) immunized with parasite Ag Sm28-GST was associated with decreased Sm28-GST-specific IgG2a Ab titer, reduced IFNγ mRNA and frequency of IFNγ-producing cells (Comoy, Pestel et al. 1998). He believed that the capacity of Der p1 to modify immune response was due to its protease activity. Inactivation of Der p1 by either heating (95°C, 1hr) or adding cysteine protease inhibitor E-64 abolished its immune regulation ability (Comoy, Pestel et al. 1998).

We found that low dose of Der p1 (2 μg/mouse) absorbed into alum *or* Freund's adjuvant containing HKL generated Th2 or Th1 dominated immunity against Der p1 respectively, which was shown by ongoing cytokine responses. Thus we first reported Th1 and Th2 immunity model against same antigen Der p1.

1.3 Comparison of the antigenicity of Der p1 and crude HDM in primary and ongoing immunity.

Serological studies revealed that Der p1 was a major allergen of whole body house dust mite (see introduction). We first compared the antigenicity of Der p1 and crude house dust mite in the context of CD4 T cell dependent cytokine responses. We found that Der p1 elicited a similar immune response to that obtained using crude whole body extracts (widely used in clinical medicine) in both primary immunization and restimulation. Therefore we proved that Der p1 can be used as model allergen to reflect sensitization to house dust mites.

2. Therapeutic significance of the capacity of IL-15 to selectively enhance Th1 immunity in allergic model

IL-15, as an IL-2 like cytokine, was identified 6 years ago. There are few and conflicting reports about its activity in Th1/Th2 responses. Ruckert et al reported that administration of IL-15 fusion protein enhanced Ag-specific IgE and IgG1 in vivo(Ruckert, Herz et al. 1998). Mori found that IL-15 induced IL-5 production by T cell lines and it also replaced IL-2 in IL-5 production by ConA-stimulated PBMC(Mori, Suko et al. 1996). Avice et al reported that IL-15 induced IFNγ production by CD4 T cells contacting monocytes in the absence of Ag (activation of bystander T lymphocyte) (Avice, Demeure et al. 1998). By using our murine allergy model, this is the first report that IL-15 enhances Ag-specific Th1, but not Th2, recall responses including both cytokine and chemokine gene expression. Because IL-15

has already been used in tumor immunotherapy as an alternative to IL-2(Waldmann, Dubois et al. 2001), our study raises the possibility of allergy treatment by IL-15.

3. The possible mechanism of IL-15 to enhance IL-12 responsiveness

Because of similar receptor distribution of IL-15 and IL-12, e.g. on NK cells and T cells, their relationships were investigated in recent years. Some investigators reported that IL-12 and IL-15 synergized to induce IFN-γ production from murine NK cell in vitro(Fehniger, Shah et al. 1999; Fehniger, Yu et al. 2000). Furthermore, some studies revealed that IL-15 can promote IL-12 production via T cell-dependent contact with monocytes in the absence of Ag (bystander activation)(Avice, Demeure et al. 1998), the mechanism was that IL-15 upregulated CD40 expression on monocyte, which engaged with CD154 on CD4 T cells (no Ag) and promote the IL-12 production from monocyte. Because CD8 T cells do not express CD154, so they have no role in such a mechanism to promote IL-12 production (Avice, Demeure et al. 1998). While other reports proved that IL-15 up-regulated the IL-12Rβ expression on PBMCs(Wu, Warrier et al. 1997), thus increased IL-12 responsiveness. With this C57 Bl/6 murine allergy model, we found that IL-12 is indispensable for IL-15 to exert its Th1-enhancing activity and it acts through up-regulating IL-12 responsiveness rather than IL-12 production. We also found that IL-12 (10pg/ml) and IL-15 (60 ng/ml) have additive, but not synergistic, effects (data not shown), but by using IL-12 knockout mice and low concentration of exogenous IL-12 (3pg/ml) we found sensitive synergic effect of IL-12 (no endogenous IL-12) and IL-15 (2.5 to 60ng/ml). Because IL-12 at high concentration can up-regulate its own receptor, we

speculate that this is the reason that we can not see the synergic effect of IL-15 and IL-12 when IL-12 concentration is high.

In logic, the increased IL-12 responsiveness could be due to two reasons. (i) IL-15 upregulates the IL-12R expression directly; (ii) IL-15 could increase IL-12 signal transduction and later biological procedure. From our finding, the simplest hypothesis is that IL-15 is capable of up-regulating IL-12Rβ2 expression on T cells, hence resulting in more IL-12 responsiveness.

To test this hypothesis, we will investigate IL-15's effect on IL12Rβ2 expression by examination of IL-12Rβ2 mRNA expression from spleen cell culture +/- exogenous IL-15 with real-time PCR. Anti-CD3 (125ng/ml) and IL-12 (50 pg/ml) stimulated spleen cell will be used as positive controls, naïve mouse spleen cell will be used as a negative control.

4. Effect of IL-15 on CD4 and CD8 T cell proliferation and differentiation

Most reports suggested that IL-15 is more important for memory CD8 T cells than naive CD8 T cell or CD4 T cells. Zhang et al found that IL-15 induced strong and selective stimulation of memory-phenotype CD44hi CD8+ (but not CD4+) cells in vivo and in vitro (Zhang, Sun et al. 1998). IL-15 transgenic mice displayed an increase of CD44(hi)CD8 memory T cells (Marks-Konczalik, Dubois et al. 2000). IL-15 or IL-15Rα deficient mice showed a selective reduction in the number of CD8 memory T cells (Lodolce, Boone et al. 1998; Kennedy, Glaccum et al. 2000).

However, recent reports revealed that IL-15's function is not limited in memory CD8 T cells. Niedbala et al reported that IL-15 can induce proliferation of naive and memory CD4 and CD8 T cells in vitro (Niedbala, Wei et al. 2002), but memory CD8 T cells are more sensitive to IL-15 because IL-15 concentration required to achieve optimal proliferation of CD4 T cells was 2-4 times higher than that needed to induce similar CD8 T cell response. The current explanation of higher responsiveness of memory CD8 T cell than naive CD8 T cell or CD4 T cell to IL-15 is that higher level of IL-2Rβ on memory CD8 T cells (Zhang, Sun et al. 1998). There is still no comparative report regarding IL-15Rα expression on these T cell subsets.

T cell proliferation is not necessary accompanied by Th1/Th2 cytokine production (i.e. differentiation). Niedbala et al used in vitro culture systems of (i) Ag-specific OVA TCR transgenic T cells and (ii) polyclonally activated normal T cells to prove that IL-15 induced CD4 and CD8 T cell proliferation but is unable to drive cytokine production in the absence of TCR activation or IL-12/IL-4 stimulation (Niedbala, Wei et al. 2002). In this thesis, we used spleen cell culture and antigen-specific restimulation and found that in the absence of *either* antigen *or* endogenous IL-12, IL-15 did not promote cytokine production, but IL-15 selectively stimulated detectable Th1 cytokine production in the presence of both Ag and IL-12. Both CD4 and CD8 T cells were strongly responsive to this concentration of IL-15 (60 ng/ml) and there was no significant difference between the responsiveness of CD4 and CD8 T cells (Fig 21). The biological significance is that IL-15 not only stimulate T cell growth but is also able to stimulate inflammatory Th1 cytokine production when

confronted with the re-infecting pathogen and thereafter IL-12 production from APC. This is the first report that gave CD4 and CD8 T cell equal importance in the responsiveness to IL-15 to produce inflammatory cytokine although there was much evidence to show a more sensitive CD8 T cell than CD4 T cell proliferation driven by IL-15. It means that IL-15 maintains memory CD8 T cell proliferation when there is no Ag, but it can stimulate both CD4 and CD8 T cell differentiation to produce inflammatory cytokine when Ag re-enters.

5. Biological significance of IL-15 replacement of IL-2 in promoting recall type 1 responses and possible mechanism.

The balance between IL-2 and IL-15 in governing T cell homeostasis is important for the consequences of immune responses. IL-2 is required for AICD involved in peripheral tolerance to self-Ag or in the normal controlling of immune responses to foreign Ag whereas IL-15 inhibits this process. IL-15 supports whereas IL-2 inhibits the survival of memory phenotype CD8 + T cells, thus maintaining a specific immune responses to pathogens(Chu, Chen et al. 1999; Sprent and Surh 2001; Sprent and Tough 2001; Waldmann, Dubois et al. 2001).

It is reported that IL-15 can upregulate IL-2Rα (low affinity receptor) expression(Korholz, Banning et al. 1997). This is the first report revealing that IL-15 directly augments consumption of IL-2 without detectably affecting its production.

We also found that IL-15 can replace IL-2 in promoting recall Th1 responses. Because it is reported that Ag-specific Th1 response was detected in IL-2(-/-) but not in IL-2/IL-15R beta(-/-) mice(Nishimura, Tagaya et al. 2001), the biological significance of our finding is: IL-15 increases the consumption of IL-2 by activated T cells and gradually takes the place of IL-2 to maintain Th1 response when more memory T cells are forming. This draws a longitudinal balance of IL-2 vs. IL-15. The purpose of augmented IL-2 consumption is to shift quickly to a IL-15-dominated pattern, rather than synergistic function because we demonstrated that IL-2 is not required for IL-15's activity and its function can be replaced by IL-15.

For the next step, our hypothesis is that IL-15 increases IL-2R expression to facilitate IL-2 internalization and consumption.

To test such hypothesis, we will determine the frequency and intensity of IL-2R positive cells following short-term culture with/without Ag, plus/minus rIL-15 used at optimal concentrations. PHA and IL-2 activated spleen cell will be used as positive controls, and naïve cell directly ex vivo as a negative control.

In subsequent experiments, two-colour staining (technique and colour compensation as previously carried out) will be used to determine if enhanced IL-2Rα expression is associated with naive or Ag experienced (CD45RA vs. CD45R0) populations.

Further, naive and Ag experienced cells will be isolated (by FACS using high speed preparative sorting) from spleen cells and cultured with Ag, irradiated (5000r) autologous spleen cells as APC and cytokines as described above to compare the results of different populations.

6. Effect of combined depletion of endogenous IL-2-like cytokine functions on recalled type 1/ type 2 responses

After investigating the selective (one cytokine at a time) effect of each of the IL2-like cytokine, the next question is: *Does combined depletion of endogenous "IL2-like" cytokine production influence recall expression of established Ag-specific type 1 vs. type 2 cytokine responses?*

It would be important to determine if combined (2 or more cytokines at a time) depletion of endogenous "IL2-like" cytokine production influences recall expression of established Ag-specific type 1 vs. type 2 cytokine responses in a different style than does selective depletion of each candidate.

One approach to do so would be to block the combinatory signaling of these cytokines and look at the impact on Th1 vs. Th2 recall responses. The methodology is two fold: (a) combinatory blocking of IL-2 and IL-15 (sharing γ- and β-chain and both promote Th1 response) or blocking IL-7 and IL-9 (sharing γ-chain and both

promote Th2 response) with anti-cytokine Ab; (b) blocking β-chain (hence abrogate IL-2 and IL-15 signaling) or γ-chain (hence abrogate all four cytokine signaling).

In summary, we established the murine type 1 and type 2 immunity models against house dust mite allergen. With such model, we compared the role of IL-2, IL-15, IL-7 and IL-9 on antigen-specific recall immunity. We analysed the cellular and molecular mechanism of their effect and their relationships with IL-2. It was found that IL-15 selectively stimulated Th1 inflammatory cytokine production when confronted with re-infecting Ags and thereafter IL-12 production by APC. In the absence of either Ag or IL-12, IL-15 could not stimulate cytokine production. Both CD4 and CD8 T cells were required equally in response to IL-15 and Ag to produce Th1 cytokines although memory CD8 T cells proliferation are more sensitive to IL-15 in the absence of Ag. IL-15 increased the consumption of IL-2 by activated T cells and gradually takes the place of IL-2 to maintain Th1 response when more memory T cells are forming. In contrast to IL-15 and IL-2, we found that γ- but not β- chain receptor cytokines IL-7 and IL-9 selectively enhanced Ag-specific Th2 response without impact on Th1 cytokines.

REFERENCES

Alam, R., J. York, et al. (1996). "Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients." Am J Respir Crit Care Med **153**(4 Pt 1): 1398-404.

Anderson, D. M., S. Kumaki, et al. (1995). "Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes." J Biol Chem **270**(50): 29862-9.

Avice, M. N., C. E. Demeure, et al. (1998). "IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-gamma secretion by CD4+ T cells in the absence of TCR ligation." J Immunol **161**(7): 3408-15.

Bamford, R. N., A. P. Battiata, et al. (1996). "IL-15: the role of translational regulation in their expression." J Leukoc Biol **59**(4): 476-80.

Bamford, R. N., A. P. DeFilippis, et al. (1998). "The 5' untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control." J Immunol **160**(9): 4418-26.

Borger, P., H. F. Kauffman, et al. (1996). "IL-7 differentially modulates the expression of IFN-gamma and IL-4 in activated human T lymphocytes by transcriptional and post-transcriptional mechanisms." J Immunol **156**(4): 1333-8.

Boulay, J. L. and W. E. Paul (1992). "The interleukin-4 family of lymphokines." Curr Opin Immunol **4**(3): 294-8.

Campbell, E. M., S. L. Kunkel, et al. (1998). "Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia." J Immunol **161**(12): 7047-53.

Carter, L. L. and R. W. Dutton (1996). "Type 1 and type 2: a fundamental dichotomy for all T-cell subsets." Curr Opin Immunol **8**(3): 336-42.

Chang, J. T., B. M. Segal, et al. (2000). "The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor beta2 subunit." Eur J Immunol **30**(4): 1113-9.

Chapman, M. D. and T. A. Platts-Mills (1980). "Purification and characterization of the major allergen from Dermatophagoides pteronyssinus-antigen P1." J Immunol **125**(2): 587-92.

Chapman, M. D., A. M. Smith, et al. (1997). "Recombinant mite allergens. New technologies for the management of patients with asthma." Allergy **52**(4): 374-9.

Chomarat, P. and J. Banchereau (1997). "An update on interleukin-4 and its receptor." Eur Cytokine Netw **8**(4): 333-44.

Chu, C. L., S. S. Chen, et al. (1999). "Differential effects of IL-2 and IL-15 on the death and survival of activated TCR gamma delta+ intestinal intraepithelial lymphocytes." J Immunol **162**(4): 1896-903.

Clarke, A. H., W. R. Thomas, et al. (1999). "Murine allergic respiratory responses to the major house dust mite allergen Der p 1." Int Arch Allergy Immunol **120**(2): 126-34.

Coffman, R. L., K. Varkila, et al. (1991). "Role of cytokines in the differentiation of CD4+ T-cell subsets in vivo." Immunol Rev **123**: 189-207.

Comoy, E. E., J. Pestel, et al. (1998). "The house dust mite allergen, Dermatophagoides pteronyssinus, promotes type 2 responses by modulating the balance between IL-4 and IFN-gamma." J Immunol **160**(5): 2456-62.

Corry, D. B. (1999). "IL-13 in allergy: home at last." Curr Opin Immunol **11**(6): 610-4.

Custovic, A., A. Simpson, et al. (1998). "Importance of indoor allergens in the induction of allergy and elicitation of allergic disease." Allergy **53**(48 Suppl): 115-20.

Davies, D. R. and A. Wlodawer (1995). "Cytokines and their receptor complexes." Faseb J **9**(1): 50-6.

DeKruyff, R. H., R. R. Mosmann, et al. (1990). "Induction of antibody synthesis by CD4+ T cells: IL 5 is essential for induction of antigen-specific antibody responses by TH2 but not TH1 clones." Eur J Immunol **20**(10): 2219-27.

Delphin, S. and J. Stavnezer (1995). "Regulation of antibody class switching to IgE: characterization of an IL-4-responsive region in the immunoglobulin heavy-chain germline epsilon promoter." Ann N Y Acad Sci **764**: 123-35.

Demoulin, J. B., C. Uyttenhove, et al. (1996). "A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9." Mol Cell Biol **16**(9): 4710-6.

Dent, L. A., M. Strath, et al. (1990). "Eosinophilia in transgenic mice expressing interleukin 5." J Exp Med **172**(5): 1425-31.

Dokter, W. H., S. J. Sierdsema, et al. (1994). "Interleukin-4 mRNA and protein in activated human T cells are enhanced by interleukin-7." Exp Hematol **22**(1): 74-9.

Dubois, S., F. Magrangeas, et al. (1999). "Natural splicing of exon 2 of human interleukin-15 receptor alpha-chain mRNA results in a shortened form with a distinct pattern of expression." J Biol Chem **274**(38): 26978-84.

Fehniger, T. A., M. H. Shah, et al. (1999). "Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response." J Immunol **162**(8): 4511-20.

Fehniger, T. A., H. Yu, et al. (2000). "Cutting edge: IL-15 costimulates the generalized Shwartzman reaction and innate immune IFN-gamma production in vivo." J Immunol **164**(4): 1643-7.

Finkelman, F. D., I. M. Katona, et al. (1988). "IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses." J Immunol **140**(4): 1022-7.

Foster, P. S., S. P. Hogan, et al. (1996). "Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model." J Exp Med **183**(1): 195-201.

Foster, P. S., A. W. Mould, et al. (2001). "Elemental signals regulating eosinophil accumulation in the lung." Immunol Rev **179**: 173-81.

Gangur, V., F. E. Simons, et al. (1998). "Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-gamma over IL-4 responses." Faseb J **12**(9): 705-13.

Gangur, V., F. E. Simons, et al. (1999). "IP-10 mediated reinforcement of human type 1 cytokine synthesis to environmental allergens among non-atopic subjects." Int Arch Allergy Immunol **118**(2-4): 387-90.

Gesbert, F., M. Delespine-Carmagnat, et al. (1998). "Recent advances in the understanding of interleukin-2 signal transduction." J Clin Immunol **18**(5): 307-20.

Giri, J. G., S. Kumaki, et al. (1995). "Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor." Embo J **14**(15): 3654-63.

Gombert, J. M., E. Tancrede-Bohin, et al. (1996). "IL-7 reverses NK1+ T cell-defective IL-4 production in the non-obese diabetic mouse." Int Immunol **8**(11): 1751-8.

Gonzalo, J. A., C. M. Lloyd, et al. (1998). "The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness." J Exp Med **188**(1): 157-67.

Hamelmann, E. and E. W. Gelfand (2001). "IL-5-induced airway eosinophilia--the key to asthma?" Immunol Rev **179**: 182-91.

HayGlass, K. T. (1995). "Allergy: who, why and what to do about it?" Immunol Today **16**(11): 505-7.

Hogaboam, C. M., C. S. Gallinat, et al. (1999). "Immunomodulatory role of C10 chemokine in a murine model of allergic bronchopulmonary aspergillosis." J Immunol **162**(10): 6071-9.

Holgate, S. T., K. S. Bodey, et al. (1997). "Release of RANTES, MIP-1 alpha, and MCP-1 into asthmatic airways following endobronchial allergen challenge." Am J Respir Crit Care Med **156**(5): 1377-83.

Hsieh, C. S., S. E. Macatonia, et al. (1993). "Pathogen-induced Th1 phenotype development in CD4+ alpha beta-TCR transgenic T cells is macrophage dependent." Int Immunol **5**(4): 371-82.

Humbert, M., G. Menz, et al. (1999). "The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences." Immunol Today **20**(11): 528-33.

Ihle, J. N., B. A. Witthuhn, et al. (1995). "Signaling through the hematopoietic cytokine receptors." Annu Rev Immunol **13**: 369-98.

Imada, M., F. E. Simons, et al. (1995). "Antigen mediated and polyclonal stimulation of human cytokine production elicit qualitatively different patterns of cytokine gene expression." Int Immunol **7**(2): 229-37.

Jiang, Y. and D. W. McGee (1998). "Regulation of human lymphocyte IL-4 secretion by intestinal epithelial cell-derived interleukin-7 and transforming growth factor-beta." Clin Immunol Immunopathol **88**(3): 287-96.

Johnston, J. A., L. M. Wang, et al. (1995). "Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases." J Biol Chem **270**(48): 28527-30.

Jose, P. J., I. M. Adcock, et al. (1994). "Eotaxin: cloning of an eosinophil chemoattractant cytokine and increased mRNA expression in allergen-challenged guinea-pig lungs." Biochem Biophys Res Commun **205**(1): 788-94.

Kalinski, P., C. M. Hilkens, et al. (1999). "T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal." Immunol Today **20**(12): 561-7.

Kao, S. T., S. D. Wang, et al. (2000). "The effect of Chinese herbal medicine, xiao-qing-long tang (XQLT), on allergen-induced bronchial inflammation in mite-sensitized mice." Allergy **55**(12): 1127-33.

Karpus, W. J., N. W. Lukacs, et al. (1997). "Differential CC chemokine-induced enhancement of T helper cell cytokine production." J Immunol **158**(9): 4129-36.

Kelso, A. (1995). "Th1 and Th2 subsets: paradigms lost?" Immunol Today **16**(8): 374-9.

Kennedy, M. K., M. Glaccum, et al. (2000). "Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice." J Exp Med **191**(5): 771-80.

Kimata, H., A. Yoshida, et al. (1996). "RANTES and macrophage inflammatory protein 1 alpha selectively enhance immunoglobulin (IgE) and IgG4 production by human B cells." J Exp Med **183**(5): 2397-402.

Kitayama, J., C. R. Mackay, et al. (1998). "The C-C chemokine receptor CCR3 participates in stimulation of eosinophil arrest on inflammatory endothelium in shear flow." J Clin Invest **101**(9): 2017-24.

Kondo, M., T. Takeshita, et al. (1994). "Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes." Science **263**(5152): 1453-4.

Kopf, M., F. Brombacher, et al. (1996). "IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses." Immunity **4**(1): 15-24.

Korholz, D., U. Banning, et al. (1997). "The role of interleukin-10 (IL-10) in IL-15-mediated T-cell responses." Blood **90**(11): 4513-21.

Kurashima, K., N. Mukaida, et al. (1996). "Increase of chemokine levels in sputum precedes exacerbation of acute asthma attacks." J Leukoc Biol **59**(3): 313-6.

Lai, Y. G., V. Gelfanov, et al. (1999). "IL-15 promotes survival but not effector function differentiation of CD8+ TCRalphabeta+ intestinal intraepithelial lymphocytes." J Immunol **163**(11): 5843-50.

Lamkhioued, B., P. M. Renzi, et al. (1997). "Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation." J Immunol **159**(9): 4593-601.

Lee, Y. L., C. L. Fu, et al. (1999). "Administration of interleukin-12 prevents mite Der p 1 allergen-IgE antibody production and airway eosinophil infiltration in an animal model of airway inflammation." Scand J Immunol **49**(3): 229-36.

Leonard, W. J. (1996). "The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling." Annu Rev Med **47**: 229-39.

Leonard, W. J., E. W. Shores, et al. (1995). "Role of the common cytokine receptor gamma chain in cytokine signaling and lymphoid development." Immunol Rev **148**: 97-114.

Lewkowich, I. P., J. D. Campbell, et al. (2001). "Comparison of chemiluminescent assays and colorimetric ELISAs for quantification of murine IL-12, human IL-4 and murine IL-4: chemiluminescent substrates provide markedly enhanced sensitivity." J Immunol Methods **247**(1-2): 111-8.

Lin, J. X., T. S. Migone, et al. (1995). "The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15." Immunity **2**(4): 331-9.

Liu, C. C., B. Perussia, et al. (2000). "The emerging role of IL-15 in NK-cell development." Immunol Today **21**(3): 113-6.

Lodolce, J. P., D. L. Boone, et al. (1998). "IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation." Immunity **9**(5): 669-76.

Lu, L., P. Chaudhury, et al. (1999). "Regulation of cell survival during B lymphopoiesis: apoptosis and Bcl-2/Bax content of precursor B cells in bone marrow of mice with altered expression of IL-7 and recombinase-activating gene-2." J Immunol **162**(4): 1931-40.

Lu, L. and D. G. Osmond (2000). "Apoptosis and its modulation during B lymphopoiesis in mouse bone marrow." Immunol Rev **175**: 158-74.

Lukacs, N. W., S. H. Oliveira, et al. (1999). "Chemokines and asthma: redundancy of function or a coordinated effort?" J Clin Invest **104**(8): 995-9.

Lukacs, N. W., T. J. Standiford, et al. (1996). "C-C chemokine-induced eosinophil chemotaxis during allergic airway inflammation." J Leukoc Biol **60**(5): 573-8.

Marks, G. B. (1998). "House dust mite exposure as a risk factor for asthma: benefits of avoidance." Allergy **53**(48): 108-14.

Marks-Konczalik, J., S. Dubois, et al. (2000). "IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice." Proc Natl Acad Sci U S A **97**(21): 11445-50.

McInnes, I. B., J. al-Mughales, et al. (1996). "The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis." Nat Med **2**(2): 175-82.

McKenzie, G. J., A. Bancroft, et al. (1998). "A distinct role for interleukin-13 in Th2-cell-mediated immune responses." Curr Biol **8**(6): 339-42.

Miyazaki, T., A. Kawahara, et al. (1994). "Functional activation of JAK1 and JAK3 by selective association with IL-2 receptor subunits." Science **266**(5187): 1045-7.

Miyazaki, T., Z. J. Liu, et al. (1995). "Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation." Cell **81**(2): 223-31.

Monteyne, P., J. C. Renauld, et al. (1997). "IL-4-independent regulation of in vivo IL-9 expression." J Immunol **159**(6): 2616-23.

Mori, A., M. Suko, et al. (1996). "IL-15 promotes cytokine production of human T helper cells." J Immunol **156**(7): 2400-5.

Moser, R., J. Fehr, et al. (1992). "IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals." J Immunol **149**(4): 1432-8.

Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-57.

Mosmann, T. R. and S. Sad (1996). "The expanding universe of T-cell subsets: Th1, Th2 and more." Immunol Today **17**(3): 138-46.

Muraille, E. and O. Leo (1998). "Revisiting the Th1/Th2 paradigm." Scand J Immunol **47**(1): 1-9.

Nakanishi, K., T. Yoshimoto, et al. (2001). "Interleukin-18 regulates both Th1 and Th2 responses." Annu Rev Immunol **19**: 423-74.

Namen, A. E., S. Lupton, et al. (1988). "Stimulation of B-cell progenitors by cloned murine interleukin-7." Nature **333**(6173): 571-3.

Namen, A. E., D. E. Williams, et al. (1990). "Interleukin-7: a new hematopoietic growth factor." Prog Clin Biol Res **338**: 65-73.

Niedbala, W., X. Wei, et al. (2002). "IL-15 induces type 1 and type 2 CD4+ and CD8+ T cells proliferation but is unable to drive cytokine production in the

absence of TCR activation or IL-12 / IL-4 stimulation in vitro." Eur J Immunol **32**(2): 341-7.

Nishimura, H., M. Tagaya, et al. (2001). "Mice lacking interleukin-2 (IL-2)/IL-15 receptor beta chain are susceptible to infection with avirulent Salmonella enterica subsp. enterica serovar choleraesuis but mice lacking IL-2 are resistant." Infect Immun **69**(2): 1226-9.

Noguchi, M., Y. Nakamura, et al. (1993). "Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor." Science **262**(5141): 1877-80.

O'Brien, R., M. A. Ooi, et al. (1996). "Immunologic responses following respiratory sensitization to house dust mite allergens in mice." Immunol Cell Biol **74**(2): 174-9.

Ohteki, T., S. Ho, et al. (1997). "Role for IL-15/IL-15 receptor beta-chain in natural killer 1.1+ T cell receptor-alpha beta+ cell development." J Immunol **159**(12): 5931-5.

Orlofsky, A., E. Y. Lin, et al. (1994). "Selective induction of the beta chemokine C10 by IL-4 in mouse macrophages." J Immunol **152**(10): 5084-91.

Pallard, C., A. P. Stegmann, et al. (1999). "Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors." Immunity **10**(5): 525-35.

Parronchi, P., M. De Carli, et al. (1992). "IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones." J Immunol **149**(9): 2977-83.

Peat, J. K., E. Tovey, et al. (1996). "House dust mite allergens. A major risk factor for childhood asthma in Australia." Am J Respir Crit Care Med **153**(1): 141-6.

Peschon, J. J., P. J. Morrissey, et al. (1994). "Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice." J Exp Med **180**(5): 1955-60.

Platts-Mills, T. A. and M. D. Chapman (1987). "Dust mites: immunology, allergic disease, and environmental control." J Allergy Clin Immunol **80**(6): 755-75.

Porter, B. O. and T. R. Malek (2000). "Thymic and intestinal intraepithelial T lymphocyte development are each regulated by the gammac-dependent cytokines IL-2, IL-7, and IL-15." Semin Immunol **12**(5): 465-74.

Purkerson, J. M. and P. C. Isakson (1992). "Interleukin 5 (IL-5) provides a signal that is required in addition to IL-4 for isotype switching to immunoglobulin (Ig) G1 and IgE." J Exp Med **175**(4): 973-82.

Quinn, L. S., K. L. Haugk, et al. (1995). "Interleukin-15: a novel anabolic cytokine for skeletal muscle." Endocrinology **136**(8): 3669-72.

Raulet, D. H. (1999). "Development and tolerance of natural killer cells." Curr Opin Immunol **11**(2): 129-34.

Rempel, J. D., M. Wang, et al. (1997). "In vivo IL-12 administration induces profound but transient commitment to T helper cell type 1-associated patterns of cytokine and antibody production." J Immunol **159**(3): 1490-6.

Renauld, J. C., A. Vink, et al. (1995). "Interleukin-9 is a major anti-apoptotic factor for thymic lymphomas." Blood **85**(5): 1300-5.

Romagnani, S. (1996). "Th1 and Th2 in human diseases." Clin Immunol Immunopathol **80**(3 Pt 1): 225-35.

Romagnani, S., P. Parronchi, et al. (1997). "An update on human Th1 and Th2 cells." Int Arch Allergy Immunol **113**(1-3): 153-6.

Rozwarski, D. A., A. M. Gronenborn, et al. (1994). "Structural comparisons among the short-chain helical cytokines." Structure **2**(3): 159-73.

Ruckert, R., U. Herz, et al. (1998). "IL-15-IgG2b fusion protein accelerates and enhances a Th2 but not a Th1 immune response in vivo, while IL-2-IgG2b fusion protein inhibits both." Eur J Immunol **28**(10): 3312-20.

Scobie, G., V. Ravindran, et al. (1994). "Expression cloning of a dust mite cysteine proteinase, Der p1, a major allergen associated with asthma and hypersensitivity reactions." Biochem Soc Trans **22**(4): 448S.

Seder, R. A. (1996). "High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4." J Immunol **156**(7): 2413-22.

Simpson, S. J., E. Mizoguchi, et al. (1995). "Evidence that CD4+, but not CD8+ T cells are responsible for murine interleukin-2-deficient colitis." Eur J Immunol **25**(9): 2618-25.

Smith, K. A. (1989). "The interleukin 2 receptor." Annu Rev Cell Biol **5**: 397-425.

Smith, R. E. (1996). "Chemotactic cytokines mediate leukocyte recruitment in fibrotic lung disease." Biol Signals **5**(4): 223-31.

Smith, R. E., R. M. Strieter, et al. (1995). "A role for C-C chemokines in fibrotic lung disease." J Leukoc Biol **57**(5): 782-7.

Snapper, C. M., L. M. Pecanha, et al. (1991). "IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4." J Immunol **147**(4): 1163-70.

Sprent, J. and C. D. Surh (2001). "Generation and maintenance of memory T cells." Curr Opin Immunol **13**(2): 248-54.

Sprent, J. and D. F. Tough (2001). "T cell death and memory." Science **293**(5528): 245-8.

Sugamura, K., H. Asao, et al. (1995). "The common gamma-chain for multiple cytokine receptors." Adv Immunol **59**: 225-77.

Sugamura, K., H. Asao, et al. (1996). "The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID." Annu Rev Immunol **14**: 179-205.

Tagaya, Y., R. N. Bamford, et al. (1996). "IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels." Immunity **4**(4): 329-36.

Tagaya, Y., J. D. Burton, et al. (1996). "Identification of a novel receptor/signal transduction pathway for IL-15/T in mast cells." Embo J **15**(18): 4928-39.

Taniguchi, T. (1995). "Cytokine signaling through nonreceptor protein tyrosine kinases." Science **268**(5208): 251-5.

Temann, U. A., G. P. Geba, et al. (1998). "Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness." J Exp Med **188**(7): 1307-20.

Temann, U. A., P. Ray, et al. (2002). "Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology." J Clin Invest **109**(1): 29-39.

Thomas, W. R. and W. Smith (1998). "House-dust-mite allergens." Allergy **53**(9): 821-32.

Tomura, M., S. Maruo, et al. (1998). "Differential capacities of CD4+, CD8+, and CD4-CD8- T cell subsets to express IL-18 receptor and produce IFN-gamma in response to IL-18." J Immunol **160**(8): 3759-65.

Tournoy, K. G., J. C. Kips, et al. (2000). "Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness." Clin Exp Allergy **30**(1): 79-85.

Tovey, E. R., M. D. Chapman, et al. (1981). "The distribution of dust mite allergen in the houses of patients with asthma." Am Rev Respir Dis **124**(5): 630-5.

Valenta, R., S. Vrtala, et al. (1998). "Recombinant allergens." Allergy **53**(6): 552-61.

Vanguri, P. and J. M. Farber (1990). "Identification of CRG-2. An interferon-inducible mRNA predicted to encode a murine monokine." J Biol Chem **265**(25): 15049-57.

Vicari, A. P., A. Herbelin, et al. (1996). "NK1.1+ T cells from IL-7-deficient mice have a normal distribution and selection but exhibit impaired cytokine production." Int Immunol **8**(11): 1759-66.

Villacres, M. C. and C. C. Bergmann (1999). "Enhanced cytotoxic T cell activity in IL-4-deficient mice." J Immunol **162**(5): 2663-70.

von Freeden-Jeffry, U., N. Solvason, et al. (1997). "The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression." Immunity **7**(1): 147-54.

Wahn, U., S. Lau, et al. (1997). "Indoor allergen exposure is a risk factor for sensitization during the first three years of life." J Allergy Clin Immunol **99**(6 Pt 1): 763-9.

Waldmann, T., Y. Tagaya, et al. (1998). "Interleukin-2, interleukin-15, and their receptors." Int Rev Immunol **16**(3-4): 205-26.

Waldmann, T. A., S. Dubois, et al. (2001). "Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy." Immunity **14**(2): 105-10.

Waldmann, T. A. and Y. Tagaya (1999). "The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens." Annu Rev Immunol **17**: 19-49.

Wang, R., T. L. Ciardelli, et al. (1997). "Partial signaling by cytokines: cytokine regulation of cell cycle and Fas-dependent, activation-induced death in CD4+ subsets." Cell Immunol **182**(2): 152-60.

Webb, L. M., B. M. Foxwell, et al. (1997). "Interleukin-7 activates human naive CD4+ cells and primes for interleukin-4 production." Eur J Immunol **27**(3): 633-40.

Wedderburn, L. R. and P. Woo (1999). "Type 1 and type 2 immune responses in children: their relevance in juvenile arthritis." Springer Semin Immunopathol **21**(3): 361-74.

Wells, T. N., C. A. Power, et al. (1996). "Selectivity and antagonism of chemokine receptors." J Leukoc Biol **59**(1): 53-60.

Wills-Karp, M., J. Luyimbazi, et al. (1998). "Interleukin-13: central mediator of allergic asthma." Science **282**(5397): 2258-61.

Wu, C., R. R. Warrier, et al. (1997). "Regulation of interleukin-12 receptor beta1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells." Eur J Immunol **27**(1): 147-54.

Yang, X. and K. T. HayGlass (1993). "IFN-gamma, but not IL-4, synthesis by antigen-primed murine T cells is IL-2 dependent. Differential requirements in de novo and established responses." J Immunol **150**(10): 4354-63.

Yin, T., S. R. Keller, et al. (1995). "Interleukin-9 induces tyrosine phosphorylation of insulin receptor substrate-1 via JAK tyrosine kinases." J Biol Chem **270**(35): 20497-502.

Zhang, X., S. Sun, et al. (1998). "Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15." Immunity **8**(5): 591-9.

Zhu, Z., R. J. Homer, et al. (1999). "Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production." J Clin Invest **103**(6): 779-88.

Ziolkowska, M., A. Koc, et al. (2000). "High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism." J Immunol **164**(5): 2832-8.