Regulation of Type 1 and Type 2 Chemokine Production in Atopic Disease

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MASTER OF SCIENCE

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4 Dedication

I would like to sincerely thank my supervisor Dr. Kent T. HayGlass for all of his support, guidance and consideration that has made my experience in the Masters' program one of the most memorable times of my life. I hold the highest degree of respect for him and his research. I would like to thank him for opening a door to me that would've otherwise been closed. The knowledge that I have gained and friends that I have made will never be forgotten and always treasured.

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The candidate, Monique J. Stinson, performed all of the experiments contained within this thesis (other than those mentioned above).

6 Abstract

Chemokines play multiple roles in immune regulation including homeostatis, cellular activation, and cellular traficking. We hypothesized that the presence of systemic levels of chemokine as well as detailed analysis of allergen-driven chemokine production by non-allergic, allergic and allergic mildly ashtmatic peripheral blood mononuclear cells (PBMC) may argue for additional roles of chemokines in atopic disease. Here we set out to: (1) Identify the presence and intensity of systemic levels of type 1 (Mig, IP-10) and type 2 (Eotaxin, TARC) chemokines as potential clinical markers of allergic disease. (2) Examine potential differences in regulation of allergen-driven type 1 and type 2 chemokine production from fresh PBMC isolated from non-allergic, allergic, and allergic mildly asthmatic subjects. (3) Determine the ability of exogenously added or endogenously produced T cell derived cytokines to regulate the intensity of allergen driven chemokine production. (4) Identify T cell and antigen presenting cell (APC) requirements for initiation of allergen-driven type 1 and type 2 chemokine production from PBMC isolated from non-allergic, allergic and allergic mild asthmatic subjects.

To examine these parameters we recruited non-allergic, allergic and allergic mild asthmatic individuals based upon skin tests and clinical history. Peripheral blood from each individual was fractionated into PBMC and plasma. Plasma was analyzed for type 1 and type 2 chemokines using chemokine specific sandwich ELISA that we developed. PBMC were placed into short term primary culture and stimulated with allergens, non-

allergenic antigens, or polyclonal activators to identify any potential differences, by ELISA, in chemokine production that may be related to clinical status.

We have shown that type 1 and type 2 chemokines are readily demonstrable in circulation *in vivo*. They are found at a wide range of concentrations (4pg/ml to 20 ng/ml) systemically in the great majority of both non-allergic and allergic individuals. However, systemic levels of these type 1 and type 2 chemokines were not indicative of clinical status as atopic groups did not differ from non-atopic subjects.

We subsequently demonstrated the ability of fresh PBMC from atopic and atopic mild asthmatic subjects to produce dramatically elevated levels of TARC, a type 2 chemokine, relative to the levels produced by healthy controls upon short term allergen specific stimulation. In addition we show the ability of exogenously added and endogenously produced IL-13, IL-10 and IFNγ to differentially modulate the intensity of allergen-driven chemokine production. We demonstrated that allergen-driven type 1 (Mig) and type 2 (TARC) production is dependent upon T cell recognition of allergen in the context of MHC class II. Interestingly, we identified differential requirements for B7-1 or B7-2 signaling to drive the initiation of allergen-stimulated Mig and TARC production depending upon clinical status.

In summary, we demonstrated that chemokines are important mediators in the maintenance of allergic inflammation as allergic and allergic asthmatic subjects display a propensity for elevated allergen-driven, but not polyclonal driven, type 2 chemokine (TARC) production. Together with the ability of type 1 and type 2 cytokines to regulate the intensity of allergen-driven, MHC class II dependent, chemokine production we

hypothesize that there exist additional roles for chemokines in promoting the maintenance of allergic inflammation.

7 Abbreviations

Antigen	Ag
Antigen presenting cell	APC
B lymphocytes	B cells
Biological allergy units	BAU
Bovine serum albumin	BSA
Cytotoxic T lymphocyte	Tc
Daltons	Da
Dermatophagoides pteronyssinus	Der p
Enzyme linked immunosorbent assay	ELISA
Ethylene diamine tetra acetic acid	EDTA
Fetal bovine serum	FBS
House dust mite	HDM
Human immunodeficiency virus	HIV
Human luekocyte antigen	HLA
Immunoglobulin (M, D, E, G, A)	Ig (M, D, E, G, A)
Interferon-gamma	IFNγ
Interferon(inducible protein of 10 kDa	IP-10

Interleukin-(1 to13)	IL-(1 to 13)
Interleukin-(1 to 13) receptor	IL-(1 to 13)R
Kilodalton	kDa
Lipopolysaccharide	LPS
Major histocompatability antigens	MHC
Macrophage derived chemokine	MDC
Microgram	µg
Microlitre	µl
Milligram	mg
Millilitre	ml
Monokine induced by IFN(	Mig
Multiple Sclerosis	MS
Nanogram	ng
Natural killer cells	NK cells
Non-idet P-40	NP-40
Penicillin G sodium, streptomycin sulfate, amphotericin B	PSF
Peripheral blood mononuclear cells	PBMC
Phleum pratense	Phl p
Phorbol 12-myristate, 13-acetate	PMA

Phosphate buffered saline	PBS
Phytohemagglutinin	PHA
Picogram	pg
Recombinant interleukin	rIL-
Rheumatoid arthritis	RA
Roswell Park Memorial Institute	RPMI
Streptokinase	SK
Sodium Azide	NaN_3
T cell receptor	TCR
T lymphocytes	T cells
Thymus- and Activation-Regulated Chemokine	TARC
Units	U

8 Introduction

8.1 Allergy and IgE

Relevance

Allergic diseases affect approximately 25-30% of the population in developed nations and are responsible for the greatest number of days lost from work and school. Treatment costs billions of dollars in North America alone each year [1], [2], [3], [4]. It is the aim of this thesis to identify key mediators critical to the maintenance of allergic disease. I will begin with a review of several current factors that have been hypothesized to be tightly associated with the development of allergic disease, but will focus on recent literature aimed at a group of key mediators involved in the maintenance of allergic disease.

Historical Development

The term 'allergy' was first coined over a hundred years ago by von Pirquet and was initially defined as an 'altered reactivity to antigenic stimulation'. Efforts to further clarify this definition of allergy have proven difficult due to the extreme complexity and multigenic nature of the disease. Identifying the key factors involved in the development and maintenance of allergy is essential for better understanding and possible treatment of these diseases. Today allergy is commonly described as a symptomatic reaction to a normally innocuous antigen. It results from the interaction between an antigen and the

antibody or T cells produced by earlier exposure to the same antigen. Allergens are antigens that can elicit specific IgE responses that are sufficiently robust to be associated with clinical evidence of IgE associated hypersensitivity responses. Allergens are typically proteins, often glycoproteins, or chemicals (haptens) that can become bound by proteins. Examples of common allergens include grass, tree and weed pollens, dust mite, animal dander, insect venoms, latex, nickel as well as pharmaceuticals such as penicillin. Allergens are often airborne particles which may be constitutively, (house dust mite, animal dander) or seasonally (grass, tree pollens) present in the air we breathe.

The first documentation of allergy was by Bostock in 1819, detailing the classical symptoms of seasonal allergic rhinitis in his patients. It wasn't until 40 years later that Salter identified the etiological agents involved in allergy and subsequently developed a test (erythematous skin reaction) to identify the environmental agents responsible. However, it was Prausnitz and Kustner in 1921 who are credited with identifying a factor found in the serum that was responsible for the allergic reaction. They hypothesized that clinical sensitivity to an allergen was a result of an unidentified serum factor. To test their hypothesis they obtained serum from a fish allergic individual (Kustner) and injected it into a fish non-allergic individual (Prausnitz). By exposing the area injected with the fish allergic serum to fish proteins they were able to show clinical sensitivity to fish in Prausnitz. The serum factor was termed 'reagin' and it wasn't until the mid 1960s that 'reagin' was classified and renamed IgE, [5].

Current Overview

IgE is found at very low levels in the circulation of adults. It makes up only 0.01% of the total serum immunoglobulin (Ig) and has the shortest serum half-life of all Ig, only 2.5 days [6]. However, once bound to Fc,R1 on mast cells or circulating basophils, IgE can remain intact and functional for days to weeks. The IgE monomer is made up of two light and heavy immunoglobulin chains similar to other Ig. IgE resembles IgM with five instead of the common four domains (four constant domains, and one variable domain) in the heavy chains. In contrast to most antibody classes IgE does not contain a hinge region, and is heat labile at a relatively low temperature (56C). IgE is believed to be important in the expulsion of parasites from the gut, because of its ability to bind Fc,R1 on eosinophils, which once activated, have cytotoxic activity. In allergy, cross-linking of normally innocuous multivalent allergens to IgE bound to Fc,R1 on mast cells or basophils results in release a wide array of inflammatory mediators. B cell class switching to IgE can result from stimulation of B cells by IL-4 or IL-13 produced by T cells [7], [8], [9]. Clearly, IgE is tightly associated with the initial wheal and flare inflammation (early-phase). However, it is the late phase response governed by cytokines and chemokines that recruit and activate inflammatory cells which is primarily responsible for the maintenance of allergic inflammation [10].

Mast cells and basophils are responsible for the development of many of the symptoms associated with allergy [11, 12]. Mast cells are present in the tissues, mucosal and connective, whereas basophils are restricted mainly to the circulation. Both express Fc,R1 constitutively at high levels and bind allergen-specific IgE with high affinity (Ka=

10^{10} M^{-1}). When IgE Ab are produced in atopic individuals upon exposure to allergen, they bind to Fc,R1 on mast cells and/or basophils. Upon subsequent allergen exposure, multivalent allergens cross-link IgE antibodies, activating the mast cell or basophil. Immediately after IgE cross-linking, enzyme cascades involving protein kinase C, and tyrosine kinase are activated, and an influx of calcium ions results in the release of preformed (histamine) and newly synthesized chemical mediators such as leukotrienes and/or prostaglandins. [11], [13], [14]. Histamine is a vasodilator, a constrictor of smooth muscle, and a potent stimulator of vascular permeability. It exerts its effects by binding H1 receptors expressed on a variety of cell types including smooth muscle cells, neurons, glandular cells, blood cells, and cells of the immune system. Leukotrienes (LT) and prostaglandins (PGD) are derived from arachidonic acid, which is made available from cell membrane phospholipids by the action of phospholipase C and diacylglycerol lipase or by phospholipase A2. LT and PGD have a similar biologic activity to histamine, but PGD can also induce smooth muscle contraction, as well as platelet aggregation and degranulation [10-12, 15]. In addition to these mediators, a great number of cytokines and chemokines are synthesized and released. They are important in regulating the duration and intensity of the immune response by promoting adhesion molecule expression, and recruiting inflammatory cells [11] among other activities.

To date, the most effective treatments for atopic diseases have relied upon corticosteroids. Corticosteroids may be administered topically, (and orally) allowing for their usage in a variety of disease manifestations. It is important to remember that corticosteroids are not curative, and inflammation reoccurs when usage is discontinued.

Mediator antagonists such as anti-histamines and anti-leukotrienes as well as tryptase inhibitors are transiently effective in neutralizing the dominant mediators responsible for the symptoms of allergic inflammation, [16] but are not preventative in the treatment of allergy. In order to address the immunological abnormalities that are responsible for allergic disease, many researchers have looked towards identification of mediators involved in the development or maintenance of atopic disease. Specifically, cytokine modulators are of much interest because of their involvement in the development and maintenance of allergic inflammation [17] [18, 19].

8.2 Potential Factors Involved in the Development of Allergic Disease

Several studies have demonstrated that the prevalence of allergic diseases in industrialized countries has increased over the past few decades. In Japan, the incidence of hay fever due to pollen from the Japanese Cedar tree tripled from 1974 to 1981 and continues to show yearly increases [20]. The incidence of allergic rhinitis in developed nations is as high as 24-38.6% in the UK [21, 22], 20.6% in Norway and 33.6% in the United States [22]. Recent estimates suggest the incidence in developed countries will approach 50% over the next two generations [23]. These studies clearly demonstrate a continuing upward trend in the incidence of allergic disease indicating a need for insight into the mechanisms involved and the development of better treatments, as none to date are curative. There are two major facets of investigation in allergy. Environmental influences as well as genetic predisposition are the most promising avenues for elucidating mechanisms involved. The majority of the literature to date focusing on potential genetic influences is complex, reflecting the polygenic nature

of allergic disease. In addition to genetic influences allergic disease is postulated to be influenced by several environmental factors. In reality, it is most likely that both genetics and environment are responsible for the increasing prevalence of atopic disorders. Therefore, I will review the potential genetic and environmental influences in allergy.

8.2.1 Genetic Influences

Genetic influences have been postulated as a predictive marker for allergy. Utilizing genetic predisposition in predicting which individuals, within a population equally exposed to environmental stimuli, will be susceptible to allergy is unlikely to explain the rapid increases over the past 200 years. However there are a number studies that do indicate the involvement, even if minor, in the development of allergic disease. Monozygotic (MZ) twins share identical genotypes whereas dizygotic (DZ) share approximately 50% of their genes. Studies of twin pairs show that the rate of concordance of hayfever, exzema, asthma, BHR, SPT responses, and serum total and specific IgE were all substantially higher for the MZ twins compared to DZ [24, 25]. Inheritance of allergic disease has been identified through the concordance of allergy in children with allergic parents [26]. The incidence of disease doubles with two allergic parents compared to one. However, the allergen sensitivity, severity of disease and time of disease onset of the parent is often different from the child, making it difficult to predict the type and time of disease onset. In contrast, the risk of a sibling of an asthmatic subject also has asthma is dramatically lower compared to cystic fibrosis or schizophrenia. [27].

Candidate Genes

Identification of genes that may indicate increased susceptibility to allergy has allowed researchers to identify several important mediators in allergy. In particular the 3q chromosome contains the 'chemokine cluster' which are important in recruiting inflammatory cells. In addition, the 5q chromosome contains numerous candidate genes including IL-4, IL-5, IL-9 and IL-13. These cytokines are critical for B cell switching of chromosome 5 contains a G-protein coupled receptor (chemokine receptor) that is found in the lung. This work demonstrates the potential importance of cytokines and chemokines in allergy. However, the immense numbers of possible candidates is extremely ineffective for research purposes. As well, there exists a very likely possibility of multiple mediators working in sequential co-ordinated patterns that would not be indentified through this technology. The importance of genetic information is useful for the identification of possible mediators involved in allergic disease but does not isolate key molecules nor does it rank their importance in allergy. The use of genetic information clearly has its uses and it's limitations. To identify the key molecules involved in the pathogenesis of the disease clearly other avenues will need to be pursued.

8.2.2 Environmental Influences

The potential environmental factors that may play a major role include early childhood infections, pollution, urban vs rural environment, as well as allergen exposure.

Hygiene Hypothesis

Due to fact that the natural mutation rate is low, altered environmental/lifestyle conditions have been heavily favored as a critical factor for the increasing prevalence of allergic diseases. Specifically, the inverse relationship between the relative lack of infectious diseases in early childhood and high incidence of allergic disorders [28] has led to the development of the 'hygiene hypothesis'. This hypothesis is based upon a large body of evidence that states the induction of cell mediated immunity (Type 1) in the early years of life (0 - 5 yrs) via viral or intracellular bacterial infections is required to deviate from the predominance of humoral immunity (Type 2) at birth. Therefore if there is a deficit in type 1 stimulation, either due to excessive antibiotics or natural decrease in bacterial infections, this will lead to activation of type 2 immunity (IgE production and mast cell activation) that is closely associated with allergic disease [27, 29].

Alternatively many argue that a lack of parasitic infections (particularly helminth) leaves a highly evolutionary-conserved immune mechanism (type 2 immunity) without its usual target antigens. This hypothesis supports the possibility that the IgE response to common aeroallergens is default present in the absence of helminths and other parasites [30, 31].

These two viewpoints of the 'hygiene hypothesis' indicate the importance of childhood infections for the development of a healthy immune system. However, both fail to identify the ideal time frame in which infections (viral, bacterial, and helminth) are required for adequate development of the immune system. A study conducted in East Germany monitored the development of allergy of children that attended day care. The study showed that if children from small families entered day care between six to eleven months (15.7%) of age compared to twelve to twenty-three months (21.8%) or after the second birthday (27%), they were significantly less likely to develop allergy. The development of allergy was monitored from five to fourteen years [32]. The presence of bacterial and/or parasitic infections after the onset of atopic disease has shown to exacerbate inflammation [33-36]. Therefore the importance of infection in preventing allergy appears to occur before or during allergen sensitization, not once the disease has been established. The point at which point infection becomes beneficial or detrimental requires elucidation.

Pollution

Another environmental factor that has received a lot of attention in recent years is the potential effect of pollution on the development and severity of atopic diseases. Specifically, diesel particles have been shown to be effective molecules in the exacerbation of allergic inflammation with and without allergen exposure [37, 38]. Diesel particles alone can induce/increase IgE, cytokine, and chemokine production. Atopic PBMC stimulated with diesel particles in combination with allergen (house dust mite) promote a 16 times greater allergen-specific IgE production compared to diesel

particles alone *in vitro* [39-41]. The basis for the synergistic effects may be due to the ability of diesel particles to provide the required cognate plus noncognate signals necessary to drive human mucosal B cells to undergo , isotype switching *in vivo* [42]. In addition, the ability of diesel particles to absorb allergens suggests a potential carrier to increase deposition in the respiratory tract, thereby increasing the allergen dose that reaches the lungs as well as enhancing their antigenicity [43, 44]. The causal relationship between allergy and air pollutants is supported by a Swiss study that demonstrated that living on busy roads is associated with a twofold higher risk for sensitization to pollen [45]. However, removal of pollution (particularly diesel) from the industrialized world has proven to be nearly impossible. Nonetheless, in an effort to reduce allergy, specific measures to reduce diesel particles, through emission controls have been implimented in a few countries around the world.

Urban vs Rural Environmental

A recent observation reported by several authors strongly suggests being raised on a farm may confer protection from the development of allergy [46-49]. A large study of the prevalence of hayfever in Bavarian children aged five to seven years raised on a farm was strongly decreased compared to their peers from the same rural area whose parents were not farmers [46].

The living conditions of farming families have significant differences from urban and non-farming rural areas. The increased presence of microbial flora present is thought to promote cell-mediated immunity in the early years of immune system development. Specifically, it is hypothesized that the presence of LPS, which is a commonly found in stables, signals through the CD14 receptor promoting the production of IL-12, a strong

cytokine mediator for the development of cell mediated immunity and inhibitor of type 2 immunity. Bacteria found in unpasteurized cow's milk may also provide a source of bacteria. Evidence for this was supported in the Bavarian survey where they were able to show a strong inverse dose-dependent relationship between exposure to livestock and the prevalence of atopic diseases (13.5% vs 34.8% p=0.01) [46].

Allergen Exposure

The degree of allergen exposure during the early years of life suggests an additional possible risk factor for the development of atopy. Specifically, the concentration of house dust mite and cat allergen has been examined by two different groups both with interesting, yet contradictory conclusions. A large multi-center study conducted by Whan et al [50] examined the possible correlation between carpet dust mite and cat allergen concentration and the development of atopy in a large birth cohort to seven years of age (MAS study). They were able to identify a strong correlation between high dust and cat allergen concentrations and the development of atopic sensitization toward that specific allergen within the first three years of life. This indicates that high antigen exposure during the first few years of life often results in sensitization towards that allergen. In contrast Platts-Mills [51] recently performed a similar study examining the prevelance of sensitization and IgG antibody to a range of concentrations of dust mite and cat allergen. Increasing exposure to dust mite was associated with increased sensitization. Interestingly, in response to high concentrations of cat allergen they demonstrated a high IgG and IgG4 response without sensitization, a finding indicating exposure to high levels of cat allergen is protective against sensitization.

Clearly, these studies have shown that environmental and genetic factors are strong candidates for the increasing incidence of atopy. However, whether an individual becomes allergic has become secondary to determining factors involved in the maintenance of allergic disease. Clearly, allergic individuals are most interested in how to manage their disease, and ultimately reach the goal of eradicating the symptoms. If we understand how allergy is maintained in an allergic individual we may open up possibilities to control the disease.

8.3 Current Treatment of Allergy

Attempts to effectively treat allergy has been an ongoing since the first documented cases. At present there is no one universally effective treatment for all types of allergies (allergic rhinitis, drug sensitivity). However, there exist three major possible management strategies for allergy that are used today with moderate success: 1) Allergen Avoidance, 2) Pharmacological solutions, 3) Allergen Immunotherapy.

Allergen Avoidance

Avoidance thus far has been a commonly used management approach to specific types of allergy. Avoidance of food allergens such as peanut or milk may be managed through screening of the ingredients found in the food consumed. However, in the case of animal danders, dust mite, and several pollens (grass, ragweed) measures to prevent exposure to allergens are not effective in the treatment of the atopic diseases, because removal of allergens from the environment is not possible. For example, grass pollen released into the atmosphere from spring to fall and cannot be prevented. Avoidance does not provide a consistent, effective treatment regime for atopic disease.

Pharmacological Solutions

Pharmacological solutions to allergy generally target the mediators formed by mast cells and basophils that result in the massive vasodilation and inflammation or they work by globally inhibiting immune responses.

Currently the most effective method of treating allergy and asthma is corticosteroids [16, 52-54]. Corticosteroids bind to a cytosolic glucocorticoid receptor. Binding results in translocation to the nucleus and formation of a homodimer that can bind to DNA to activate genes, including anti-inflammatory genes. However, the important anti-inflammatory effect of corticosteroids is their ability to suppress multiple inflammatory genes, such as inflammatory cytokines, enzymes, adhesion molecules, and inflammatory mediator receptors [16, 52, 53]. Recently, it was demonstrated that glucocorticoids are able to prevent the upregulation of nasal mucosal secretion of eotaxin, a chemoattractant and activator of eosinophils, that is normally seen in allergic rhinitis patients during the grass pollen season [55-57]. Pullerits et al were able to demonstrate the benefit of systemic and topical glucocorticoids respectively on suppressing multiple components of allergic airway inflammation [55]. This broad immunosuppressive effect is the reason why corticosteroids are so effective at treating the complexity of atopic disorders. However, corticosteroids are not curative and inflammation returns after their usage is discontinued.

β2 agonist are molecules that bind to β2 adrenergic receptors. Their binding results in bronchodilation, which has made β2 agonists a popular choice for the treatment of acute exacerbation of allergy and/or asthma. Much work has been done to develop a

long acting β2 agonist that would be able to replace corticosteroid treatment [58-61]. Concern has been expressed about whether long-acting β2 agonists may, by their bronchodilator and symptom-relieving effects, mask the development of persistence of airway inflammation and put the subject at risk of more serious disease. [62].

Similar to corticosteroids, β2 agonists only provide temporary relief from the symptoms, but do not address the underlying mechanisms responsible.

Antileukotrienes and antihistamines are two classes of mediator antagonists aimed at blocking mediators produced by cells involved in atopic disease. Leukotrienes are powerful bronchoconstrictors and inducers of plasma exudation and may promote eosinophilic inflammation [63]. Unfortunately, recent clinical trials of antileukotrienes in allergic rhinitis display little benefit compared with nasal corticosteroids [55]. Histamines are potent vasodilators and promoters of mucus production and constriction of smooth muscle. They are commonly produced when FcεR1 on mast cells is cross-linked [10]. Antihistamines are H1-receptor (histamine receptor) antagonists and have been used to treat atopic diseases for many years, [64]. Mediator antagonists may be effective at relieving the symptoms of ongoing inflammation but their usage is required daily if not more frequently and symptoms return shortly after their usage is stopped.

Unfortunately, to date there is no preventative and/or long lasting immunological treatment of most allergies. To provide this type of therapy insight into the mechanism of ongoing inflammation is required. At present we have a wide variety of pharmacological treatments to block the symptoms of inflammatory mediators or to inhibit global immune responses thereby allowing the possibility of opportunistic

infections. More work is needed to identify mediators that will promote remodeling of the immune system towards a protective and preventative response to allergens.

Allergen Immunotherapy

Immunotherapy is a technique that has been used for decades as a method of 'desensitizing' a susceptible individual towards an allergen. The basic protocol uses increasing doses of allergen that the atopic individual is sensitive towards. The goal is to prevent the production of IgE and other inflammatory mediators in response to allergen. Whether immunotherapy works to remove, inhibit, or alter the immune response is not well understood. It has the disadvantage that it is not consistently effective. Presently, there are several hypotheses that focus on determining the factors and/or processes involved in immunotherapy with the goal of improving this strategy. The first two hypotheses focus on the cytokine mediators produced by T cells specific for the allergen. Differences between these two possibilities lies in the "type" of cytokine that is involved. The last hypothesis focuses on the possibility that the allergen specific T cells are inactivated or become 'anergic'.

To begin with, there is an increasing body of evidence that claims that immunotherapy can result in the development of a population of T helper cells that predominantly produce IL-10 and TGFβ, [65-67]. Initially these T cells were identified in a mouse model [68] but were recently identified in human PBMC [69-71]. This subset of Th cells arises after chronic antigen T cell stimulation. However, they fail to proliferate after TCR stimulation, possibly through downregulation of IL-2, [70, 71] but are characteristically producers of large quantities of IL-10, [72-76]. It is believed that through the production of IL-10 and TGFβ but not IL-4 [77] surrounding Th cells are transformed into

"regulatory T cells" (Tr). Tr cells express $CD45RB^{low}$ and CD25+ and have been shown to be potent inhibitors of polyclonal T cell activation [78], allogeneic responses [65], and experimental autoimmune disease [79]. However, this powerful inhibitory property was demonstrated to be restricted to a contact-dependent mechanism [73].

Alternatively, Umetsu et al was able to reverse established allergen-induced airway hyperreactivity in a mouse model through the usage of an IL-18 expressing adenovirus [80]. Administration of IL-18 was able to reduce airway hyperreactivity, and IL-4 production. In addition Hamid et al demonstrated the ability of IL-12 (a potent mediator driving cell-mediated immunity) to promote eosinophil apoptosis *in vitro* [81]. Each of these approaches utilize the well known documentation of long term allergen-specific T cell clones from the peripheral blood of nonallergic individuals have been shown to produce Th1 cytokines [82, 83]. In addition the ability of Th1 cytokine producing cells to cross regulate the production of cytokines (eg IL-4, IL-13) that promote the development of atopy [84] was shown to be a very useful technique for treatment purposes. Through using specific allergens in combination with cytokines (Th1) that inhibit the development of allergy one may be able to achieve positive results with immunotherapy [85]. However, studies to date utilizing recombinant cytokines such as IL-12 have not been very promising. Administration of IL-12 was able to reduce circulating eosinophils, but did not reduce airway hyperreactivity. This disappointing finding was compounded by the toxic effects seen in human systems [86, 87]. Although administration of rIFNγ to sensitized animals resulted in inhibition of eosinophilic inflammation, a similar effect was not seen in a comparible human model [88, 89]. Monoclonal antibodies against IL-5, an important type 2 cytokine key for development of eosinophils, were used to attempt to

reduce systemic eosinophils, the late asthmatic response and airway hyper-reponsiveness. Similar to rhIL-12, αIL-5 mAb reduced the number of circulating eosinophils but did not significantly alter the late asthmatic response or the airway hyper-reactivity [90]. Clearly, there are multiple mechanisms working concertedly, thereby requiring more than one mediator to reverse an unwanted, yet ongoing immune response.

Lastly, a hypothesis that has gained much support over the past few years focuses on the possibility that chronically activated allergen specific T cells or T cells stimulated with greatly elevated allergen concentrations results in long-term unresponsiveness or anergy [91, 92]. Through this mechanism, repeated treatment of relatively large quantities sensitizing allergen results in the short-term production of cytokines that promote the development of allergy (IL-4). Shortly after activation allergen specific Th cells enter a state of non-responsiveness whereby the cells do not produce cytokines and do not replicate. Transition to this state is conditional upon the presence of cytokine mediators present during TCR stimulation. Specifically, the presence of IL-4 may prevent T cell anergy and there is evidence for dependence upon the presence of allergen to maintain the unresponsive, anergic CD4+T cell population [93]. The time, dosage, and route are all factors that may alter the time of onset of anergy and how long it may last. Whether this is merely a temporary state, or whether it is possible to administer large quantities of certain allergens (eg peanut) for anergy to occur remains unknown.

The potential ability of immunotherapy to act upon and deviate the immunological mechanisms involved in allergy makes this technique a beneficial tool. However, there

are several caveats to allergen immunotherapy, such as the inability of some allergens, (eg. Peanut), to be administered, even at low doses, due to the likelihood of fatal anaphylaxis. Additionally some allergens, such as grass pollen, are much less effective at 'desensitization' in comparison to others, such as hymeroptera (insect venom). What physical and chemical characteristics constitute a 'good allergen' for immunotherapy remain unknown. Additionally, the long term protection that is provided is not well documented and tends to vary with allergen, model system, and individual subject. In general, more work needs to be done to determine how anergy may be induced in Th cells, how Tr develop from a heterogenous population and why non-atopic and atopic individuals have different ratios of Tr cells.

The treatment of allergic disease over the past 200 years has yielded some beneficial results. Indeed, most allergic diseases may be managed through the use of pharmacologics, or at times immunotherapy. However, the restrictions placed on these treatments, such as length of relief from allergy symptoms, cost and quality of life has driven the demand towards more cost effective and permanent solutions. Specifically, treatment should directly act upon the cells involved in the maintenance of an allergen specific immune response. Due to the chronic inflammation that is commonly seen in several atopic diseases there is much evidence for positive feedback loops. We need to identify these key mediators driving ongoing inflammation. Additionally, comparing allergic and non-allergic individual responses to allergens within a system similar to that seen in vivo is still required. Clearly, allergy is a multifaceted disease involving key mediators driving downstream events that are not immunologically beneficial to the

population as a whole. Identification of a treatment that effectively treats the underlying causes of allergy for long-term would be a great advantage to allergy sufferers.

8.4 Grass Allergy

Grass pollen allergens belong to the most frequent and potent elicitors of allergic symptoms. At least 40% of allergic patients are sensitized against grass pollen allergens, [94]. It is commonly referred to as allergic rhinitis as well as hayfever. Certainly in Manitoba, grass pollen represents the major seasonal allergen. Allergy to grass is a result of pollen grains released into the atmosphere during pollination in the summer (peaking June/July). The atmospheric pollen grain counts remain at relatively high levels until the fall (September). It is during the grass pollen 'season' (spring to fall) that sensitized individuals are continuously exposed towards greatly elevated levels of allergen. (Lesley ShyJak, Aerobiology Research Laboratories 1999, 2000) The majority of the grass allergic population produces IgE specific antibodies against the timothy grass allergens: Phl p 1, 2, 3, 4, and 5. Over 94% of grass pollen specific IgE from grass atopic Canadians, Europeans and Japanese individuals will bind Phl p1, and 5 [95, 96]. The isolation and production of recombinant allergens using *E. coli* has been useful for identifying the major grass allergens that the majority of individuals are sensitized against. However, the use of recombinant allergens is controversial specifically for analysis of *in vivo* responses to grass pollen (ie skin testing and identification of IgE specific Ab). A recent study examining the efficacy of diagnosis of clinical hypersensitivity to grass pollen compared recombinant (Php 1-5) and natural allergens [95]. Interestingly, they demonstrated that a proportion (~30%) atopic

individuals produce IgE Ab against minor allergens within the Phl p family (Phl p 2, Phl p 4). In addition a proportion (~25%) of atopic individuals produce IgE specific to antigens prevalent in their environment (eg. Red top or Kentucky Blue grass). The presence of bacterial contaminants in the recombinant preparation as well as inappropriate folding or inconsistencies in glycosylation of the protein has favored the use of the natural allergen extracts for testing of allergy as well as for use *in vitro*.

Allergic rhinitis is one of the most prevalent allergic disorders. Immunotherapy and pharmacological treatments have not provided consistent, long-lasting, or preventative solutions. Efforts to elucidate the key mediators involved in the pathogenesis of maintenance of grass pollen specific allergic inflammation are essential to build towards universally effective treatment.

8.5 Classification of T cell subsets based upon the profile of functional ability and cytokine profiles

8.5.1 CD4+ and CD8+ subsets

T cells are key regulators of the immune response and are indispensable for the maintenance of a healthy immune system. Antigen-specific T cells are able to perform a variety of immunoregulatory functions including: Recognition of foreign, pathogenic organisms, effective removal of the pathogen, maintenance of a memory population, downregulation of the immune response once the infection is cleared and most importantly regulate the intensity of each individual ongoing immune response. To accomplish these tasks there exists more than one specialized form of T cell. This subdivision is based upon the T cells ability to effectively respond to various types of antigens and the effector mechanisms they employ.

T cells are characterized as circulating mononuclear cells that express a TCR-CD3 complex on their extracellular surface. The TCR complex is made up of two polypeptide immunoglobulin-like chains. Two different combinations of Ig-like polypeptide chains have been described: αβ and γδ. The main purpose of the αβ or γδ chains is to physically recognize and bind antigen. The αβ TCR can bind a seemingly endless array of antigens whereas γδ TCR have been shown to be restricted to evolutionarily conserved motifs such as peptidoglycan, a major component in bacterial cell walls. The TCR contains several conserved sequences that recognize MHC molecules commonly

found on APC. Through hydrophobic and hydrophilic interactions as well as salt bridges and hydrogen bonds a TCR-CD3 complex can bind antigen in the context of MHC [97, 98].

The CD3 complex consists of two epsilon chains as well as a gamma chain, a delta chain and a homodimer of zeta chains. The CD3 complex forms a critical part of the peptide-MHC-TCR trimer and is essential for signaling the T cell. The cytoplasmic tails of the CD3 complex contain multiple ITAM sequences which upon activation recruit intracellular signaling molecules such as Src and ZAP70 (tyrosine kinases) to initiate an intracellular cascade of (de) phosphorylation events which lead to nuclear translocation of transcription factor and ultimately transcription of genes. The CD3 complex is essential for the TCR to efficiently bind and signal the T cell once the TCR has 'recognized' a foreign peptide in the context of MHC [6].

Co-stimulatory molecules CD4 and CD8 are expressed on the cellular surface of αβ T cells. CD4+ T cells preferentially bind MHC class II molecules on the surface of antigen presenting cells (APC). CD4 polypeptide recognizes a conserved region on the MHC II complex and is essential in stabilizing the trimer [99-101]. MHC II molecules are expressed on professional APC and display exogenous antigens. The MHC II expression pattern is usually constitutive at low levels and is upregulated through stimulation from cytokines such as IFNγ [102]. Antigens that are taken up by the cell either through pinocytosis or phagocytosis are digested within endocytic compartments through fusion with lysosomal vesicles with a low pH and digestive enzymes. The digested peptides bind to conserved residues on the MHC II complexes and are displayed on the APC cell surface. Therefore, CD4+ T cells recognize exogenous

antigens (eg. Grass pollen) in the context of MHC II [103-106]. Upon stimulation, CD4+ T cells generally respond by synthesis of inflammatory mediators such as cytokines or they may stimulate other cells to produce a tightly orchestrated pattern of cytokines that can function to amplify maintain or suppress an immune response. Due to the ability of CD4+ T cells to regulate the immune response they have been collectively termed 'helper T cells' (Th).

Alternatively αβ T cells may express CD8 on their cell surface. CD8 co-stimulatory molecules exclusively recognize MHC I molecules that are expressed on every nucleated cell. MHC I molecules generally present peptides from intracellular pathogens, such as intracellular bacteria and viruses. [6, 107] [108]. Due to the infectious nature of most intracellular pathogens, the response of CD8+ T cells is to directly kill the target cell. Killing is accomplished through Fas-FasL expression, release of perforin and granzymes and secreted cytokines to recruit cells to ensure the infection is cleared [109-111]. Due to their ability to effectively kill viral and bacterial infected cells CD8+ T cells are often referred to as 'cytotoxic T cells' (Tc).

8.5.2 Type 1/Type 2 Hypothesis

When naïve T cells encounter antigen for the first time, they begin a process of differentiation that involves a commitment to a specific pattern of cytokine production. Naïve T cells produce IL-2 when they first recognize antigen in the context of MHC class I or II in the periphery but are incapable of producing large quantities of other cytokines, such as IL-4 and IFN(. Three stages of differentiation may occur in naïve T cells after initial stimulation through the TCR-CD3: 1) the Initiation Phase, 2) the

Commitment phase, and 3) the Acute phase [112]. The initiation phase is highly dependent upon the 'type' of antigen, cytokine and/or cytokine-induced STAT transcriptional proteins present during stimulation. The ability of extracellular factors such as cytokines to direct T cell differentiation will be reviewed in detail later. The commitment phase involves the transcription factors that will determine the commitment of T cells into two different populations. It is at this stage due to selected transcription factors that differentiated T cell phenotypes are stabilized and maintained in the absence of further stimulation. Finally an acute phase of gene transcription is initiated through secondary contact of the differentiated T cell with the specific antigen [113, 114]. It is during the acute phase that one can distinguish between two populations based upon their cytokine profiles. Mosman [115] and Del Prete [116] were able to demonstrate in mice and humans respectively that two types of cytokine profiles could be elicited from stimulated Th cell clones. The two Th cell populations were termed 'Th1 and Th2 cells" and the resulting cytokine profiles elicited from these cellular isolates has led to the generation of the Th1/Th2 hypothesis. The Th1/Th2 hypothesis follows that the cytokine profiles from differentiated T cells can mould the type of immune response generated against antigen [117-120]. Shortly after the discovery of Th1 and Th2 clones, cytotoxic T cells (Tc1/Tc2) and dendritic cells (DC1/DC2) with similar cytokine profiles were isolated [121-124]. This is important because it demonstrates the involvement of APC as well as cytotoxic cells in directing the development of an immune response. As a result the 'Th1/Th2 hypothesis' has been more accurately termed the 'Type1/Type 2 hypothesis'. Type 1 cells produce cytokines IFNγ, IL-12 and TNFβ. Type 1 cytokines are highly protective against infections

mounted by the majority of microbes, because of the ability of type 1 cytokines to activate phagocytes and to promote the production by B lymphocytes of opsonizing and complement-fixing antibodies. Type 1 immunity is also known as cell mediated immunity (CMI) [115]. However, when the antigen is not rapidly removed from the body, the type 1 response may become dangerous for the host due to the strong and chronic inflammatory reaction evoked (eg Tuberculosis, Multiple Sclerosis). Type 2 cells produce the cytokines IL-4, IL-5, IL-6, IL-9, and IL-13 which induce the differentiation, the activation, and the survival of eosinophils, promote the production of IgE as well as other Ig isotypes from B lymphocytes, as well as the development of mast cells and basophils. In addition type 2 cytokines inhibit several macrophage functions and the development of Th1 cells. Thus the phagocyte-independent type 2 response is usually less protective than the type 1 response against the majority of infectious agents, with the exception of helminths. Type 2 immunity is often referred to as humoral immunity [125]. In addition to cytokine production profiles there are additional markers for type 1 and type 2 cells. Chemokine receptors are preferentially expressed on T cell clones with type 1 or type 2 cytokine profiles. Specifically CXCR3 and CCR5 are found upon on Th1 cells, and CCR4 as well as CCR8 are exclusively expressed on Th2 cell lines [126].

T cells expressing cytokine of both patterns have been designated as type 0 (Th0). Th0 cells usually mediate intermediate effects depending upon the ratio of cytokines produced and the nature of the responding cells. [127]. It is possible that type 0 cells are involved in eliminating many pathogens, where a balance of both regulated cell-mediated immunity and an appropriate humoral response will eradicate an invading

pathogen with minimum immunopathology. To what numerical extent type 1 and type 2 cells dominate such in vivo responses is as yet not clear, but their ability to influence chronic disease or pathology by their production of high levels of regulatory cytokines is not in doubt [120, 128].

8.5.3 Factors involved in directing type 1 versus type 2 development

It has become evident over the past few years that Th1 and Th2 cells are not derived from distinct lineages but rather develop from the same T helper cell precursor under the influence of both environmental and genetic factors acting at the level of antigen presentation. The strength of interaction mediated through the TCR and MHC/peptide complex or the dose of antigen can directly affect lineage commitment of CD4+ T cells [129-131]. The nature of the antigen [132] as well as the relative involvement of co-stimulatory molecules B7-1 and B7-2 may differentially regulate Th1 and Th2 development [133-137]. Interestingly, Th cells that do not express CD4 co-receptor are unable to mount a type 2 response against an antigen *in vivo* and in [138, 139]. However, there exist many conflicting reports and additional work is required to elucidate the mechanisms for the aforementioned phenomena.

The most clearly defined factor determining Th1 and Th2 differentiation from the Th cell precursor is the cytokine environment present during the initiation of the immune response [118, 128]. Bacterial (LPS) stimulation of macrophages results in the production of type 1 cytokines IL-12 and IFNγ that promote the development of type 1 immunity. In contrast, early IL-4 production possibly by NK1.1+ cells or basophils in response to helminth infection favors development of the type 2 lineage. Interestingly,

sufficient levels of IL-4 present during allergen sensitization results in the commitment of type 2 immunity even if significant levels of IFNγ are present as well [140]. Type 1 and type 2 cytokines can effectively cross-regulate the development and intensity of each other. For example, IL-4 and IL-13 inhibit the actions of type 1 cytokines, IFNγ and IL-12. Conversely, IFNγ inhibits the development of type 2 cells and humoral immunity in the absence of sufficient levels of early IL-4 [141]. Cytokines that are either directly related towards the development of type 1/ type 2 immunity or are critical mediators of established type 1/ type 2 responses will be reviewed in detail.

IL-4 is an important cytokine in the development of type 2 immunity. It binds to a 140Kd transmembrane protein, IL-4Rα, in combination with the common γ chain. However, γc KO cells may use the IL-13Rα as an accessory factor to allow IL-4 signaling [142]. IL-4R binds IL-4 with high affinity (Kd=400pmol) and recruits tyrosine kinase signal transduction molecules STAT6 and Janus Kinases. IL-4R is expressed on macrophages, NK cells, fibroblasts, eosinophils, airway smooth muscle, and endothelial cells highlighting its pleiotropic ability. IL-4 is initially produced from as yet unidentified source. Reports suggest that early IL-4 production is from NK1.1+ (NK T), naïve CD4 T cells or basophils, [143-146] however this remains to be elucidated. Early IL-4 production results in naïve T cell expression of IL-4R, creating an amplification of IL-4 production from Th2 cells. IL-4 promotes B cell class switching to IgE and inhibits pro-inflammatory cytokine production including IL-12, which promotes type 1 immunity. Most importantly IL-4 promotes Th 2 cell differentiation [118, 147-149]. IL-4 present at threshold levels will preferentially drive type 2 differentiation regardless of the presence of sufficient type 1 cytokines [118, 150]. IL-4 expression from Th2 cells and mast cells

is transient and is often undetectable after 12 hours [151-153]. Interestingly, IL-4 may not be the only mediator driving type 2 immunity because IL-4 deficient mice are able to mount Th2 responses towards allergens, and parasitic infections [154, 155]. However, IL-4 KO mice do display reduced type 2 immunity in response to helminth infection. Therefore, IL-4 is regarded as a key molecule mainly involved in the initiation of type 2 immunity, but is not required after T cell activation.

IL-13 was first cloned in the mouse in 1989 and its' human counterpart in 1993. It shares a number of structural as well as functional characteristics with IL-4. However, these similarities are often due to the finding that IL-13R is a multi-subunit structure that requires the IL-4Rα chain for efficient signaling [156-158]. IL-13R is found upon macrophages, fibroblasts, eosinophils, basophils, mast cells, airway smooth muscle, human B cells, mouse T cells, endothelial cells, dendritic cells and NK cells. [152, 159, 160] The IL-13R complex consists of either the IL-13Rα1 or IL-13Rα2 chain in conjunction with the IL-4Rα chain. The IL-13R binds IL-13 with varying affinities but is most effective when it binds IL-13Rα plus IL-4Rα (Kd=400pmol). IL-13Rα alone can bind IL-13 but with much lower affinity (Kd=4-50pmol) and no signaling capacity. The IL-4Rα chain is absolutely required for IL-13R signaling. Using the identical signaling chain (IL-4 also uses γc) results in very similar signal transduction pathways. Specifically, signaling through IL-4Rα results in the phosphorylation of STAT6 as well as Janus kinases [161, 162]. Activation of these signal transduction pathways results in increased expression of MHC II, CD80 and CD86, which allow for enhanced capacity to stimulate antigen specific T cells. [163, 164] In addition, IL-13 signaling promotes integrin expression on endothelial cells, T cells, monocytes (CD11b, CD11c, VLA-4),

and eosinophils ($\alpha 4\beta 1$) [152, 164]. Integrin up-regulation results in the cellular recruitment and eosinophil survival at sites of allergic inflammation further emphasizing the importance of this cytokine in the pathogenesis and maintenance of allergic responses. IL-13 up-regulates IgM, IgG, IgA, IgE and CD23 (low affinity FcεR) expression by B cells, as well as promote B cell growth and prevents apoptosis hence, the association with humoral immunity. IL-13 expression remains at high levels for extended periods after T cell activation (+72 hours *in vitro*, *in vivo* duration unknown) [151]. In contrast, IL-4 expression is transient, at 100-1000 fold lower levels, and is rarely seen more than 12 hours after T cell activation. However, human T cells and mouse B cells do not express a functional IL-13R and therefore IL-13 cannot directly influence Th2 cell differentiation nor IgE or IgG4 class switch in mice [152, 159, 165]. It is therefore hypothesized that IL-13 is important in maintaining allergic inflammation through B cell survival, Ig production (IgE) as well as recruiting key inflammatory cells through intergrin expression. In addition, IL-13 promotes type 2 immunity through inhibiting type 1 responses. IL-13 inhibits proinflammatory cytokines and chemokines IL-12, IL-1α, IL-1β, IL-6, TNFα, IL-8, MIP-1α, MIP-1β, and MCP-3 [152]. In addition, stimulation by IL-13 decreases NO production from macrophages [152, 153, 163]. Recently, Barner M. et al demonstrated the importance of IL-13 in the development of type 2 immunity. Specifically, they were able to shown that IL-4Rα deficient mice (no IL-4 nor IL-13 signaling) were more severely affected by nematode *Nippostongylus brasiliensis* infection compared to IL-4 deficient mice [166].

IL-10 was originally described as a Th2 cytokine in mice, inhibiting cytokine synthesis by Th1 cells [167]. Today there is increasing evidence that IL-10 acts as a general inhibitor of proliferation and cytokine responses [168-171]. IL-10 is produced by monocytes, macrophages [170], NK cells [172], as well as type 1 and type 2 CD4 as well as CD8 cells [173]. The effects of IL-10 have been demonstrated to be directed primarily against APC. IL-10 down-regulates MHC class II expression thereby diminishing their effect on the antigen-specific T cell proliferation [170, 171]. Moreover, IL-10 down-regulates the B7 expression on Langerhans cells, dendritic cells and macrophages [168, 174-177]. Recently, IL-10 was shown to downregulate LPS induced production of MCP-1 by human blood monocytes [178, 179]. In contrast whole blood culture treated with IL-10 resulted in an increase in MCP-1 production [180]. Recently, a Tcell subset has been designated as T regulatory 1 cells, which produce high levels of IL-10 but little or no IL-2 and IL-4 suggesting IL-10 may play a role in tolerance [75].

IFNγ is an important mediator of type 1 immunity. NK and T cells as well as NKT cells produce IFNγ. Levels are enhanced by the addition of IL-12 and IL-18 [181-185]. As well, IFNγ promotes itself through an autocrine pathway thereby making it a potentially important cytokine in treating ongoing inflammatory responses. IFNγ functions to enhance IL-12Rβ2 (receptor for IL-12) expression on mouse CD4+ T cells which provides evidence to its role in regulating the magnitude of the Th1 response [186-188]. In addition, IFNγ promotes IgG2a class switching (in mice) and interferes with the class switching regulatory effects of IL-4 [140]. Because IFNγ is produced shortly after Th1 cell activation and continues for an extended period, the involvement of IFNγ in maintaining type 1 responses is an attractive hypothesis. Although treatments with

rIFNγ have not been as successful as one would have hoped (Section 5.3) mediators enhanced or inhibited by IFNγ are now ideal targets.

Clearly, cytokines are important regulators of type 1 and type 2 immunity. However, the degree to which one cytokine can be the sole instigator of an ongoing allergic inflammation is slight. As well, the use of recombinant type 1 cytokines as a method to reverse and/ or deviate the immune response has met with limited success. Due to our inability to knock out genes in human subjects compounded with the fact that removal of signaling molecules (eg B71 or B72) is immunologically inappropriate results in our need for understanding the pathways involved in the maintenance of allergy. Key molecules involved in recruiting type 2 cells such as Th2, mast cells, basophils, and eosinophils may help us to identify a possible target for therapy. Type 2 cells as well as the cytokines and chemokines they produce that are directly responsible for the recruitment of inflammatory cells in atopic disease are currently of great interest especially in terms of a possible therapeutic.

8.6 Polyclonal and allergen driven immune responses

To measure cytokine/chemokine production by allergen-specific Th2 cells there are many benefits in utilizing antigen, as opposed to polyclonal activators such as anti-CD3 and PHA. Firstly only the antigen-specific cells will respond to the antigen which accounts for approximately 0.01-0.001% of the T cell repertoire. In contrast, polyclonal activators often stimulate virtually all of the T cell population. Thus, stimulation of cells that would not normally be stimulated *in vivo* drastically compromises the relevance of the data. Secondly, the receptor through which activation by antigen is signalled is the same pathway as the one activated in vivo. In contrast different receptors and pathways are involved in signaling of polyclonal activators. When different receptors and pathways are used for activation the result can be qualitatively and quantitatively different.

Differences in antigen stimulation and polyclonal stimulation were shown directly by Imada et al in a study of grass pollen allergic and non-allergic subjects [189]. In response to allergen the atopic subjects displayed an enhanced IL-4 and IL-10 levels as well as decreased IFNγ production in comparison with non-atopic subjects. However, these differences were not seen with the use of polyclonal activator. In addition, they demonstrate that specific polyclonal activators display global type 1 or type 2 bias in terms of cytokine production. PHA elicited a Th2 dominated response, whereas anti-CD3 elicited a Th1 dominated response regardless of atopy. These results indicate the importance of using antigen stimulation to allow for interpretation of the relative importance of cytokines/chemokines in atopic disease.

8.7 Chemokines

8.7.1 Chemokine Biology

Chemokines (*chemo*tactic cyto*kines*) are relatively small molecular weight proteins (8-12k Da) that were first identified approximately 20 years ago due to their critical involvement in cellular trafficking. [190-194] They are highly pleiotropic proteins produced by virtually every cell under appropriate stimulation and are able to act upon almost every cell type resulting in often overlapping, but not always redundant functions [194-196]. Chemokines are produced by both structural and inflammatory cells under a range of stimuli (LPS, cytokines) or are produced constitutively to maintain homeostasis within microenvironments such as lymph nodes [191, 194, 197-201]. A remarkable feature of chemokines is their promiscuity with respect to receptor ligand interactions. In contrast to cytokines, chemokines may bind multiple chemokine receptors (eg RANTES binds CCR1, CCR3 and CCR5) as well as multiple chemokines may be specific for one receptor (eg IP-10, Mig, I-TAC bind CXCR3). Multiple possible receptor ligand interactions may be due to redundancy or may represent a tightly regulated system resulting in discrete functions based on time or location of expression.

Chemokines demonstrate a range (20-70%) of homology with each other, but retain striking similarity in their tertiary structures such as 3 conserved β-pleated sheets and a C-terminus α-helix [200, 202]. In addition to structural motifs they exhibit conserved primary sequences distinguishing the two largest subfamilies by the presence (CXC) or

absence (CC) of an intervening amino acid between the first two conserved N-terminal cysteine residues [194]. Currently there are approximately 40 chemokines classified into four families CC, CXC, C, and CXC3C (Table 1) [195, 203]. In addition the CXC family is divided into 2 subfamilies based upon the presence/absence of a tri-peptide sequence consisting of a glutamine-leucine-arginine (ELR). Presence of this tri-peptide has been shown to selectively chemoattract neutrophils, whereas, CXC chemokines lacking the ELR sequence primarily attract lymphocytes [197, 199, 204, 205]. Interestingly, ELR containing chemokines demonstrate angiogenic properties such as promoting endothelial cell growth and chemotaxis. Whereas, non-ELR chemokines tend to promote angiostasis through ill defined mechanisms. [193, 195, 206]

One recent classification system broadly characterizes chemokines based upon the site of production and the eliciting stimuli. Functionally, chemokines can be distinguished as either "inflammatory/inducible" and "homeostatic/constitutive" chemokines [194, 207, 208]. Inflammatory chemokines are produced by structural and inflammatory cells in response to stimuli (LPS, cytokines). Through receptor-ligand interactions they attract effector and memory lymphocytes as well as other cell types of the innate immune system (neutrophils, monocytes) to the site of inflammation. [194, 209-212]

Tissue cells such as stromal cells and endothelial cells primarily produce homeostatic chemokines. They function primarily in maintaining homeostasis in primary and secondary lymphoid organs via chemotaxis (reviewed in [194, 201, 213]. In addition, they are responsible for cell compartmentalization, leukocyte trafficking and hematopoiesis. BCA-1 and SLC are both examples of homestatic chemokines. The importance of these chemokines is evident when a mouse model deletion of SDF-1,

produced a lethal phenotype [214]. A third group within this classification contains both inflammatory and homeostatic characteristics. MDC and TARC are examples of chemokines that are capable of dual roles. They are induced in a variety of cell types (mainly APC) by appropriate stimuli and are constitutively produced at low levels in primary and secondary lymphoid organs. In both systems these molecules are important in bringing APC and T cells together for TCR sampling of MHC-peptide complexes. This merging of inflammatory and homeostatic properties is one of many examples demonstrating the diverse role chemokines play in maintaining a healthy immune system.

A new nomenclature system was recently established to rename chemokines. Chemokines were previously identified based on a range of characteristics often chosen by the individual investigator that has resulted in a patchwork of functionally defining and tissue specific names. To bring order to the nomenclature the CC chemokines are now designated CCL1-27 and the CXC chemokines CXCL1-15. [193, 215]. As this nomenclature is relatively recent, the historic names of chemokines are still commonplace (ie TARC=CCR17). The more widely recognized previous nomenclature is used here for simplicity.

The role of chemokines in directing cellular movements is well established. Chemokines ensure migration of APC to the site of inflammation and then are able to direct movement to lymphoid organs as the APC matures. Within the lymph nodes, chemokine receptor interactions bring T cells and APCs together and are ultimately responsible for directing effector T cells to sites of inflammation [194, 207, 208]. In addition to trafficking, chemokines are key players in angiogenesis/angiostasis [204,

216], homeostasis [217], hematopoiesis [197, 218], inhibition of tumor growth [219], modulation of cytokine responses [220, 221], degranulation of inflammatory cells [194], as well as activation and regulation of the immune system [195, 197, 222, 223]. Although cellular trafficking is an important role within these additional roles, they clearly go above and beyond chemotaxis. The ability of chemokines to influence ongoing immune responses has been of much interest. Specifically, the possible role of chemokines in maintaining inflammation as well as exacerbations may reveal critical new insights into the pathogenesis of inflammatory disorders. Clearly, more work is required to clarify the extent of the involvement of key chemokines in directing inflammatory responses beyond chemotaxis.

Table 1 Chemokine Families and their Receptors

Chemokine Family	Receptor	Subfamiles	Examples (New nomenclature)
CXC (α)	CXCR	ELR NON-ELR	IL-8 (CXCL8) IP-10 (CXCL10)
CC (β)	CCR		Eotaxin (CCL11)
C (γ)	CR		Lymphotactin (CL1)
CX3C (δ)	CX3CR		Fractaline (CX3CL1)

8.7.2 Chemokine Receptors

Chemokines bind seven transmembrane domain receptors coupled to *B. pertussis* toxin-sensitive G-proteins for signal transduction. [190]. In addition, they bind to sulphated proteoglycans present along the surface of endothelial cells forming a gradient and activate chemokine receptors on rolling leukocytes. This is important in leukocyte extravasation and localization at sites of inflammation but does not induce intracellular signaling [191, 204, 224]. To date there are 15 chemokine receptors identified numerically as CCR1-7 and CXCR1-5, as well as XCR1, and CX3CR1 [200, 203, 217, 225, 226].

Chemokine receptors display conserved structural motifs including two conserved cysteines, one in the NH2-terminal domain and the other in the third extracellular loop that are thought to form a disulfide bond critical for the conformation of the ligand-binding pocket [200]. Chemokine receptors are unique in that they only bind chemokines exclusively often displaying selectivity as well as redundancy. However, families of chemokines (CXC, CC, C, CX3C) will only bind the same family of receptors (CXCR, CCR, CR, CX3CR). In addition different chemokine receptors expressed on the same cell can induce specific signals, suggesting receptors are coupled to distinct intracellular pathways. This phenomenon is apparent with the ability of IL-8 to bind to neutrophils via CXCR1 and/or CXCR2. Binding to CXCR1 results in a vastly different cellular response compared to binding CXCR2 regardless of the similar affinity IL8 has for both receptors [227]. While the majority of chemokines may interact with more than one receptor, there are a few examples of ligand-receptor restrictions. Specifically, IP-

10, Mig and I-TAC are restricted to CXCR3 as well as TARC and MDC are exclusive for CCR4.

Binding of chemokine receptors results in the internalization of the receptor ligand complex, proteolytic degradation of the ligand and recycling of receptor to the surface [196, 224]. The intensity of receptor expression may be up-regulated at different stages of maturation, in response to antigenic stimuli or in response to chemokine binding. Loetscher et al demonstrated the ability of IL-2 to up-regulate chemokine receptor expression in lymphocytes [228]. Interestingly, there exists a non-signaling seven transmembrane receptor expressed on erythrocytes and endothelial cells that binds chemokines from both CXC and CC subfamilies and is also a receptor for *Plasmodium vivax* and *Plasmodium knowlesi*. This receptor was named Duffy antigen receptor for chemokines (DARC) [229]. Approximately 70% of African Americans are Duffy negative, most likely as a evolutionary response to malaria infection [230]. Initially, DARC was postulated as a sink for systemic chemokines. Today there is interest in whether relative expression of DARC is related to disorders characterized by excessive inflammation. However, population studies have not found a correlation between lack of DARC expression in African Americans and asthma [197, 231].

Chemokine receptors are unique in that they are part of a select group of cell surface markers that are exclusively expressed on Th1 and Th2 cells. Although this is useful for identification of cell populations, it also indicates a possible mechanism through which chemokine receptor interactions may influence T cell mediated immune responses. Specifically, CXCR3 and CCR5 are restricted to Th1 cell lines [232, 233]. CCR3 and CCR4 are exclusively expressed on Th2 cell lines [234, 235]. In addition the ability of

type 1 cytokines (IFNγ) to enhance CXCR3 ligands as well as type 2 cytokines (IL-4, IL-13) to enhance production of CCR3 and CCR4 ligands has led to the designation of type 1 and type 2 chemokines respectively [60, 236, 237]. In addition elevated IP-10 and Mig (CXCR3 ligands) levels have been documented in type 1 dominated disorders such as multiple sclerosis and rheumatoid arthritis. [238]. MDC, TARC, and eotaxin (CCR4 and CCR3 ligands respectively) levels are elevated in type 2 dominated disorders such as atopy [239, 240]. This existence of chemokine receptor restriction upon Th cell surfaces suggests an active role for chemokines in maintaining ongoing inflammation. The specificity of key chemokine receptors for Th1 and Th2 cells is useful not only as a marker but as an indicator of key chemokines involved in maintaining inflammation.

8.8 Chemokines in Inflammation

The ability of chemokines to recruit various cell types represents a mechanism through which chemokines could mould the development and maintenance of inflammation. The recruitment of Th2 cells, mast cells, eosinophils and neutrophils to the site of initial inflammation results in what is termed the late phase reaction. The influx of these cell types results in inflammation that is often more severe than the initial inflammation caused by IgE cross-linking on mast cells. Recruiting inflammatory cells that contribute to the damage recognized by the late phase reaction is a critical step in which chemokines are directly involved. The chemokine eotaxin has generated considerable interest because of its lineage-specific effect on eosinophils, as opposed to other cell types. Studies of eotaxin-deficient mice demonstrate that eotaxin is important in the early recruitment of eosinophils after allergen challenge [241]. However, eosinophil recruitment at later time points after allergen challenge is eotaxin-independent, suggesting an important role for several additional chemokines.

Over the past five years there is growing evidence that chemokines may participate in much more than cellular trafficking. Specifically, chemokines are able to induce cellular activation and inflammatory mediator release in T cells, mast cells, basophils, and eosinophils. In addition cytokines and chemokines are involved in a tightly controlled amplification loop in which chronic inflammation and/or exacerbations may be manifested. These will be discussed in detail as they relate to the involvement of chemokines in inflammation.

Chemokine Induced Cellular Activation & Mediator Release

Mediators released from T cells, basophils/mast cells, and eosinophils are directly responsible for the intense inflammation experienced upon subsequent exposure to allergen in atopic individuals. Preformed and newly synthesized mediators such as histamine, and prostaglandins produced by activated basophils and masts cells are capable of inducing contraction of smooth muscle, mucus secretion and subsequently participate in eliciting an inflammatory cell infiltrate [11, 12, 242]. Eosinophils release a variety of preformed cytoplasmic granule mediators, such as major basic protein (MBP) and eosinophil cationic protein (ECP) upon activation resulting in significant tissue injury. In addition to these potent mediators, basophils, mast cells and eosinophils release cytokines and chemokines [11, 12, 243-245]. Cytokines and chemokines work to promote cellular infiltrate by promoting adhesion molecule expression.

T cells recruited to the site of inflammation are capable of releasing a range of mediators. Cytokines such as IL-3 and IL-5 work to promote hematopoiesis of CD34+ progenitor cells in the bone marrow to differentiate into eosinophils and mast cells [11, 12, 246]. Chemokines such as TARC, MDC, Eotaxin, IP-10 specifically recruit inflammatory cells. In addition to cellular recruitment, chemokines are capable of promoting maturation and activation of various cell populations involved in the late phase response. The majority of the work in this area has focused on the ability of chemokines to activate basophils and eosinophils as well as promote their development from the bone marrow.

MCP-1, MCP-4, MCP-3, RANTES, MCP-2, MIP-1a and eotaxin are capable of inducing basophil degranulation with varying degrees of potency [240, 247-252]. Interestingly,

chemokine-induced histamine release is rapid (seconds) compared to allergen-IgE interaction [253-255]. Co-stimulation with IL-5, a known promoter of basophil development, primes basophils to further augment histamine release by chemokines [254, 256]. RANTES, MCP-3, MCP-4, eotaxin, and eotaxin-2 induce eosinophils to release granule proteins and reactive oxygen species [247, 248, 257-261].

The ability of chemokines to stimulate activation and maturation of key cell populations brings forth an important new role for chemokines in inflammation.

Amplification of Type 1/Type 2 Immune Responses by Chemokines

The discovery of discrete sets of chemokine receptors on Th1 (CXCR3, CCR5) and Th2 (CCR3, CCR4) cell lines has led investigators to speculate about a possible mechanism through which chemokines could be involved in the development and maintenance of polarized type 1 and type 2 immune responses. In addition to receptor expression, certain chemokines are regulated by type 1 and type 2 cytokines. Specifically, Mig and IP-10 are induced by IFNγ produced primarily by T cell and NK cells [236]. Preferential recruitment of Th1 IFNγ producing cells via CXCR3 to the site of inflammation will induce an increase in IP-10 and Mig expression. This characteristic is thought to be important in maintaining an amplification circuit that retains Th1 cells at the site of inflammation. A similar feedback system is thought to operate in type 2 disorders such as atopy. MDC production by monocytes is enhanced by exogenous IL-4 and IL-13 [262]. The exclusive expression of CCR4 on Th2 cell lines in combination with the restriction of MDC for CCR4 has led many investigators to speculate on the involvement of MDC in atopic disorders. Unfortunately, to date the majority of studies have

exclusively examined systemic levels of chemokines as well as receptor expression on tissue sections from sites of inflammation [240, 262-271].

Investigation of the relative intensity of type 1 and type 2 chemokine production within individuals with type 1 or type 2 disorders is essential to determine the extent of the involvement of these potential mediators in chronic inflammation.

Type 1 associated diseases

Multiple sclerosis (MS) is a chronic relapsing neuroinflammatory disease in which inappropriate recognition of an autoantigen in myelinated nerve fibres recruits T lymphocytes and APC into the central nervous system [272]. Th1-associated immunopathology often results and is exacerbated by injection of IFNγ [273]. Expression of Th1 chemokine receptors, CXCR3 and CCR5 is up-regulated on fresh CD3+ cells in the circulation of MS patients [274, 275]. In addition, brain tissue samples from MS cadavers revealed marked up-regulation of these receptors and their cognate ligands, IP-10, Mig, and RANTES [276]. Similar to MS, rheumatoid arthritis is characterized by autoimmune responses resulting in T cell mediated inflammation mainly associated with a type 1 response. Inflammation often occurs in the synovium resulting in stiffness and swelling in the joints. CXCR3 and CCR5 are highly expressed in both circulating and synovial fluid lymphocytes. In addition, CCR5 and it's ligands are elevated in the synovial fluid of RA patients [277-279].

Interestingly, there exists a naturally occurring 32 basepair deletion in the CCR5 gene termed CCR5 Δ32. Individuals homozygous for this mutation do not express functional

CCR5 receptors and heterozygous individuals have reduced levels. Surprisingly, there is no significant difference in the allele frequency of the CCR5 Δ32 gene between MS or RA patients and the control population suggesting that CCR5 (and it's corresponding ligands) may not be key for the development of MS or RA. However, there are few homozygous individuals and heterozygous individuals do experience reduced symptoms, slower progression and delayed onset of disease suggesting that CCR5 may be important but not critical to the development of RA or MS [280-282].

Type 2 associated disorders

As mentioned in detail earlier, allergy is a result of a type 2 biased cytokine response towards an allergen. The involvement of chemokines in the late phase response of allergic inflammation has been studied extensively. Biopsies of mucosa from allergic asthmatic individuals contain elevated eotaxin levels [240]. Eotaxin production by multiple cell types including lung epithelium, smooth muscle cells and eosinophils can induce signals in both tissue and blood eosinophils [240, 257, 283, 284]. T. Pullerits et al demonstrated elevated eotaxin mRNA in the nasal mucosa of patients with allergic rhinitis after provocation with allergen [271]. However, an independent study examining the nasal mucosa of allergic rhinitis patients did not see an increase in eotaxin positive cells after nasal provocation with 10 000 BU of grass pollen extract [285]. Nonetheless, Teixeira M et al and Heath et al demonstrate in a mouse and human model respectively the ability to inhibit eosinophil recruitment by blocking the CCR3 receptor with an antibody [286, 287]. As well, Rothenberg et al demonstrated that deletion of the eotaxin gene (-/-) resulted in a significant, but not total, (70%) reduction of airway eosinophilia in

ovalbumin-sensitized mice, and a 50% reduction in antigen-induced corneal eosinophilia [241]. Although the reductions in eosinophilia are substantial they do indicate the involvement of other molecules, most likely alternative ligands for the CCR3 receptor such as RANTES, MCP-1, MCP-3, MCP-4 and MIP-1α [241]. It is important to remember that prevention of eosinophil recruitment does not ensure prevention of atopy symptoms, a finding clearly demonstrated by the clincal trials using blocking mAb to IL-5 [90]. Mast cell and basophil degranulation is still capable of causing many of the symptoms of atopy. In addition, blocking CCR3 does not prevent type 2 cytokine production and memory generation, thus a constant level of circulating blocking antibody to is required to prevent eosinophil recruitment.

The involvement of specific chemokines in inflammatory disorders has led to selection of several candidates for development of potential therapeutics to treat atopic disorders. The identification of the regulatory mechanisms driving the initiation and intensity of these candidate chemokines is required to allow critical analysis of their involvement in inflammatory disorders such as atopy.

Possible Candidate Chemokines

To study the involvement of chemokines in maintaining inflammatory responses such as atopy, selection of candidates that contain the following properties are essential:

i) Documented elevated/decreased levels of chemokine or cognate receptor in type 1 or type 2 dominated disorders (Multiple Sclerosis, asthma)

ii) Ability to specifically recruit Th1 or Th2-like cells as well as other relevant effector cells to the site of inflammation via chemokine receptors.

With such restrictions there exist a limited number of possible candidates that are known promoters of type 1 and type 2 immunity through discrete mechanisms.

IP-10 and Mig

IFN(inducible protein of 10 kDa (IP-10) and monokine induced by IFNγ (Mig) are related chemokines of the non-ELR CXC subfamily. Both induce T cell chemotaxis (*in vitro*) exclusively through CXCR3 at concentrations within 10-50 ng/ml [288-290]. As their names suggest, they are induced by IFNγ in various cell types such as keratinocytes, neutrophils, endothelial and epithelial cells [291-298].

Approximately 40% of resting T lymphocytes and a low number of B cells and NK cells stain positive for CXCR3 but these cells did not respond to IP-10 or Mig. However, treatment with IL-2 for 10 days resulted in fully responsive CXCR3+/CD45RO+ T cells [88, 290, 299]. CXCR3 expression is elevated in Th0 and Th1 lymphocytes [232] and IP-10 levels are elevated in Th1-type disorders such as RA and MS [275, 300-302]. Interestingly, a recent study by our lab clearly demonstrates the ability of exogenous rIP-10 to enhance allergen-stimulated IFNγ production in PBMC from non-atopic, but not

atopic, subjects. Interestingly, rIP-10 addition to allergen-stimulated PBMC did not alter IL-4 levels in non-atopics or atopics [220]. Due to these observations, it has been suggested that locally high concentrations of IFNγ up-regulate IP-10 and Mig, which then results in the recruitment of activated/effector T cells, thereby initiating and maintaining the effector arm of type 1 immunity [303].

TARC and MDC

Thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) are recently identified members of the CC family [304, 305]. TARC and MDC are both homeostatic and inflammatory chemokines. TARC is expressed constitutively in the thymus and transiently in PHA-stimulated PBMC and LPS stimulated dendritic cells [209, 304].

MDC is constitutively produced by dendritic cells, B cells, macrophages, and thymic medullary epithelial cells [306, 307]. Monocytes, NK cells and CD4+T lymphocytes produce MDC only upon appropriate stimulation [306]. TARC and MDC binds exclusively to CCR4, which is found primarily on T cells, dendritic cells, macrophages and to a lesser degree NK cells [304, 305, 308-311]. Interestingly, CCR4 is exclusively expressed upon Th2 cell lines and chronically activated Th2 cells [232, 233, 306]. MDC production from human monocytes is elevated in response to exogenous IL-13 and IL-4 and is suppressed by IFN(, as well as IL-12 [237, 268, 306, 312]. In addition, MDC was detected in the sera of individuals suffering from Th2 disorders such as atopic dermatitis [268].

Eotaxin

Eotaxin is a member of CC family of chemokines and is selective in binding specifically to CCR3. Additional chemokines are able to bind to CCR3 such as RANTES, MCP-1, MCP-3 and MCP-4 [240, 250, 251]. CCR3 is primarily expressed on eosinophils, basophils, mast cells and to a lesser degree T cells [239, 249, 313, 314]. Today there is a compelling amount of information indicating that CCR3 ligands are involved in both the migration and the activation of eosinophils and basophils during an allergic response [247-249]. However, eotaxin is the most selective and effective inducer of eosinophil chemotaxis [239]. Extensive infiltration of eosinophils into the lung is not only a hallmark of allergic asthma but also contributes to much of the damage of respiratory epithelium during the late phase response [315, 316]. In addition, atopic asthmatic subjects have high concentrations of eotaxin in the BAL fluid and an increase in the expression of eotaxin mRNA and protein in the epithelium and submucosa of their airways when compared to normal controls [252]. The expression on CCR3 on type 2 cellular mediators combined with the presence of elevated levels of eotaxin within type 2 inflammatory environments makes eotaxin an attractive candidate for pathogenesis of allergic disorders. Interestingly, several groups have demonstrated the ability of type 2 cytokines, IL-4 and IL-13, to elevate eotaxin levels from human dermal fibroblasts as well as murine airway epithelial cells [317]. Together these characteristics demonstrate a key mediator of the late phase response most likely through a combination of chemotaxis of inflammatory cells and their subsequent activation.

Rationale

A common characteristic of these possible candidates is a lack of information concerning the intensity of allergen driven chemokine responses in non-atopic versus atopic individuals. Atopic disorders are complex with multiple factors involved. Analysis of direct ex vivo responses of PBMC to allergen is critical for defining possible associations between atopic disease and altered chemokine production or responsiveness. The relative intensity of type 1 and type 2 chemokines in atopic disease requires elucidation as numerous studies have exclusively examined single specific chemokines only. The possibility that all "inflammatory" chemokines are upregulated during an immune response to allergen has not been addressed. Analysis of the intensity of allergen driven type 1 and type 2 chemokine production in atopic and non-atopics may indicate an underlying mechanism by which inflammatory disorders are regulated. As well, regulatory factors involved in driving possible differences between these two populations should be addressed.

8.9 Summary of Project

We hypothesize that type 1 and type 2 chemokine production may determine the ability of representative type 1 and type 2 chemokines to serve as potential markers of atopic disease *in vivo* and *in vitro*. In addition, we hypothesized that there are several parameters involved in regulating the initiation and intensity of type 1 and type 2 chemokine production *in vitro*. We speculate that identification of the differences between type 1 and type 2 chemokines *in vivo* and/or *in vitro* is critical to understanding the involvement of chemokines in atopic disease.

Utilizing clinical status as a parameter we investigated whether the presence and intensity of type 1 (IP-10, Mig) and type 2 (Eotaxin, TARC) chemokines *in vivo* could be used as a potential marker of allergic rhinitis. Additionally, we developed a novel *in vitro* culture system to quantify allergen-driven type 1 and type 2 chemokine production from PBMC taken directly *ex vivo*. We made use of subjects exhibiting a spectrum of grass pollen sensitivity by examining (1) non-atopic, (2) grass atopic and (3) grass atopic mild to moderate asthmatic subjects allowing for detailed examination of a range of disease severity.

One strength of this study lies in the use of fresh PBMC directly *ex vivo* to analyze allergen-driven (as opposed to polyclonal driven) analysis of chemokine production from a mixed population of T cells, B cell and APC. Utilizing an allergen-driven system enabled us to examine if type 1 and type 2 chemokine production was differentially regulated in response to an allergen in allergic versus non-allergic subjects. We tested a range of allergen concentrations for grass pollen and house dust mite, as well as a

non-allergenic bacterial antigen, (streptokinase) to determine the optimal concentration of antigen for chemokine production. We compared antigen driven chemokine production to commonly used polyclonal activators (TSST-1 and PHA) to determine whether the balance and intensity of type 1 and type 2 chemokine production was dependent upon the type of stimulus used. Usage of allergen-driven PBMC directly *ex vivo* allowed detailed analysis of allergen-specific type 1 and type 2 chemokine production based upon clinical status. Specifically, we were able to critically examine: 1) Potential differences in allergen-driven chemokine production between non-atopic and atopic/ atopic mild asthmatics. 2) Regulation of representative type 1 (Mig) and type 2 (TARC) chemokine production by exogenously added and endogenously produced cytokines (IFNγ, IL-10, IL-13 and IL-4). 3) Activation requirements to allow for the initiation of allergen-driven chemokine production. 4) Immunocytochemical analysis of populations within PBMC to determine which are responsible for Mig production *in vitro*.

9 Materials and Methods

9.1 Subjects

Blood was obtained from healthy non-atopic, grass atopic, and grass atopic mild asthmatic subjects, randomly recruited subjects between 23 and 45 years of age. Clinical diagnosis of allergy was determined using: 1) skin prick test to 14 different allergens including a grass pollen mixture (Mixture #1649 containing June/Kentucky Blue grass, Timothy grass, Orchard grass, Brome grass and Red-top grasses, Hollister-Stier/Miles, Canada, Toronto, Ontario) and 2) A minimum two year documented history of seasonal allergic rhinitis. We recruited 20 grass atopic subjects as well as 20 non-atopic volunteers who had no history of asthma or other allergic disorders including allergic rhinitis. Non-atopic volunteers exhibited negative epicutaneous tests to the grass pollen mix used in this study.

We recruited 20 mild to moderate asthmatics based upon prior diagnosis of asthma as well as substantial improvement in spirometry tests after administration of Ventolin. Mild asthmatics chosen for this study were not currently (3 days prior to blood collection) using anti-inflammatory medication such as anti-histamines, corticosteroids or β2 agonists. All participants were between the ages of 23 and 45 years of age. This study was approved by the University of Manitoba Faculty Committee on the Use of Human Subjects in Research and written informed consent was obtained from each individual.

9.2 Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Plasma

Peripheral blood (40 ml) was collected in March/April (1999, 2000, 2001), July/August (1999/2000, 2001) and November (2000) by venipuncture into 2ml of 2.7% EDTA (Sigma Oakville, ON, Canada). Peripheral blood was diluted by ½ in 0.85%NaCl (40ml blood + 20 ml 0.85% NaCl). Diluted blood (10ml) was layered onto a 3.5 ml Ficoll gradient (Sigma) and centrifuged at 460 x g for 30 minutes (Eppendorf Centrifuge 5804) to separate mononuclear cells from polymorphonuclear cells, platelets and red blood cells. Platelet-poor plasma was collected after centrifugation. Usually, plasma was treated with 0.5% NP40 (Sigma) and stored at –20 C until analysis of plasma chemokine levels. Preliminary experiments demonstrated that NP40 treatment or freeeze-thawing did not affect the ability to quantify plasma chemokines, (Table 3). "Buffy coat" of PBMC between plasma and Ficoll fluid was carefully collected and washed twice in 0.85% NaCl. Cells were resuspended in 10% fetal calf serum in RPMI-1640 (Sigma) with Penicillin/Streptomycin/Fungisome (P/S/F) and 2-mercaptoethanol. PBMC concentration was calculated using a hemocytometer. Trypan blue was used for viability calculations. >98% viability was obtained in all samples collected.

9.3 Chemokine ELISAs

Human plasma chemokine levels were determined using specific sandwich ELISAs for IP-10, Mig, TARC, Eotaxin, MCP-1 and MDC developed by the candidate. Recombinant human chemokines, anti-chemokine capture MAbs and biotinylated anti-chemokine detection Abs were obtained from R&D Systems (Minneapolis, MN) and Peprotech (Rocky Hill, NJ). ELISAs were optimized using monoclonal capture

antibodies, (mouse anti-human IgG) and polyclonal biotinylated detection antibodies, (goat anti-human IgG). Mouse anti-human and goat anti-human antibodies were developed by the manufacturers and were used to identify and capture human chemokines specifically. ELISAs were developed using streptavidin-alkaline phosphatase (Jackson –Immunoresearch). Using a 3-fold range of both capture and biotinylated antibodies we tested each antibody concentration independently to determine the optimal concentration of antibody for maximum sensitivity (Figure 2). Chemokine protein was quantified in reference to serial dilutions of recombinant standards falling on the linear part of the standard curve for each specific chemokine measured. Unknown samples were quantified by averaging the serial dilutions (4-8X) of each sample against the standard curve. The sensitivities of the chemokine ELISAs' are as follows:

Table 2 Chemokine ELISA development time and sensitivity

CHEMOKINE	DURATION OF ASSAY DEVELOPMENT (HOURS)	STANDARD CURVE	ASSAY SENSITIVITY
MDC	2	2000-15.6pg/ml	31.3 pg/ml
TARC	3	500-3.9pg/ml	3.9 pg/ml
IP-10	4	1000-7.8pg/ml	7.8 pg/ml
MIG	5	4000-31.3pg/ml	31.3 pg/ml
MCP-1	5	4000-31.3pg/ml	62.6 pg/ml
EOTAXIN	3	1000-7.8pg/ml	15.6 pg/ml

Sensitivity of an ELISA assay refers to the ability of the coating and biotinylate antibody pair to detect the presence of a known amount of recombinant chemokine (standard

curve) with absorbance values above background/control conditions. Control wells contain both coating and biotinylated antibody but did not contain any recombinant chemokine specific for that antibody pair. For example the control wells for the MDC ELISA display absorbance values comparable to those seen when 15.6pg/ml of recombinant human MDC were added to the standard curve well. However, absorbance values for 31.3pg/ml are well above those seen in the control wells. Thus, the MDC ELISA can not measure levels of MDC below 31.3pg/ml and is therefore stated to have an assay sensivity of 31.3pg/ml.

9.4 PBMC culture

PBMC were suspended in complete media (RPMI-1640, 10% heat inactivated fetal bovine serum, P/S/F, and 2-mercaptoethanol) at a concentration of 2.5 x 10^6 cells/ml. 250 000 cells were cultured with optimized antigen concentrations. Time course and antigen concentration was determined by preliminary experiments (Figure 9).

Antigens: Grass Pollen Mixture--Timothy, Kentucky Blue, Red Top and Orchard grasses (Hollister-Stier/Miles, Canada, Grass Mix 1649)

Crude House Dust Mite—D. pteronyssinus (Meridian Biomedical Inc.)

Streptokinase—Streptase (Hoechst, New York, NY)

Recombinant human cytokines: rhIL-10, rhIL-13, rhIL-4 (Peprotech), and rhIFNγ (NIH Standard)

Blocking antibodies: mouse anti-human IL-10 (ATCC, Manassas, VA)

Mouse anti-human IL-13 (Peprotech)

Mouse anti-human IL-4 (Pharmingen)

Mouse anti-human IFNγ, CD4, HLA-DR, HLA-DR/DP/DQ, B7-1, B7-2 (BD Pharmingen, Mississauga, ON)

Mouse anti-human CTLA-4Ig—(Gift from Dr. P. Nickerson, University of Manitoba)

Mouse IgG1 isotype matched antibody (Pharmingen)

Mouse IgG2a isotype match antibody (Pharmingen)

Cells were cultured for 16 hours to 7 days depending on the chemokine analyzed or antigen used (e.g. Grass stimulation-day 5, TSST-1 stimulation-day 3) and were kept at 37°C with 5% CO_2. Supernatants were harvested and stored at –20°C until chemokine concentrations could be quantified.

9.5 Flow Cytometry

Whole blood (40ml) was collected by venipuncture into 2 ml of 2.7% EDTA. 10ul of whole blood was allocated for flow cytometry. Antibodies that were used in flow cytometry were as follows: Mouse anti-human Fy6b mAb (BD Pharmingen) was used to identify an isotype of DARC. FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used as a secondary antibody to detect Fy6 MAb antibody. Isotype matched control Ab (BD Pharmingen) were used with each blood sample to determine specificity of goat anti-mouse mAb-FITC.

Each 10ul sample of whole blood was stained with MAb (Fy6 or Isotype match IgG) for 45 minutes at 4 C and then washed twice with FACS washing solution (1% BSA in PBS with 0.02% NaN3). Secondary FITC-Ab was then added for 45 minutes at 4 C in the dark and then washed twice with FACS washing solution. Cells were then resuspended in 0.5ml 2% paraformaldehyde in PBS prior to analysis on a Beckman-Coulter Flow Cytometer-EPICS ULTRA CELL SORTER that was calibrated daily with CaliBRITE beads. Acquisition and analysis of data was determined using EXPO 2 software. Staining of duplicate blood sample aliquots revealed less than 0.5% variation inherent in the procedure (data not shown).

Chemokine receptor expression on CD4+ antigen experienced (CD45RO+) PBMC were measured by 3 color flow cytometry. Cy-Chrome-labeled anti-human CD4 and PE-labeled anti-human CD45RO (both from Pharmingen) were used to identify CD4+/CD45RO+ cells. Mouse IgG1 anti-human CXCR3 and mouse anti-human IgG2b anti-human CCR3 (both FITC-labeled antibody from R& D Systems) were used to determine chemokine receptor expression levels. FITC-labeled control mAbs (R&D Systems), isotype matched to the anti-chemokine receptor mAbs, were used with each blood sample to determine background fluoresence and set quandrant markers for scatter plots. For each sample, 50ul of whole blood was stained with combinations of mAbs for 15 minutes at room temperature and red blood cells lysed with 1X FACS lysing solution (Becton Dickenson, San Jose, CA) according to the manufacturer's protocol. Cells were then resuspended in 0.5 ml 2% paraformaldehyde in PBS prior to analysis on a FACSCalibur flow cytometer (Becton Dickenson) calibrated daily with CaliBRITE beads. For each sample, lymphocytes (1 X104 events) were gated based

on forward and side scatter parameter. A secondary gate was drawn around the CD4+ population to allow analysis of chemokine receptor expression on the CD45RO+/- subsets on CD4+ cells. Data were analyzed using CellQuest software (Becton Dickenson).

9.6 Immunocytochemistry

PBMC were obtained from healthy and atopic individuals (described above). Golgi plug (Pharmingen) was added to cells 6 hours prior to harvest to prevent export of the chemokine from the Golgi Apparatus to the endocytic vesicles and out of the cell. Cells were washed twice and resuspended in Hanks' buffer (Sigma, St. Louis, MO). 1-2 x 10^6 cells were placed on adhesive slides, (slides treated with poly-L-lysine from Sigma) for 10 minutes at room temperature. Cells were then fixed with 2%paraformaldehyde for 20 minutes at 4°C and then washed three times with Hanks buffer. Slides were then blocked with 2% FCS in Hanks buffer for 10 minutes at 37°C. Slides were then washed three times with Hanks buffer, dried and placed at –20°C until staining. Cells were hydrated and stained for surface CD3 using mouse anti-human CD3-FITC MAb (Pharmingen) for 2 hours at room temperature. Cells were then washed three times with buffer. Cells were permeabilized using 0.1% saponin (Sigma) in Hanks buffer. Cells were first stained with goat anti-human polyclonal anti-Mig Ab or isotype matched goat IgG for 1 hour at room temperature. Slides were washed three times and stained with donkey anti-goat Cy3 conjugated MAb (Jackson ImmunoResearch Laboratories Inc.) for 2 hours. Slides were then washed three times and mounted using mounting media containing Mowiol (CALBIOCHEM, LaJolla CA). Observed using an OlympusBX60 EpiFluorescence microscope fitted with a CCD camera (Melville, NY).

Acquisition and analysis of data was performed using Image Pro Version 4.1.0.2 (Media cybernetics, Silver Spring Maryland).

9.7 Statistical Analysis

Chemokine values were converted to base-10 logarithms to satisfy normality assumptions for statistical analysis. Correlations were determined with parametric statistics (Pearson product-moment correlation coefficient). Associations were determined with non-parametric (Mann-Whitney U) and parametric statistics (Student's T test) with raw and log converted data respectively. All statistics were performed using SPSS version 10.0 software (SPSS Inc., Chicago, IL) and/or PRIZM (PRIZM Inc., San Diego CA). All p values shown are 2-tailed.

10 Results

10.1 Characterization of Subjects and Isolation of PBMC

Characterization of Subjects

We randomly recruited healthy non-atopic, grass atopic, as well as grass atopic mild asthmatic individuals before (winter), during (summer) the grass pollen season for this study. The atopic status of the subjects was characterized using a skin prick test including a panel of 14 seasonal and perennial allergens as well as clinical history. A grass atopic individual was defined as a subject with a positive skin prick test as well as a clinical history of sensitivity to grass pollen such as itchy eyes, sneezing, or bronchoconstriction. As a precaution any anti-inflammatory medications (eg anti-histamines) utilized by the subjects were screened to prevent any false negative skin results. The intensity of the inflammatory response experienced during the grass pollen season was combined with the skin test results to determine a severity score that was given to each atopic subject ranging from 1-5. All of the atopic individuals enrolled displayed severity symptoms equal to or above 3+ with the majority of the recruited subjects displaying symptoms equal to 4+.

The presence of allergy induced asthma or mild asthma was determined by examining clinical history (previous diagnoses) as well as a spirometry test before and after administration of Ventolin. Ventolin is a bronchodilator and was administered to the atopic patients who reported wheezing during exposure to allergen. In all cases, spirometry tests after administration of ventolin improved. The combination of clinical history of asthma and grass pollen sensitivity and significant improvement in forced

expired volume (FEV) after treatment with Ventolin, and skin test reactivity to grass pollen determined atopic asthmatic classification.

Isolation of PBMC

Peripheral blood was taken via venupuncture into a 50ml sterile polypropylene tube containing 2% EDTA final. A Ficoll density gradient was used to separate peripheral blood mononuclear cells (PBMC) from RBC, polymorphonuclear cells as well as plasma. In figure 1 we identify the layers of blood components resulting from centrifugation of whole peripheral blood on a Ficoll density gradient. Within this thesis we identified the presence and the intensity of type 1 and type 2 chemokines within each layer. Specifically:

(1) The top layer contains plasma, which is the fluid that the circulating cells are immersed in. It contains several secreted proteins from the tissue and/or from the circulating cells. We were interested in the presence of chemokines within the plasma. Upon separation of peripheral blood we allocated several milliliters of plasma for the purpose of chemokine analysis. Plasma was treated with NP-40 and NaN_3 for safety reasons and to allow for non-sterile identification and measurement of plasma chemokines in an ELISA. NP-40 is a surfactant that disrupts the plasma membrane and results in lysis of bacteria, enveloped viruses, as well as eukaryotic cells. NaN_3 interferes with cellular respiration resulting in arrest of cell division and eventually results in prokaryotic and eukaryotic cell death. The potential ability of NP-40 and NaN_3 to alter chemokine levels (ie as an assay confounder) was determined by comparing both CXC and CC chemokine levels in treated and untreated plasma samples (Table 3) via a chemokine specific ELISA that we developed (Figure 2). Mig, IP-10, Eotaxin and

TARC levels were not significantly altered by the addition of NP-40 and NaN3 therefore allowing usage of these chemicals for safety purposes of the investigator and the plasma sample from bacterial contamination.

There are several studies to date that examine the presence and/or intensity of plasma chemokines from peripheral blood treated with a range of possible anti-coagulants such as heparin, EDTA or sodium citrate. Additionally, allowing peripheral blood to coagulate and separate results in isolation of serum which has been demonstrate to contain several types of chemokines [266, 268, 318]. We examined the ability of different anti-coagulants to alter the presence or intensity of plasma chemokines. Peripheral blood from the same subject was left to coagulate (serum) or treated with EDTA, Heparin or sodium citrate. Treatment of peripheral blood with different anti-coagulants resulted in strikingly different chemokine levels. Plasma chemokines (IP-10, Mig, Eotaxin, TARC) isolated from blood treated with EDTA, Heparin or nothing (serum) revealed very similar levels. Surprisingly sodium citrate treatment resulted in substantially lower plasma chemokine levels compared to peripheral blood treated with EDTA, Heparin, or nothing (serum). Further subjects are required to confirm the statistical significance of these results (Table 4).

Therefore we argue that the addition of NP-40 and NaN_3 to plasma isolated from peripheral blood treated with EDTA does not alter systemic CC or CXC chemokines.

Table 3 NP-40 and NaN3 addition to plasma does not alter analysis of Mig, IP-10, TARC or Eotaxin levels

Subject	+NP-40+NaN3	Mig (pg/ml)	IP-10 (pg/ml)	Eotaxin (pg/ml)	TARC (pg/ml)
# 067	No	4140	140	85	45
# 067	Yes	4450	180	90	50
# 066	No	420	105	40	10
# 066	Yes	400	115	35	10

Table 4 Choice of anti-coagulant for peripheral blood may alter evaluation of plasma chemokine levels

Condition	Mig (pg/ml)	IP-10 (pg/ml)	Eotaxin (pg/ml)	TARC (pg/ml)
Subject #066				
Heparin	500	135	45	20
Citrate	320	100	20	10
EDTA	400	115	35	10
Serum	430	135	40	15
Subject # 067				
Heparin	4370	190	235	60
Citrate	3510	145	75	35
EDTA	4450	180	90	50
Serum	4650	155	100	110

(2) PBMC are comprised of mainly CD4+ and CD8+ T cells, B cells, NK cells, monocytes, macrophages and possibly dendritic cells. To examine whether polymorphonuclear cells such as basophils or neutrophils were contaminants within the PBMC, we randomly selected a freshly isolated sample of PBMC. Staining the cells with Wrights' stain and using physiological markers we counted the number of mononuclear cells and polymorphonuclear cells based upon

morphology (Table 5). We observed virtually no (<3%) polymorphonuclear cells within the sample. This is important because it indicates that any chemokine production measured *in vitro* is restricted to the mononuclear cell populations. PBMC were placed into short-term primary culture immediately after isolation. We utilized a range of different types of antigens to compare the differences between the intensity of stimuli and resulting chemokine production. Specifically, PBMC were stimulated with either grass pollen, house dust mite, streptokinase, TSST-1 or PHA. Grass pollen is a seasonal allergen peaking in atmospheric concentrations during the summer months. House dust mite is present throughout the year and is commonly at high concentrations in mattresses as well as carpets [319, 320]. Streptokinase is an enzymatic component of extracellular streptococcal bacteria and is a commonly used recall antigen. The great majority of the population have been previously exposed to this antigen are able to mount a memory response resulting in an expanded antigen specific T cell population. TSST-1 is a superantigen that binds simultaneously to the TCR (Vβ chain) and the (α chain) of MHC II. TSST-1 targets specific types of TCR chains as well as MHC II resulting in activation of approximately 2-20 % of the CD4+ cell repertoire. PHA (phytohemagglutinin) is a polyclonal activator that binds to carbohydrate chains found on the surface of T cells. αCD3 Ab binds to the CD3 complex of the TCR. Aggregation of the Ab results in the activation of the TCR.

Chemokines present in the supernatants of stimulated and untreated cells were analyzed using a chemokine specific sandwich ELISA that we developed to allow

for ultra-sensitive measurement of extremely low concentrations of chemokine (<5pg/ml) (Figure 2). Analysis of polyclonal as well as allergen-driven chemokine and cytokine production was performed on all three subject groups. We examined the underlying mechanisms regulating the intensity of allergen-driven chemokine production such as exogenous and endogenous type 1 and type 2 cytokines. In addition, we examined the requirements of critical co-receptors and co-stimulatory molecules involved in antigen presentation for the initiation of chemokine production.

(3) Using flow cytometry we were able to analyze the presence and frequency of chemokine receptors present on PBMC in atopic and non-atopic populations. Specifically, CXCR3 and CCR3 expression on CD4+/CD45RO+ and CD8+/CD45RO+ T cells was analyzed using four color flow cytometry. Freshly isolated PBMC were stained with the appropriate monoclonal antibodies and kept on ice until analysis could be performed.

(4) Erythrocytes (RBC) were examined for the presence of the Duffy antigen receptor for chemokines (DARC). DARC is a seven transmembrane cell surface protein that indiscriminately binds both CXC and CC chemokines, reviewed in [197]. DARC is also a critical receptor for entry of malaria into RBC. Approximately 70% of African Americans are negative for the gene responsible for DARC probably as an evolutionary acquired resistance to malaria. We were interested in examining differences, if any, in the frequency and/or intensity, of DARC expression on atopic and non-atopic individuals. Differences in frequency or intensity of DARC expression is important because of the possibility that

reduced expression of DARC on RBC may result in extended exposure of the vascular endothelium to systemic chemokines. We obtained a monoclonal antibody specific DARC (Fy6) and examined the frequency and intensity in comparison to isotype matched control antibodies via flow cytometry in both atopic and non-atopic populations.

Table 5 Percentages of cell types found within PBMC isolated from human peripheral blood via Ficoll gradient purification

Subject	Lymphocytes	Monocytes	Basophils	Neutrophils
# 068	71.3 %	27 %	0.6 %	0.9 %
# 002	65.84 %	31 %	1.4 %	1.6 %

Figure 1 Overview of isolation of human Plasma, PBMC, and RBC from peripheral blood

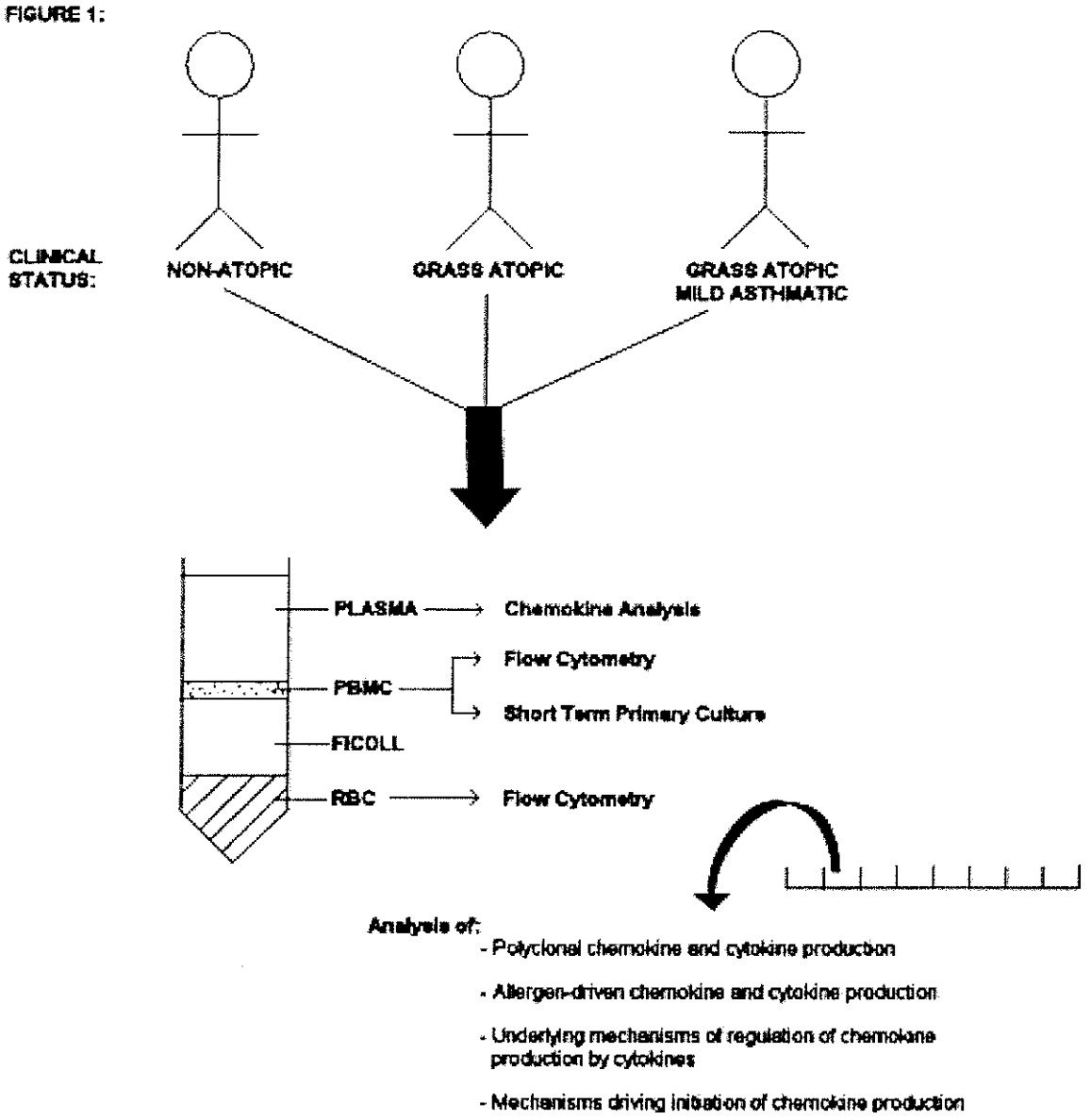

Figure was courtesy of Karen Morrow (Department of Immunology)

10.2 Development of Sensitive Chemokine ELISAs

The goal of this study was to examine allergen-driven chemokine production in human PBMC isolated from atopic and non-atopic populations. Considering the limitations discussed earlier that are associated with the usage of polyclonal activation of PBMC we decided to perform direct *ex vivo* analysis of allergen-driven chemokine production in atopic and non-atopic populations, an approach we believe to be more reflective of the *in vivo* immune response to allergen. Identification of a predisposition towards type 1 or type 2 chemokine production in response to allergen would be beneficial towards understanding the role of chemokines in allergic disease. Due to the fact that allergen-driven chemokine production is of much lower intensity compared to polyclonal activation in long term T cell clones or hybridomas, a prerequisite for successful detection of chemokine synthesis in this study was the availability of highly sensitive and specific chemokine measurement techniques. We therefore optimized a chemokine specific sandwich ELISA to allow for measurement of low chemokine concentrations as low as 4 pg/ml.

Optimizing TARC ELISA: Representative Chemokine ELISA

Highly sensitive chemokine ELISA's were developed for the measurement of IP-10, Mig, TARC, Eotaxin, MCP-1 and MDC. I will describe in detail how the TARC ELISA was optimized as it is representative of how additional chemokine ELISAs were optimized.

Using purified anti-TARC mAb (R & D systems MAB 366) and biotinylated anti-TARC polyclonal Ab (R & D systems BAF 366) as capture and developing mAbs respectively, this assay quantitates concentrations as low as 4 pg/ml. To determine the optimal concentration of coating and detection antibodies we tested a range of concentrations of capture and detection antibody. In figure 2 we demonstrate the absorbance values obtained during the optimization of the TARC ELISA. Beginning with the manufacturers' suggested concentrations of antibody we tested four decreasing amounts of capture antibody as well as three decreasing amounts of detection antibody in concert. Well 1A was used as a blank to account for any absorbance due to the buffers used during each stage of the ELISA protocol. Recombinant human TARC was added to each lane at 500 pg/ml. Seven doubling dilutions were performed resulting in a calculated 7.8 pg/ml of TARC in the second last well of each column. Row H is a control lane and did not receive recombinant chemokine but did receive capture and detection antibody at concentrations allocated to that column. This control lane was used to examine any non-specific binding by the SAAP-PNPP conjugate. The SAAP-PNPP conjugate is the enzymatic cleavage of streptavidin alkaline phosphatase bound to the biotin marker on the biotinylated antibody by PNPP. This cleavage results in a flouresence that is quantified as absorbance at 405-690nm. The optimized concentration of capture antibody and detection antibody was chosen based upon the ability of the antibody pair

to recognize the lowest concentration of recombinant chemokine with an absorbency reading above the normal background absorbency providing a linear standard curve. For TARC assay we decided upon 0.25 ug/ml of capture antibody and 35 ng/ml of detection antibody. The TARC standard curve in our ELISA is able to detect 3.9 pg/ml (n>500).

Figure 2 Optimization of TARC ELISA

BIOTINYLATED ANTIBODY (in pg/ml)

	150	75	37.5	150	75	37.5	150	75	37.5	150	75	37.5
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.025 blank	3.767	3.515	3.859	3.737	3.384	3.802	3.724	3.267	3.701	3.504	2.961
B	3.853	3.149	2.354	3.564	3.024	2.344	3.432	3.056	2.283	3.176	2.989	2.194
C	3.664	2.230	1.429	2.721	2.153	1.577	2.771	2.246	1.516	2.568	2.141	1.449
D	3.220	1.463	0.951	2.013	1.463	0.969	1.953	1.450	0.978	1.820	1.471	1.120
E	2.434	0.953	0.610	1.498	1.019	0.664	1.493	1.023	0.633	1.343	1.032	0.689
F	1.650	0.697	0.429	1.161	0.778	0.513	1.089	0.826	0.474	1.008	0.699	0.437
G	1.273	0.630	0.420	1.057	0.654	0.451	1.075	0.730	0.413	0.771	0.555	0.359
H	0.582	0.425	0.272	0.771	0.479	0.289	0.824	0.545	0.311	0.588	0.351	0.218
	2.0 ug/ml			1.0 ug/ml			0.5 ug/ml			0.25 ug/ml		

COATING ANTIBODY

Figure 2: Representation of the data obtained during the optimization of the TARC ELISA. Using 96 well ELISA plate we coated with a range of "coating" antibody concentrations (2.0, 1.0, 0.5, and 0.25 ug/ml). Blocking solution containing 1% BSA was added to the wells to ensure that any rhTARC present will bind to the mouse anti-human TARC antibodies present. Standard recombinant human TARC was added to each of the wells in Row A at 500pg/ml. The rhTARC was then serially diluted 7 times down each column. A "secondary" biotinylated antibody was then added at a range of concentrations (150, 75, and 37.5 ng/ml). Using SAAP-PNPP to identify any boitinylated antibody bound to "coating" antibody we were able to determine the optimal amount of "coating" and biotinylated antibody required to detect as little as 3.9 pg/ml.

10.3 Analysis of Samples via Chemokine-Specific Sandwich ELISA

An allergen-driven culture system allowed us to analyze the intensity of type 1 and type 2 chemokine production in PBMC obtained from atopic and non-atopic populations. However, due to the low number of allergen-specific T cells (0.1-0.01% or less of circulating lymphocytes) we required a system that could measure extremely low levels of chemokine. Most chemotaxis assays utilize 10-100ng/ml. Utilizing the optimized chemokine specific ELISA (section 7.2) we developed, we were able to measure concentrations of chemokine as low as 4 pg/ml. Stimulating PBMC with grass pollen allergen produced supernatants that contained various levels of type 1 and type 2 chemokines. To accurately determine the concentration of chemokine within each sample we performed four doubling dilutions of the same sample. Absorbencies that fell onto the linear portion of the standard curve were accepted as representative concentrations within the sample. If all four dilutions produced absorbencies that fell within the linear portion of the standard curve we averaged all four concentrations calculated from each dilution. In figure 3 we provide an example of concentration determination using four doubling dilutions. We can see that 4460, 4883, 4966, and 4339 pg/ml were all contained within the linear part of the standard curve and are within an acceptable range (~10% variation). An average of these four concentrations results in a value of 4662 pg/ml. This concentration is then used as the representative concentration of TARC within that sample. Occasionally the top well or the bottom well does not fall within the linear part of the standard curve and is removed from the analysis. On average we were able to measure 3-4 dilutions per sample allowing for a considerable amount of confidence in the determined concentration. The chemokine

assays that we developed were tested for interassay variation as well. To test ths concentrations were assessed on two or more independent days demonstrating an interassay variation ranging from 5-12%.

One strength of this study lies within the ultra-sensitive chemokine specific sandwich ELISA and allergen driven primary culture system we developed to study the presence and relative intensity of type 1 and type 2 chemokines in atopic disease.

Figure 3 Sample calculation of "unknown" TARC concentration using multiple dilutions

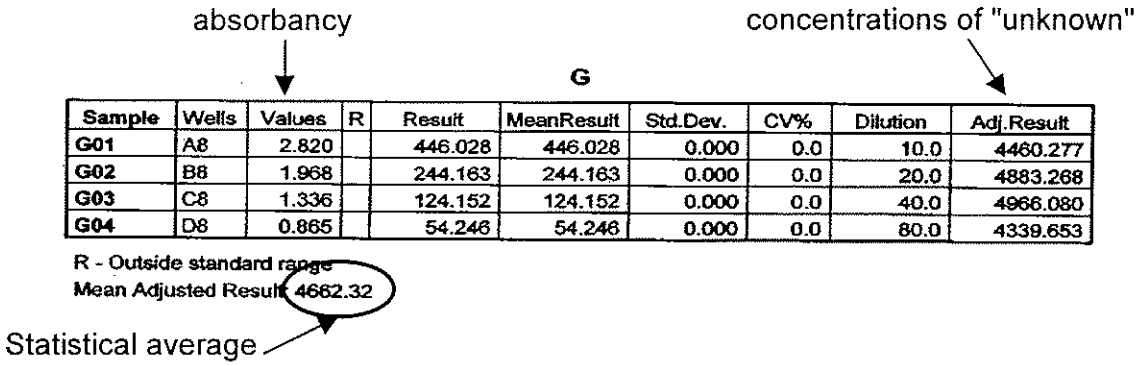

Figure 3: Demonstration of data obtained from analysis of an unknown sample using chemokine specific sandwich ELISA. Using a 96 well plate coated with a monoclonal mouse anti-human TARC antibody we added the "unknown" sample into row G at a 1 in 10 dilution (ie 10ul sample + 90 ul buffer). The "unknown" was then serially diluted at a 1:2 ratio 3 times (note wells A8, B8, C8, and D8). Therefore, the "unknown" sample is measured at 4 different dilutions (1:10, 1:20, 1:40 and 1:80). Softmax Pro software calculates the amount of chemokine in the "unknown" sample by comparing the "unknowns" absorbancy at 405-690nm to a standard curve. The standard curve is created using recombinant chemokine at known concentrations (500pg/ml -- 3.9pg/ml). This "unknown" displays concentrations of TARC at 4460.277, 4883.268, 4966.080, and 4339.653 pg/ml. A statistical average of all four determined concentrations is then calculated to be 4662.32 pg/ml. Assuming all dilutions of the "unknown" fall onto the linear part of the standard curve a concentration of 4662.32 pg/ml of TARC is given to the "unknown".

10.4 Stability of Plasma Chemokines

There has been a considerable amount of work demonstrating that the upregulation of plasma chemokine levels are directly correlated with intense inflammatory disorders [268, 318]. Up-regulation of chemokine receptors (CXCR3, CCR5) has been demonstrated at sites of chronic inflammation such as rheumatoid arthritis and multiple sclerosis [275]. A critical examination of the degree of variation naturally present within plasma chemokines as well as their receptors is required to accurately interpret cross-sectional and longitudinal data. To address this we examined the expression of a range of type 1 and type 2 plasma chemokines (IP-10, Mig and Eotaxin, TARC) as well as Th1 and Th2 associated chemokine receptors (CXCR3 vs CCR3) from six healthy individuals over a three week period. Platelet-poor plasma was isolated from whole blood treated with 2% EDTA using a Ficoll density gradient (see Figure 1). Utilizing a chemokine specific sandwich ELISA that we developed, we were able to quantitate plasma chemokine at levels as low as 4pg/ml. Within our subject groups we observed a wide range in the amount of plasma chemokines (4-5 log) from <4pg/ml to >30 ng/ml. Regardless of the range of plasma chemokines present within the 5 subjects examined, the levels of each chemokine examined were extremely stable over the three-week period. In figure 4a and 4b we see that representative type 1 (IP-10, Mig) and type 2 (Eotaxin, TARC) plasma chemokines demonstrate median variation levels between 4.3-5.5% over the time period.

Figure 4a Systemic levels of type 1 chemokines (IP-10 and Mig) are stable

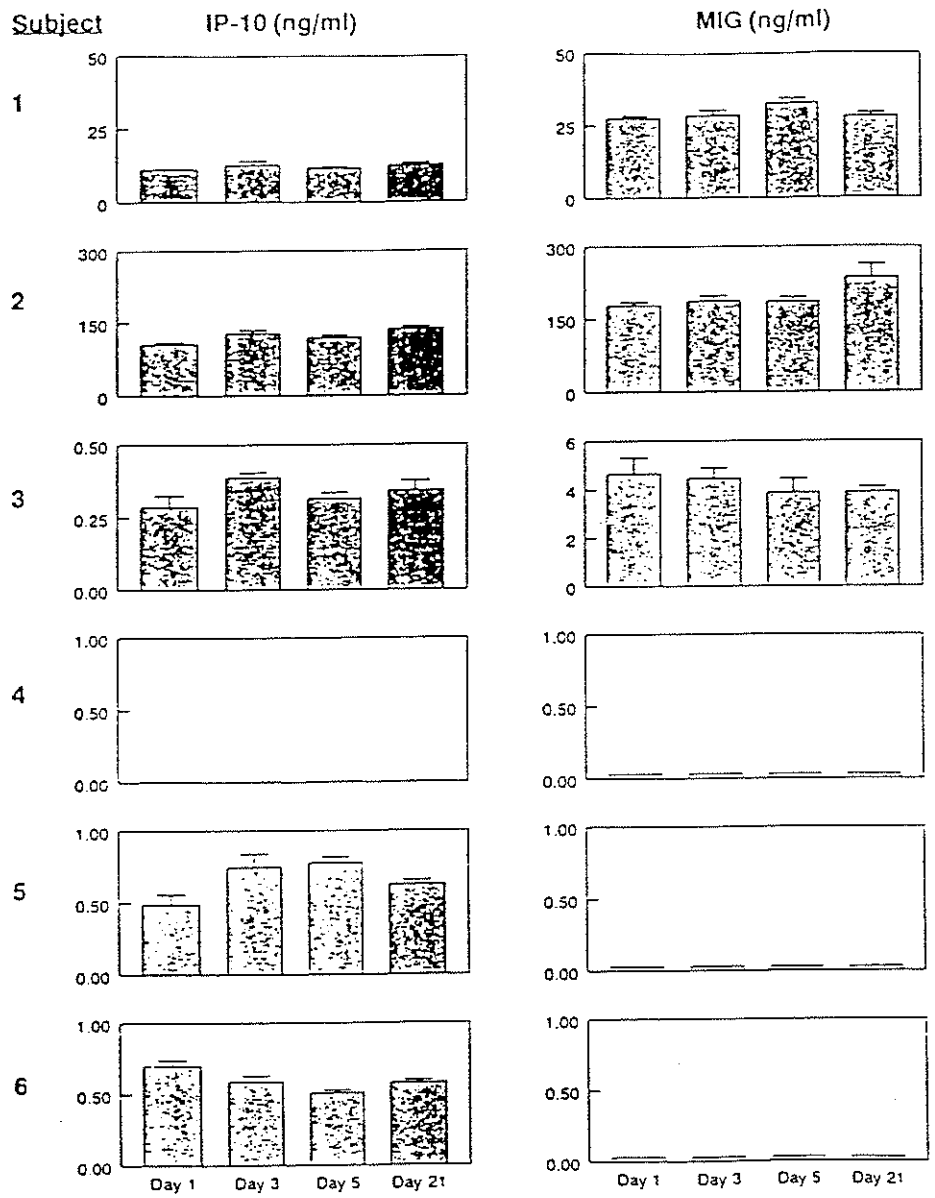

Figure 4a: Plasma IP-10 and Mig protein levels are stable in healthy subjects. IP-10 and Mig protein levels in human plasma were determined on day 1, 3, 5, and 21. On each day, plasma was separated from cells within 30 min of blood collection by centrifugation of EDTA-treated whole blood. IP-10 and Mig levels were quantified by specific ELISAs as described in Materials and Methods. Each data point represents readings from two independent assays performed in triplicate. Data are expressed as mean +/- SEM.

Figure 4b Systemic levels of type 2 chemokines (Eotaxin and TARC) are stable

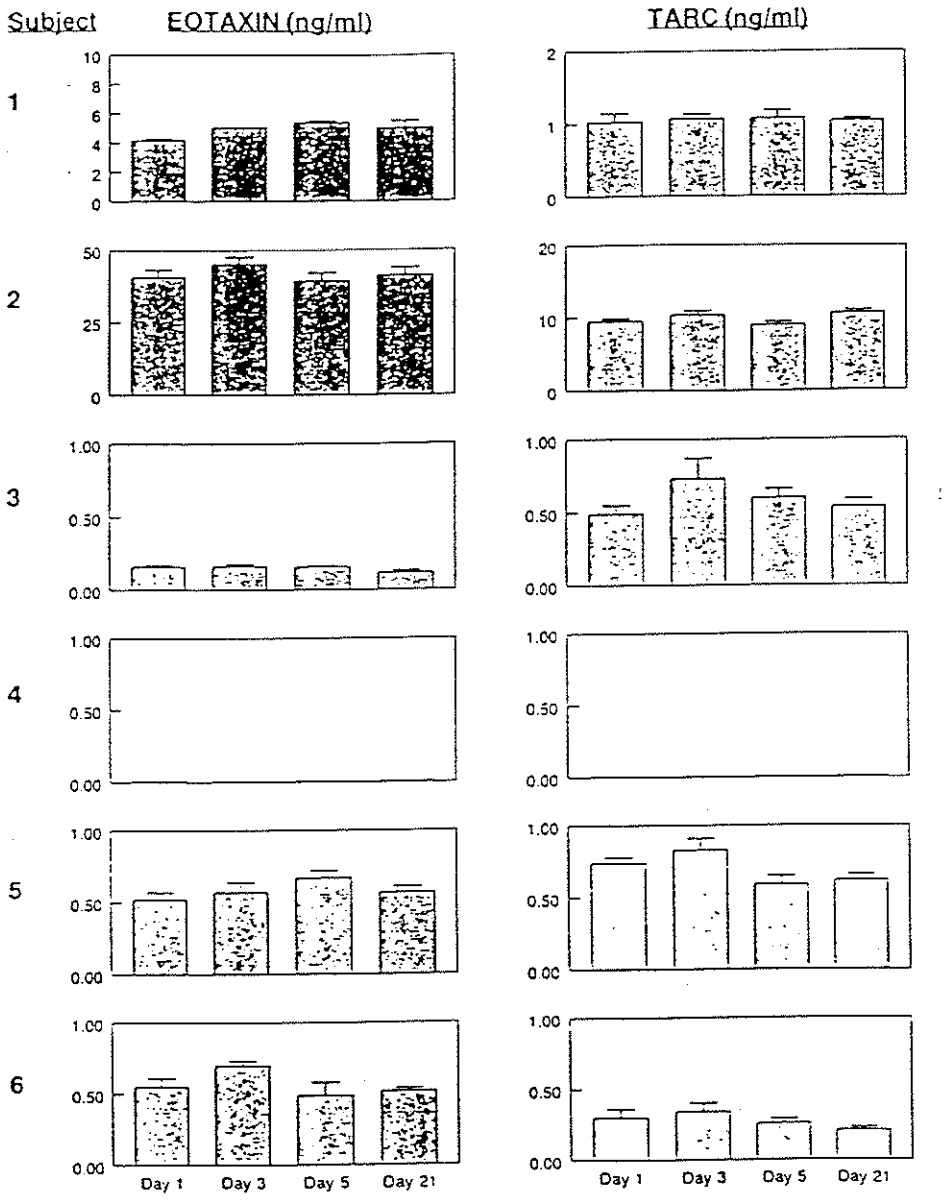

Figure 4b: Plasma Eotaxin and TARC protein levels are stable in healthy subjects. Eotaxin and TARC protein levels in human plasma were determined on day 1, 3, 5, and 21. On each day, plasma was separated from cells within 30 min of blood collection by centrifugation of EDTA-treated whole blood. Eotaxin and TARC levels were quantified by specific ELISAs as described in Materials and Methods. Each data point represents readings from two independent assays performed in triplicate. Data are expressed as mean +/- SEM.

In concert we examined the frequency of cells bearing CXCR3 (Th1 associated) as well as CCR3 (Th2 associated) on CD4+/CD45RO+ T cell populations using four color flow cytometry. The levels of CXCR3 and CCR3 we observed fall within the range of those reported previously for T cells in single time point cross-sectional analysis of healthy individuals: 1-10% for CCR3 [232, 234], 40-80% for CXCR3 [321]. Similar to plasma chemokines, we found that the percentage of CXCR3 and CCR3 positive cells were remarkably stable over a three week period (<5% variation).

Taken together these data indicate: (1) Type 1 (IP-10, Mig) and type 2 (Eotaxin, TARC) plasma chemokines are present in the circulation of healthy subjects but vary dramatically between subjects (100pg/ml vs 20 ng/ml). Similarly, CXCR3 (Th-1 associated) and CCR3 (Th-2 associated) expression on PBMC vary markedly between normal healthy subjects. Due to the high levels of intersubject variability a large sample size is required to identify significant differences between disease and control groups. Plasma chemokines as well as chemokine receptors CXCR3 and CCR3 expressed on PBMC of any given individual displayed very little variation over the three week period (<5%). This is important because it indicates a reliable baseline of in vivo plasma chemokine and receptor expression among healthy subjects. These results support the potential for cross sectional examination of plasma chemokine levels and their cognate receptors for the purpose of determining the involvement of plasma chemokines or their receptors as markers of inflammatory disease.

10.5 Plasma Chemokines and Atopic Disease

Cross Sectional Comparison of Plasma Chemokines in Non-atopic and Atopics

Plasma chemokines have been suggested to be important clinical markers of atopic disorders. Specifically in allergic disease, elevated type 2 plasma chemokine (eotaxin, MDC) levels have been reported in asthmatics as well as atopic dermatitis subjects compared to normal controls [268, 318]. Additionally, serum TARC levels were closely related with disease activity in atopic dermatitis (AD). However, type 2 plasma chemokines were exclusively measured and only during acute exacerbation of disease (eg. emergency room admission, severe AD). We sought to determine the relative intensity of type 1 and type 2 chemokines in the circulation of healthy and stable atopic individuals. We hypothesized that type 1 and type 2 plasma chemokines would prove to be useful markers of allergic rhinitis and that their levels of expression would correlate with allergen exposure before (winter) and during (summer) the grass pollen season. Subjects were analyzed over two grass pollen seasons to ensure the results were not representative of a single weak or strong grass pollen season.

We randomly recruited 34 non-atopic and 53 grass atopic individuals and studied their plasma chemkokine levels in the winter and summer of 1999 and 2000. Platelet-poor plasma was isolated and chemokine levels were measured utilizing chemokine specific sandwich ELISA. Mig and IP-10 levels in the plasma were selected representative type 1 chemokines due to exclusive expression of CXCR3 on Th1 clones and the ability of IFN(to enhance their production. Eotaxin and TARC were examined as representative type 2 chemokines. Eotaxin is directly linked to eosinophil and basophil recruitment

whereas TARC has been shown to attract primarily CCR4 expressing Th cells to the site of allergen exposure. [202, 311, 322, 323]

Within both atopic and non-atopic populations there exists relatively high (>1000 pg/ml) levels of plasma chemokines as well as extremely low levels (<3.9pg/ml) of plasma chemokine. A significant portion of the population displayed plasma chemokine levels ranging between these two extremes. All four chemokines measured displayed a remarkable range of concentrations (4-5 log). We found that through analysis of plasma chemokine levels in atopic and non-atopic subjects:

1) No significant differences between a representative type 1 plasma chemokine, IP-10, in non-atopic and atopic individuals (Figure 5a). Mig, an alternative type 1 chemokine, also did not demonstrate different systemic levels in either subject group (data not shown).
2) Representative type 2 plasma chemokine, Eotaxin, was not elevated in atopic subjects (Figure 5b). In addition an alternative type 2 plasma chemokine, TARC, did not differ between groups (data not shown).
3) Seasonal exposure to greatly elevated levels of sensitizing allergen (grass pollen) did not generate disparities between type 1 or type 2 plasma chemokines within either atopic or non-atopics. Specifically, after extensive exposure to grass pollen (Summer) plasma type 1 and type 2 chemokine levels did not differ between atopic and non-atopics.

Figure 5a Levels of type 1 plasma chemokine IP-10 do not differ between atopic and non-atopics

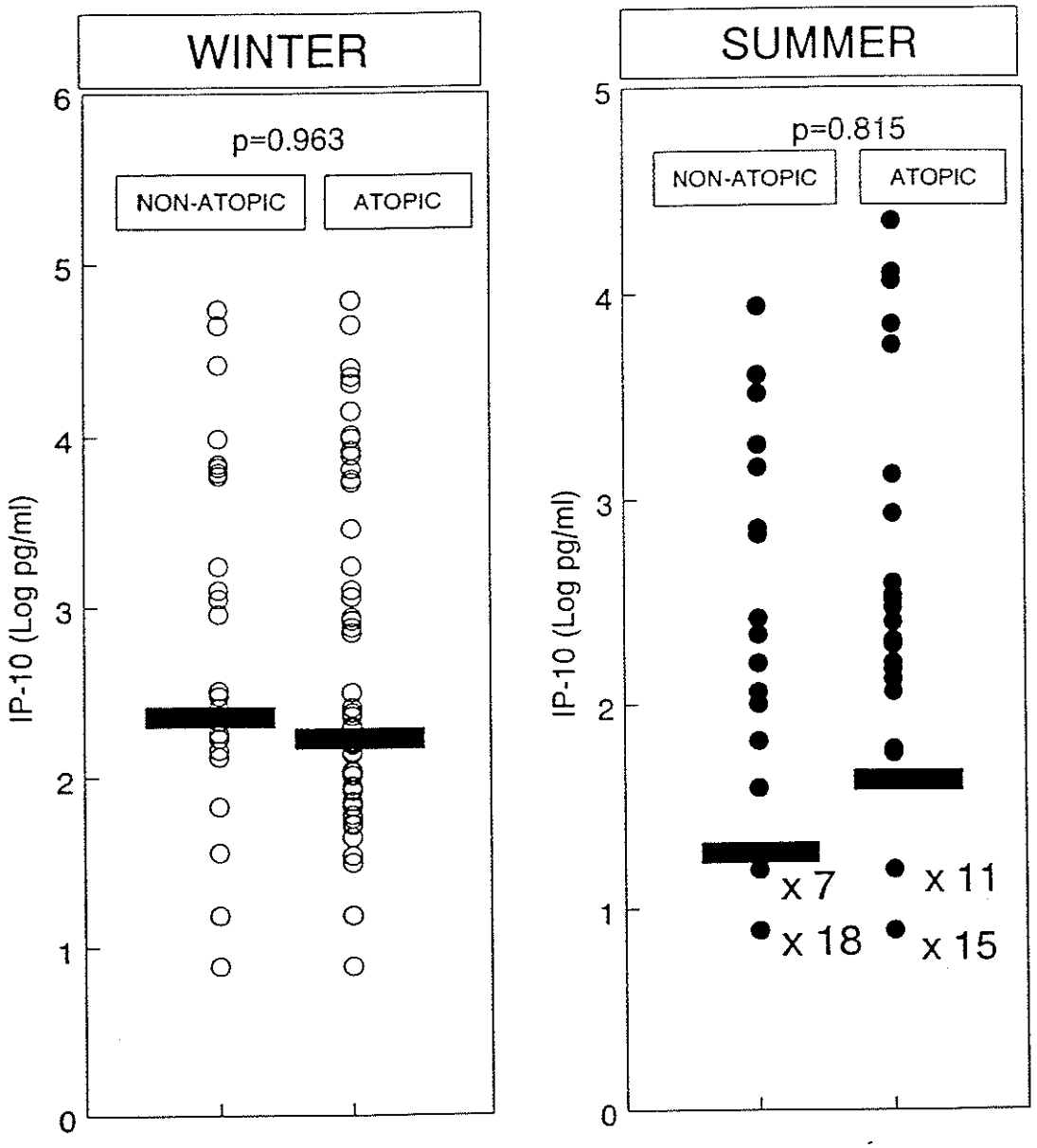

Figure 5a: Cross-sectional analysis of systemic levels of IP-10 and Mig, are not indicative of atopy. Type 1 chemokines, IP-10 and Mig (data not shown) were quantified via ELISA in platelet-poor plasma from healthy, non-atopic (n=34) and grass atopic (n=53) individuals in the winter and summer of 1999 and 2000. Data were converted to logarithmic equivalent to allow for parametric analysis using a Students T test. Median values are indicated by a bar.

Figure 5b Levels of type 2 plasma chemokine Eotaxin do not differ between atopic and non-atopics

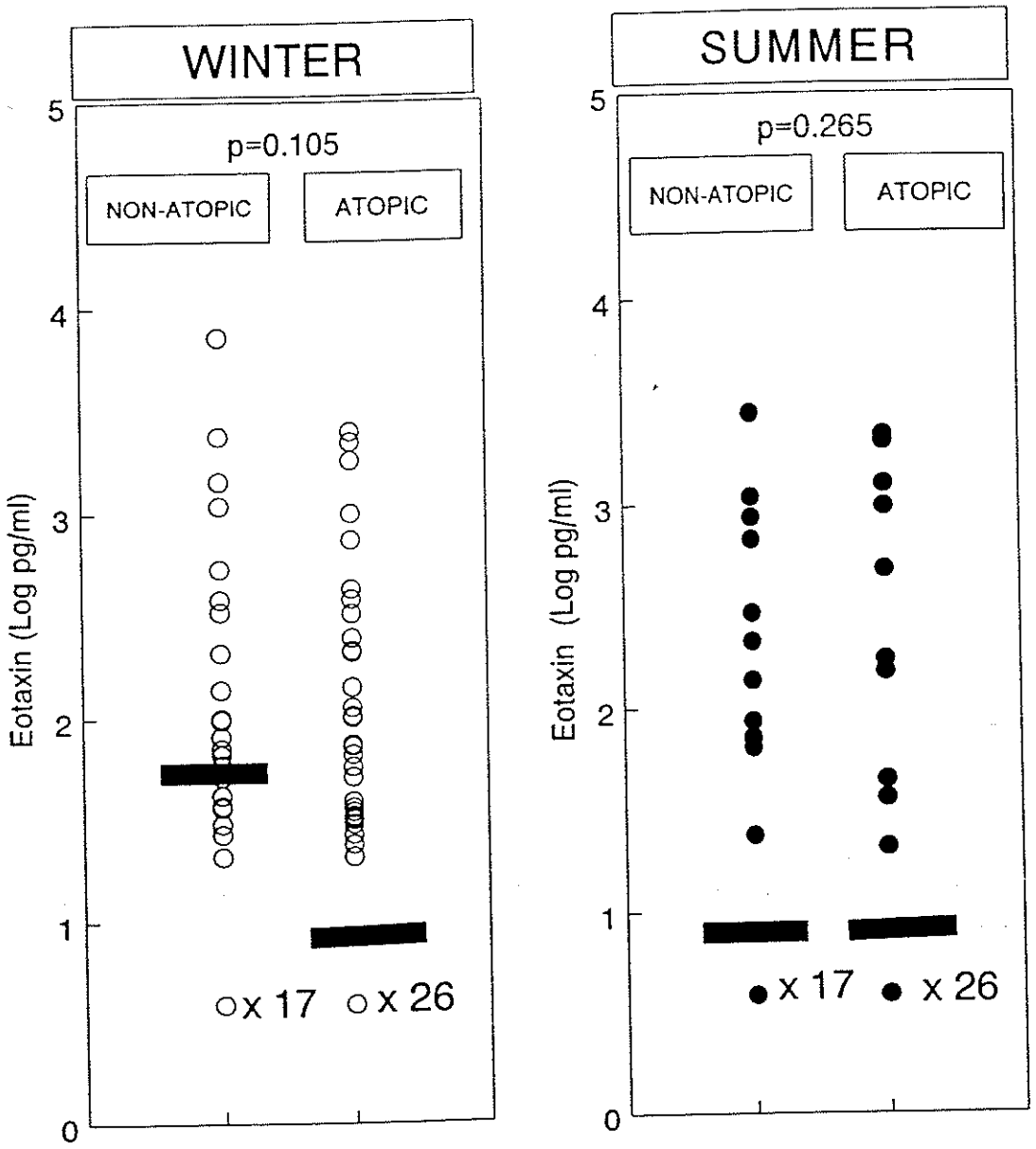

Figure 5b: Systemic levels of Eotaxin and TARC, are not indicative of atopy. Type 2 chemokines, Eotaxin and TARC (data not shown) were identified in platelet-poor plasma isolated from healthy, non-atopic (n=34) and grass atopic (n=53) individuals in the spring and summer of 1999 and 2000. Data were converted to logaritmic equivalent to allow for parametric analysis using a Students T test. Median values are indicated by a bar.

We believe that this data clearly shows that systemic levels of IP-10, Mig, Eotaxin and TARC are not correlated with allergic rhinitis. This does not exclude the possibility that alternative chemokines such as RANTES, or MIP-1a could be utilized as markers of allergic rhinitis. We argue that conclusions drawn from published data suggesting a direct relation between atopic disorders and altered levels of plasma chemokines are not applicable to allergic rhinitis.

Longitudinal Analysis of Plasma Chemokine Levels

Cross-sectional analysis of type 1 and type 2 plasma chemokines (above) did not demonstrate any significant differences that correlated with clinical sensitivity. In a slightly different approach we examined non-atopic and stable atopics longitudinally to determine the ability of elevated grass pollen exposure to modulate systemic chemokine levels. Exposure to grass pollen occurs at its highest in Manitoba during the summer (peaking in June/July). Grass atopic individuals often experience intense type 2 mediated inflammatory reactions during this period.

We hypothesized that circulating levels of Eotaxin and TARC, (type 2 chemokines) in atopic subjects would be elevated in comparison to healthy controls due to extensive exposure to greatly enhanced levels of grass pollen. Platelet-poor plasma was isolated from atopic (n=56) and non-atopic (n=34) subjects. Levels of IP-10, Mig, Eotaxin and TARC within the plasma were measured using a chemokine specific sandwich ELISA. As observed in Figure 1, a noteworthy range in type 1 and type 2 plasma chemokine levels (4-5log) ranging from 4pg/ml to 20 ng/ml within atopic and non-atopic populations exists. Surprisingly, a decrease in plasma IP-10 (type 1 chemokine) levels during the

grass pollen season (summer) was observed both atopic and non-atopics (Figure 6a). Interestingly, the non-atopics demonstrated a significant decrease in plasma eotaxin levels in the summer compared to winter (Figure 6b).

Figure 6a Systemic levels of type 1 chemokine IP-10, but not Mig, are significantly decreased over the grass pollen season

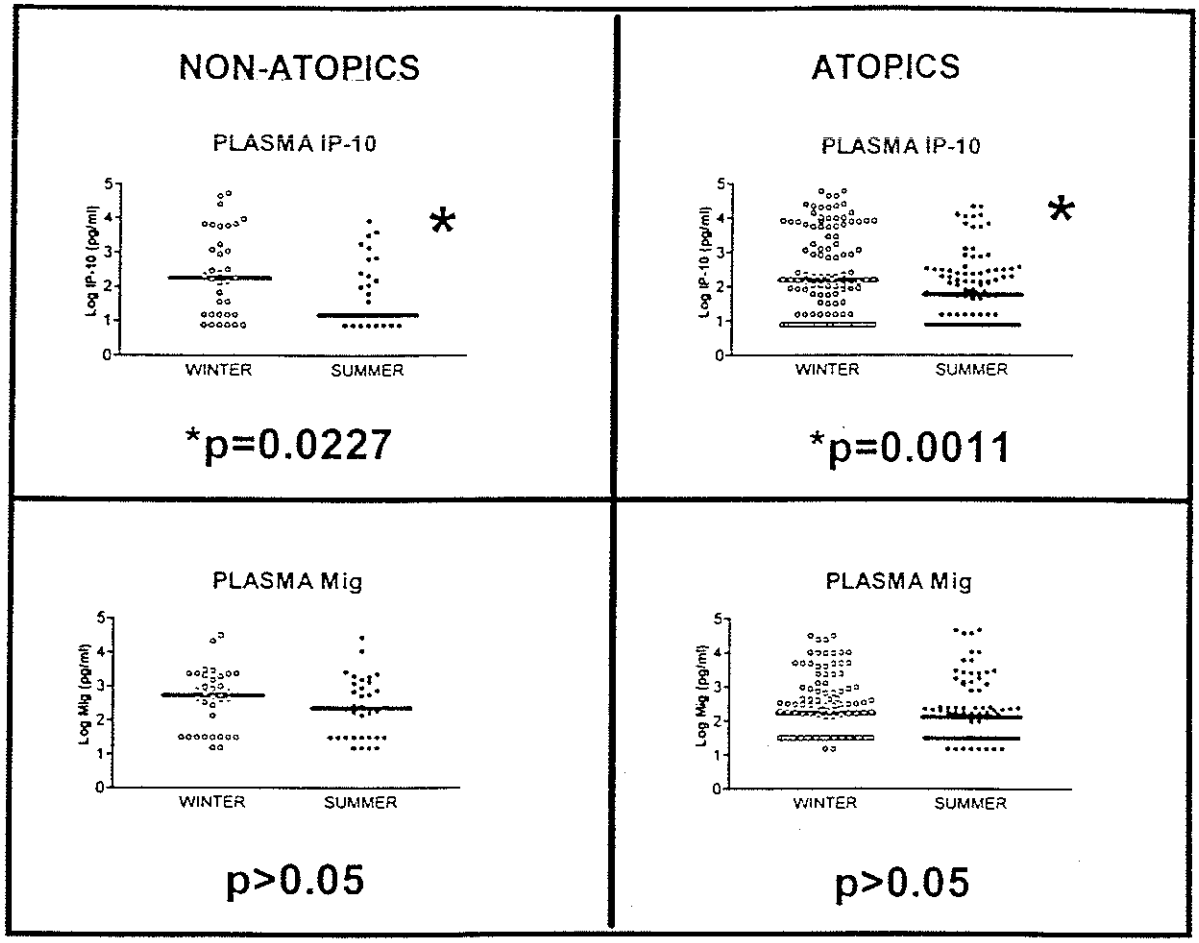

Figure 6a: Longitudinal analysis of systemic levels of circulating IP-10 reveals significantly decreased over the grass pollen season. Platelet-poor plasma was isolated from atopic (n=53) and non-atopic (n=34) individuals during the winter and summer of 1999 and 2000. IP-10 and Mig were measured using ELISA. Data were converted to logarithmic equivalent to allow for parametric analysis using a Paired T test. Median values are shown as a bar.

Figure 6b Systemic levels of type 2 chemokine Eotaxin, but not TARC, are significantly decreased over the grass pollen season in non-atopics only

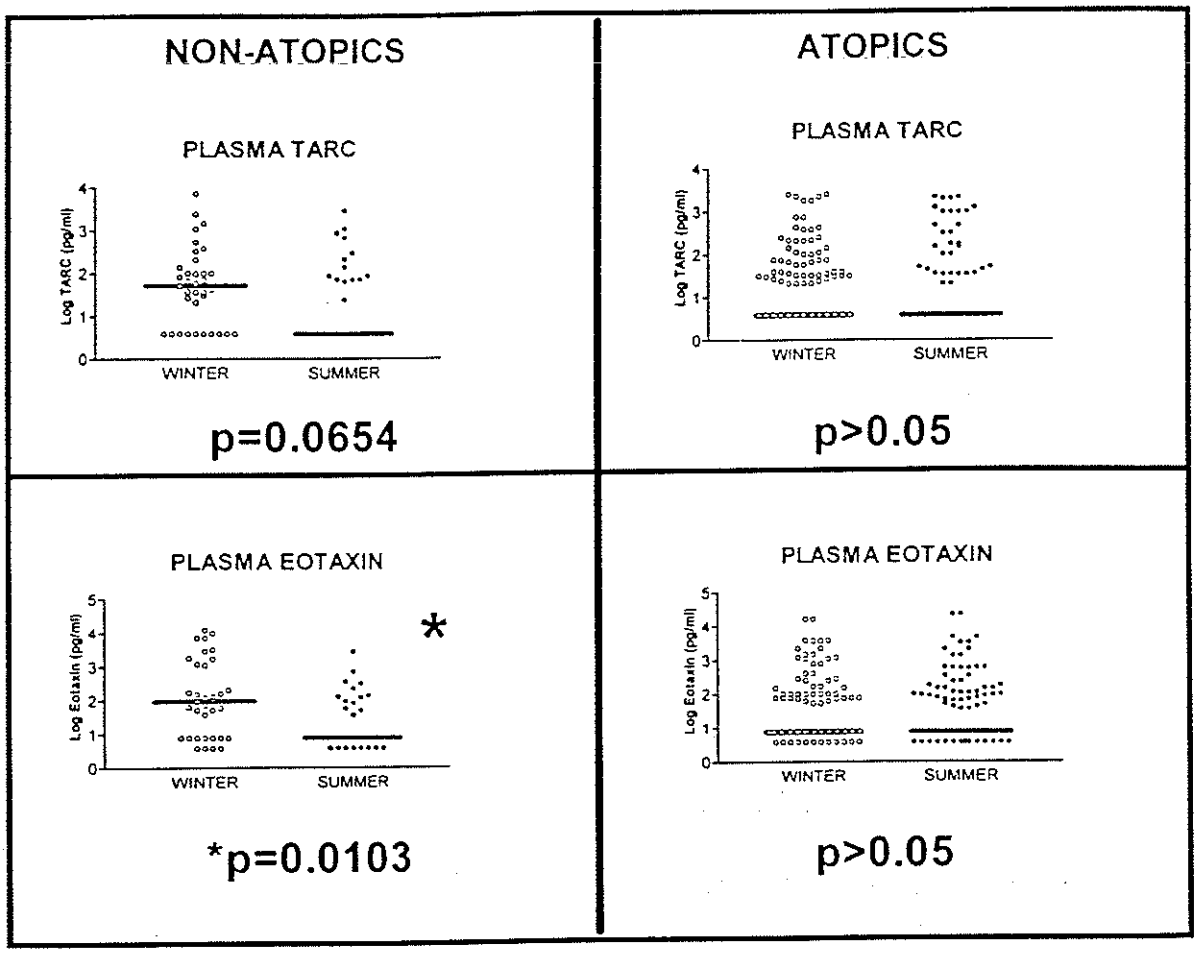

Figure 6b: Longitudinal analysis of systemic levels of circulating Eotaxin reveals a decrease in non-atopics over the grass pollen season. Platelet-poor plasma was isolated from atopic (n=53) and non-atopic (n=34) individuals during the winter and summer of 1999 and 2000. Eotaxin and TARC were measured using ELISA. Data were converted to logarithmic equivalent to allow for parametric analysis using a Paired T test. Median values are indicated by a bar.

The cognate receptors to IP-10 and Mig (CXCR3) as well as eotaxin (CCR3) on PBMC were examined to complement the plasma chemokine results and possibly shed light into the mechanisms driving the unique patterns we observed. We hypothesized that alteration in the intensity of CXCR3 and CCR3 during significant allergen exposure may provide evidence for a mechanism through which systemic chemokines may promote type 1 or type 2 immunity. We measured CXCR3 and CCR3 expression levels on CD4+ CD45RO+ T cells by flow cytometry. CXCR3 is able to recruit antigen experienced Th1 (IFNγ secreting) cells to the site of inflammation via Mig, IP-10 and I-TAC. [290, 293, 299] CCR3 recruits eosinophils, basophils and even Th2 (IL-4 secreting) cells to the site of inflammation via eotaxin as well as other chemokines [42, 250, 324]. CXCR3 and CCR3 expression on CD4+ antigen experienced (CD45RO+) PBMC was measured by four-color flow cytometry.

Non-atopics demonstrate a significant increase in CXCR3 expression during the grass pollen season whereas atopics did not (Campbell et al, submitted Sept 2001). CCR3 levels observed on CD4+ CD45RO+ cells were present at extremely low levels (~5%). Therefore we did not observe significant changes in CCR3 expression on CD4+/CD45RO+ T cells during the grass pollen season. An increase in CXCR3 expression levels on CD4+/CD45RO+ T cells is important because it indicates a mechanism through which clinical tolerance may be maintained within non-atopics. Specifically an increase in CXCR3+ CD4+ antigen experienced cells and systemic levels of the cognate ligand, IP-10, suggests evidence for an active mechanism by which IFNγ secreting cells are recruited to the site of allergen exposure. Decreased

eotaxin levels in non-atopic subjects may represent fewer eosinophils, basophils, and mast cells releasing eotaxin in response to allergen.

We conclude that exposure to significant levels of natural allergen (grass pollen) results in up-regulation of systemic IP-10 levels as well as CXCR3 expression on antigen experienced Th cells in non-atopics. Due to the preferential expression of CXCR3 on Th1 cell lines we argue that preferential up-regulation of CXCR3 on Th cells may represent enhanced recruitment of IFNγ secreting cells to the site of allergen exposure promoting type 1 immunity and clinical tolerance.

Type 1 and Type 2 Plasma Chemokine Correlation

Since the discovery of T cell clones that preferentially express type 1 or type 2 cytokines [115, 116] in response to antigen, it has become clear that type 1 and type 2 cytokine production (ie IFN γ vs IL-4) is often negatively correlated. This imbalance has been hypothesized to be responsible for immune dysregulation and unnecessary inflammation in response to innocuous allergens. Due to the importance of chemokines in immune regulation, as well as preferential expression of chemokine receptors on Th1 and Th2 clones, we hypothesized that type 1 and type 2 plasma chemokines exhibit a negative correlation. To test this hypothesis, plasma chemokines, IP-10 and Mig (type 1) as well as Eotaxin and TARC (type 2) were measured in atopic (n=106) and non-atopics (n=68) using ELISA.

We observed a positive correlation between our representative type 1 chemokines IP-10 and Mig in both atopic and non-atopic populations. (Figure 7a). Similarly, representative type 2 chemokines, eotaxin and TARC were positively correlated

regardless of atopic status. These results are consistent with cytokine data demonstrating a positive correlation within type 1 as well as type 2 cytokines [127, 325].

Surprisingly, we observed a strong positive correlation between representative type 1 and type 2 plasma chemokines, (Figure 7b). Individual analysis of atopic and non-atopics resulted in equally strong positive correlations (r=0.679 and 0.481 respectively). This indicates a tendency towards maintaining similar levels of type 1 and type 2 plasma chemokines regardless of clinical status. These results are in stark contrast to negative correlations observed when comparing type 1 and type 2 cytokines (eg IFNγ and IL-4) [326]. Interestingly, a decrease in the IP-10 and eotaxin plasma levels in non-atopics over the grass pollen season (Figure 6a) did not alter the postive correlation existing between type 1 and type 2 chemokines in non-atopics.

We conclude that IP-10 and Mig (type 1) as well as Eotaxin and TARC (type 2) are positively correlated. We report a positive correlation between type 1 and type 2 chemokines that we examined. These results may be indicative of an unidentified regulatory mechanism responsible for regulating systemic chemokine levels. Further work is required to determine the mechanisms underlying this unique finding. The potential implications of several elevated plasma chemokine levels include inappropriate activation of leukocytes and immune dysregulation.

Figure 7a Type 1 and type 2 plasma chemokines are positively correlated within atopics and non-atopics

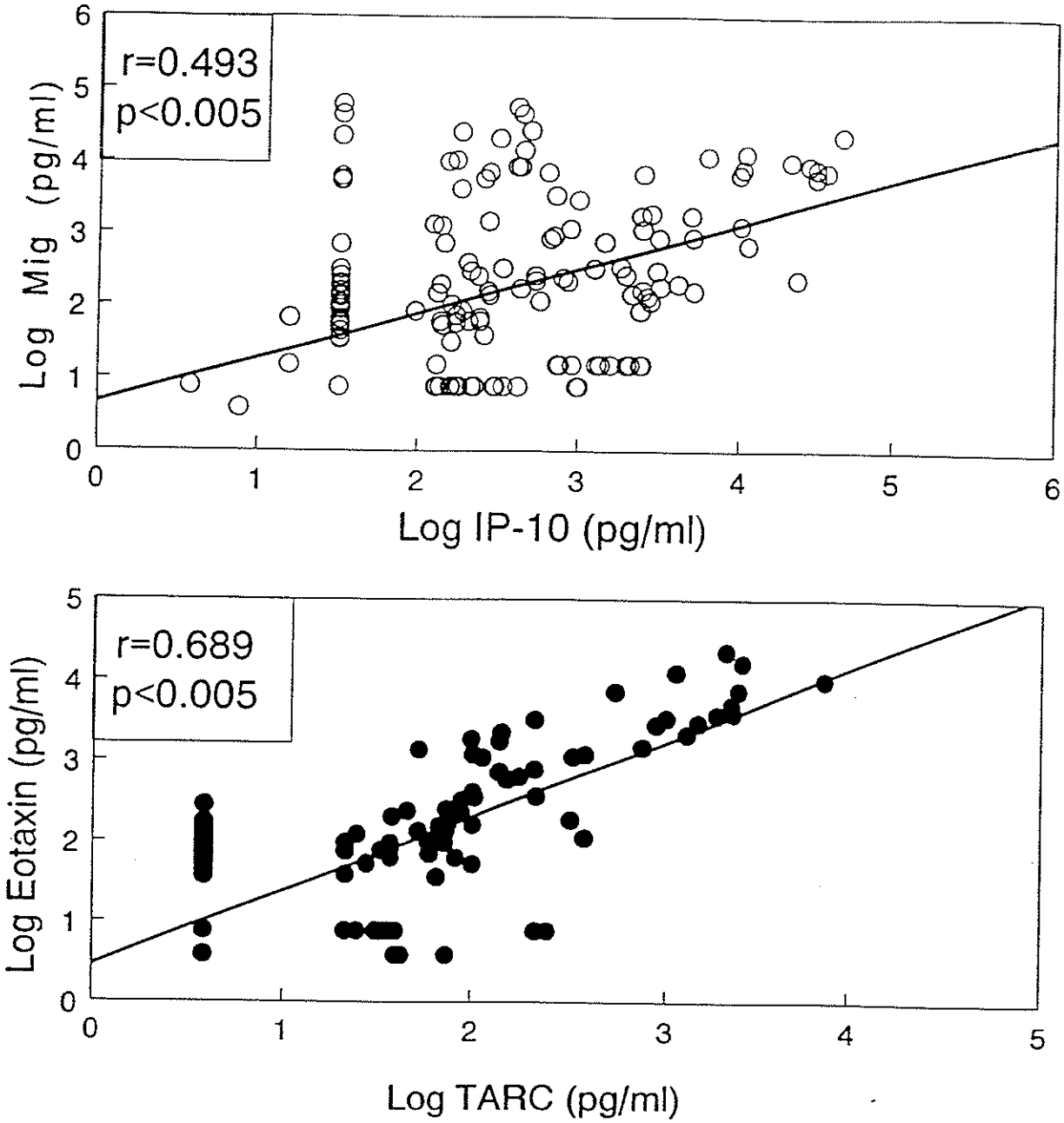

Figure 7a: Type 1 chemokines (IP-10, Mig) as well as type 2 chemokines (Eotaxin, TARC) are positively correlated. Chemokines were measured from platelet-poor plasma isolated from 68 non-atopic, and 106 atopic individuals using ELISA. Data were converted to logarithmic equivalent to allow for parametric analysis using a Spearman's test. Individuals selected for non-atopic status displayed similar correlations (IP-10 vs Mig, $r=0.413$, $p<0.005$ and Eotaxin vs TARC, $r=0.791$, $p<0.005$). Individuals selected for atopic disease displayed similar correlations (IP-10 vs Mig, $r=0.493$, $p<0.005$, Eotaxin vs TARC $r=0.607$, $p<0.005$).

Figure 7b Type 1 and type 2 plasma chemokines are positively correlated in atopic and non-atopics

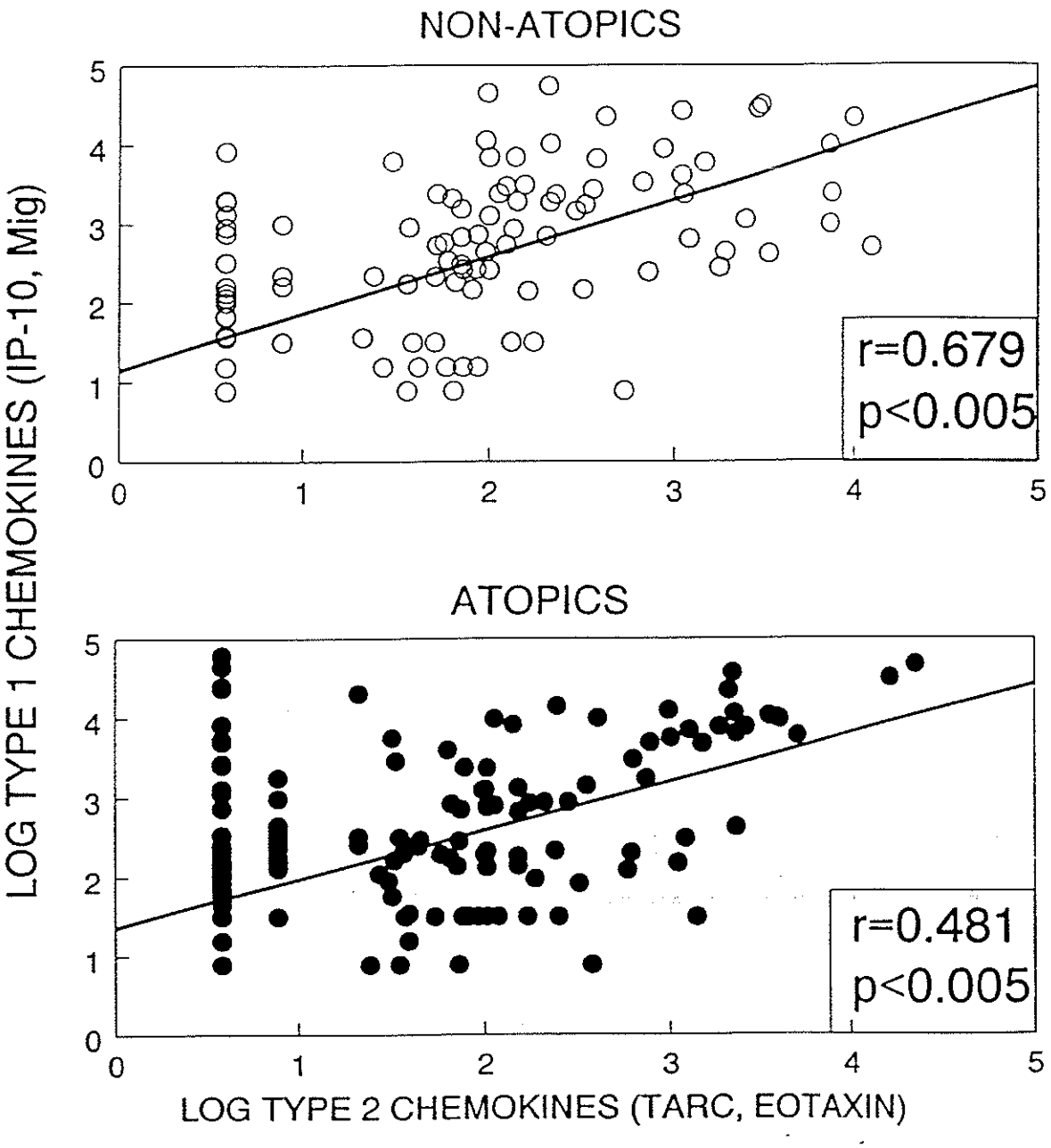

Figure 7b: Representative type 1 (IP-10, Mig) and type 2 (eotaxin,TARC) plasma chemokines are positively correlated in non-atopic and atopic individuals. Plasma was isolated from 68 non-atopic healthy, and 106 grass atopic individuals. Chemokine levels were determined using a chemokine specific sandwich ELISA. Data were log converted to allow for parametric analysis using a Spearman's test.

DARC and Plasma Chemokines

DARC is a chemokine receptor found on red blood cells (RBC), endothelial cells of postcapillary venules, and Purkinje cells of the cerebellum. DARC is unique in that it binds both CXC and CC chemokines with equal affinity [327]. It has been postulated to act as a sink for systemic chemokines intended to prevent inappropriate cellular trafficking or activation. Given the wide (>4 log) variation in plasma chemokine levels seen, we hypothesized that the intensity of DARC expression on RBC was negatively correlated with plasma chemokine levels. Analysis of the frequency of DARC on human RBC by flow cytometry revealed DARC expression on virtually all RBC (97%) in each of the individuals studied (Table 2). Due to the unique ability of DARC to bind a wide range of CXC and CC chemokines and the high frequency of DARC expression on RBC the possibility that DARC expression influences the amount of free circulating chemokine proved extremely likely. We isolated platelet-poor plasma from peripheral blood of atopic (n=10) and non-atopic (n=10) and measured a range of type 1 (IP-10, Mig) and type 2 (MDC, MCP-1, Eotaxin, TARC) chemokines via chemokine specific ELISA. In addition the mean fluoresence intensity (MFI) of DARC expression on RBC was quantified.

Table 6 There is no detectable difference in the frequency of DARC expression on RBC in atopic and non-atopics

Subject/Status	Isotype Match IgG (MFI)	DARC mAb (MFI)	FITC Ab alone (MFI)
060 MLS /atopic	1.7	22	1.9
005 A-V / atopic	3.7	13.9	4.4
048 JIN / atopic	1.8	27.1	1.9
036 NAS / atopic	4.0	20.0	2.3
058 MSL / atopic	1.9	25.7	1.8
061 LLO / atopic	3.4	15.6	3.6
047 DAH / atopic	3.0	23.3	1.6
034 KAB / atopic	3.6	19.1	2.8
062 CHD / atopic	3.5	16.6	3.0
046 RGD / atopic	5.0	18.8	4.4
012 BMK / atopic	2.4	20.9	2.1
024 GWG / non-atopic	4.2	24.6	3.0
019 DGT / non-atopic	3.1	19.0	2.3
041 BWS / non-atopic	4.7	23.5	4.4
013 CMK / non-atopic	3.9	17.9	2.6
015 LFB / non-atopic	2.8	17.6	2.0
008 SMZ / non-atopic	1.8	16.5	2.7
055 CPT / non-atopic	3.3	27.1	2.8
022 NCG / non-atopic	2.6	36.4	1.8
014 JDC / non-atopic	2.5	20.1	2.8

We observed a range in the MFI of DARC expression (10-40 MFI) on RBC. This is important because it demonstrates a difference in the intensity of a key chemokine receptor, therefore suggesting a range in the ability to bind free chemokine. Isotype matched controls yielded MFI < 5. Surprisingly, there is no apparent correlation between the level of plasma chemokines and the frequency and intensity of DARC expression on RBC (Figure 8).

We examined the possibility that higher or longer exposure of blood vessel endothelial cells to chemokines due to differences in the intensity of DARC expression on RBC might contribute to the pathogenesis of atopy. Independent analysis of atopic and non-atopic groups did not reveal a correlation between the intensity of DARC expression and the chemokines analyzed (data not shown).

To determine whether clinical sensivity was correlated with DARC expression, as opposed to type 1 and type 2 plasma chemokines, we compared the intensity of DARC expression with the presence of atopic disease (skin tests, clinical history). Similarly, we observed no correlation between the intensity of DARC expression and clinical sensitivity (data not shown).

Figure 8 Plasma chemokine levels are not correlated with the intensity of DARC expression on RBC

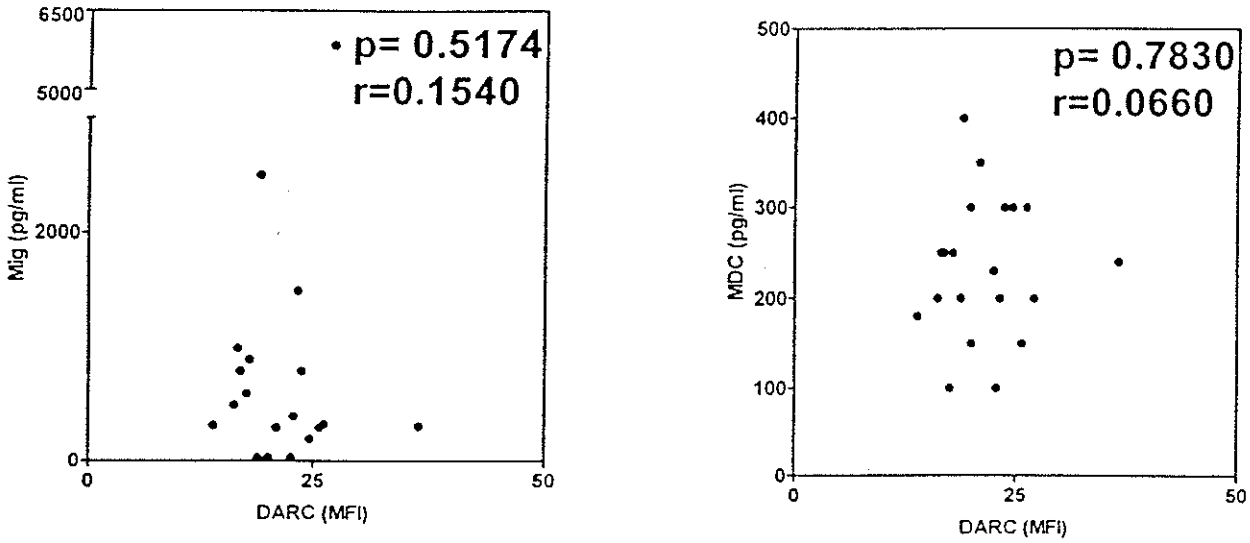

Figure 8: Plasma chemokine are not correlated with the intensity (MFI) of DARC expression on human RBC. RBC from individuals selected for atopic disease (n=10) as well as healthy non-atopic individuals was isolated from whole blood. RBC were stained with a primary mAb (IgG1) against DARC (Fy6) followed by a secondary polyclonal FITC conjugated goat-anti-mouse IgG1. Plasma levels were converted to logarithmic equivalent to allow for parametric analysis using a Pearsons' Test for correlation with intensity of DARC on RBC.

Taken together these data demonstrate a range in the intensity of DARC expression (10-40 MFI) on a substantial percentage (~97%) of RBC implicating the majority of RBC in binding systemic chemokines. DARC expression on RBC is frequently absent (>70% population) in African individuals as a result of resistance to malaria (reviewed in [197]). Taken with the high prevalence of allergic airway disease among African Americans [328, 329] there is reason to speculate a possible correlation between DARC expression and atopic disorders. We argue that the intensity or frequency of DARC expression does not correlate with systemic levels of type 1 and type 2 plasma chemokines within grass non-atopic and atopic individuals. More importantly the intensity or frequency of DARC expression does not correlate with clinical sensitivity to grass pollen (ie allergic rhinitis).

10.6 Kinetics of Allergen and Polyclonal Driven Chemokine Production

Several chemokines have been implicated in the exacerbation of inflammation. Information regarding key chemokines involved in recruiting polarized T cell populations to the site of inflammation has been limited. What is known is the existence of exclusive chemokine receptors on Th1 or Th2 cell lines suggesting that up-regulation of the cognate ligands may represent a possible positive feedback loop. (Reviewed in [195]) The majority of information regarding chemokine production from isolated cell populations or cell lines has been restricted to the use of polyclonal activators.

We hypothesized that allergens represented sufficient stimuli to elicit chemokine production in a primary culture system containing freshly isolated PBMC. In addition we wished to compare the intensity and kinetics of chemokine production elicited by allergen stimulation (grass pollen) and polyclonal stimulation (TSST-1). The ability of our ELISA to measure chemokine levels as low as 4pg/ml in culture supernatants was essential to determine the ability of allergen to stimulated chemokine production from PBMC.

We stimulated freshly isolated PBMC (5×10^6) from five healthy non-atopic individuals with 0.1ng/ml of TSST-1 or 400 ug/ml of grass pollen (concentrations which represent previously optimized concentrations for cytokine production). We chose to examine the IP-10 (type 1) and TARC (type 2) production by PBMC due to the ability of these chemokines to preferentially bind Th1 and Th2 clones respectively. Supernatants were collected 24 hours, 3 days, 5 days and 7 days after stimulation and IP-10 as well as

TARC levels were measured using chemokine specific sandwich ELISA. This analysis revealed a time dependent expression pattern of both chemokines (Figure 9). Allergen driven chemokine production was measurably lower than polyclonal stimulation but was present at modest levels (300 pg/ml TARC, 8000 pg/ml IP-10) peaking at later time points. Specifically, allergen-driven chemokine production was optimal on day 5 whereas polyclonal stimulation resulted in optimum chemokine levels 3 days post stimulation. For both IP-10 and TARC, the concentrations detected in the supernatants decreased significantly by day 7. PBMC stimulated with lower concentrations of grass pollen (40 ug/ml and 4ug/ml) displayed a slight increase in both IP-10 and TARC production (data not shown). Similar results were found with addition of type 1 chemokine, Mig, (data not shown). Therefore, an optimization of the optimal allergen concentration for chemokine production was required.

We demonstrated the ability of allergens to stimulate representative type 1 and type 2 chemokines from PBMC directly *ex vivo*. Comparison of allergen and polyclonal stimuli reveals characteristically different times of optimal chemokine production. Differences in intensity and kinetics of chemokine production are most likely due to the ability of polyclonal activators to stimulate a greater proportion of the cells present compared to allergens (stimulate <0.1% T cells). These results reflect the qualitative and quantitative differences in polyclonal and allergen activation. Based upon this finding we will continue to harvest allergen cultures five days after stimulation and polyclonal stimulated culture three days after culture.

Figure 9 Kinetics of allergen and polyclonal driven chemokine production

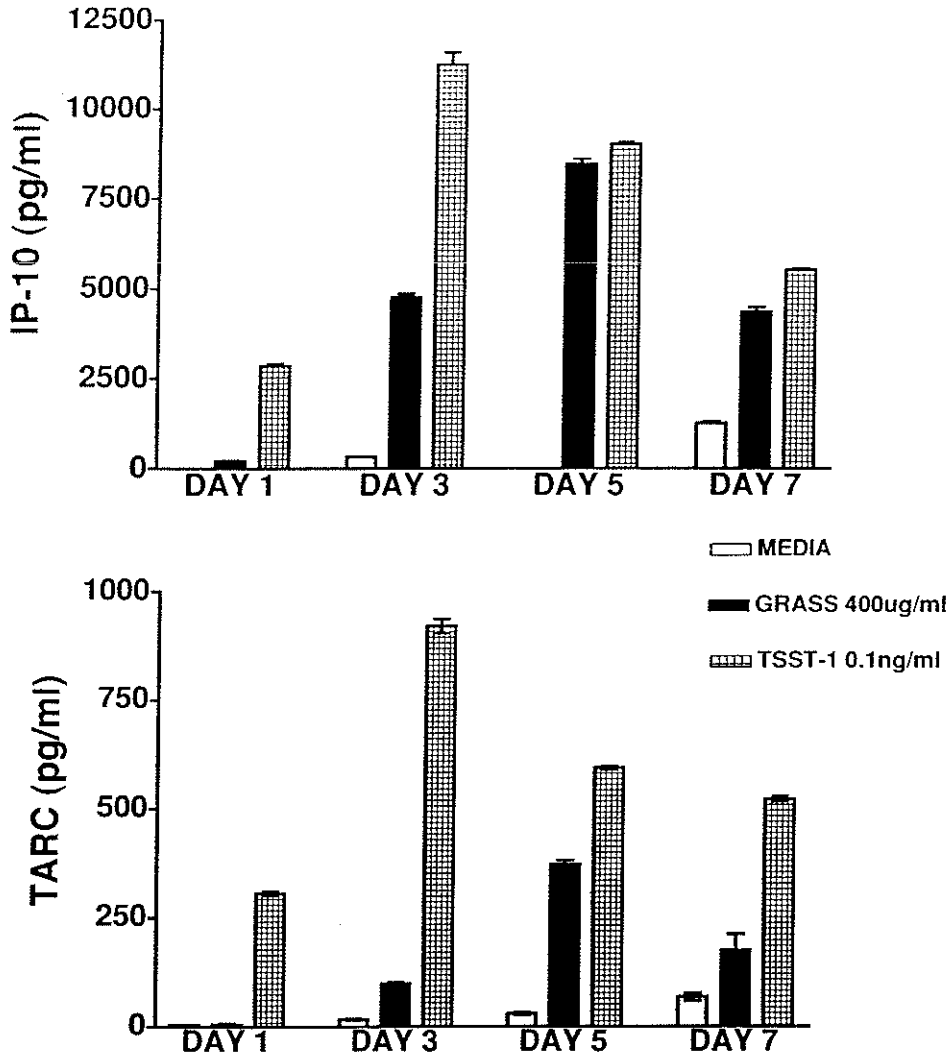

Figure 9: Allergen and polyclonal driven chemokine production in short term primary culture of fresh PBMC directly ex vivo is optimal at different time points. PBMC from one of 5 non-atopic, healthy individuals was stimulated with grass pollen allergen (400ug/ml) or TSST-1 (0.1ng/ml) for seven days. Individual cultures were examined for each time point. Supernatents were quantified for IP-10 and TARC levels using chemokine-specific sandwich ELISAs. Samples were analyzed on two separate days with four doubling dilutions for each ELISA to determine SEM.

10.7 Polyclonal Stimulation does not Differentiate Between Atopic Status

The majority of information regarding chemokine production from isolated cell populations or cell lines is restricted to the use of polyclonal activators. As mentioned previously, chemokines have been implicated in key stages of atopic disease. To our knowledge there is little information detailing differences in intensity of polyclonal-driven chemokine production between atopic and non-atopic PBMC. A recent paper from our lab clearly shows that *cytokine* production from polyclonal stimulated PBMC does not differentiate between atopic and non-atopic status. However, allergen driven cytokine production demonstrated enhanced IL-13 production (type 2 cytokine) in atopic subjects only [330].

We hypothesized that polyclonal-driven type 1 and type 2 chemokine production does not differentiate between atopic and non-atopics. We recruited 20 grass atopic and 10 non-atopic healthy individuals for this study. PBMC were isolated and placed into primary culture with either PHA (2ug/ml) or TSST-1 (0.1ng/ml) for three days. We chose to examine representative type 1 (IP-10) and type 2 (TARC) chemokines in the culture supernatant via the chemokine specific ELISAs we developed.

Stimulating PBMC with either PHA or TSST-1 resulted in enhanced levels of both IP-10 and TARC levels compared to media alone in culture. In Figure 10 we demonstrate no difference in polyclonal-driven type 1 or type 2 chemokine production between atopic and non-atopics. Interestingly, PHA driven chemokine production was significantly lower than TSST-1 stimulation. We speculate that this is representative of functionally

different stimuli suggesting that TCR stimulation may be more important in IP-10 and TARC production from PBMC. TSST-1 is a superantigen that binds to the TCR and the MHC class II simultaneously resulting in activation of the T cell regardless of the antigen presented in the MHC class II peptide-binding groove. Thus TSST-1 stimulation is TCR specific whereas PHA enters the T cell regardless of the presence of the TCR. Chemokine production through TCR-MHC class II crosslinking operates through similar pathways that allergen specific T cell activation would occur. TSST-1 was therefore chosen as a positive control for future experiments.

Therefore, we argue that polyclonal driven IP-10 and TARC production does not differentiate between atopic status. This is important because it demonstrates the requirement for analysis of physiologically relevant stimuli to determine differences, if any, between atopic and non-atopic subjects in terms of chemokine production.

Figure 10 Polyclonal stimulation does not differentiate between atopic status

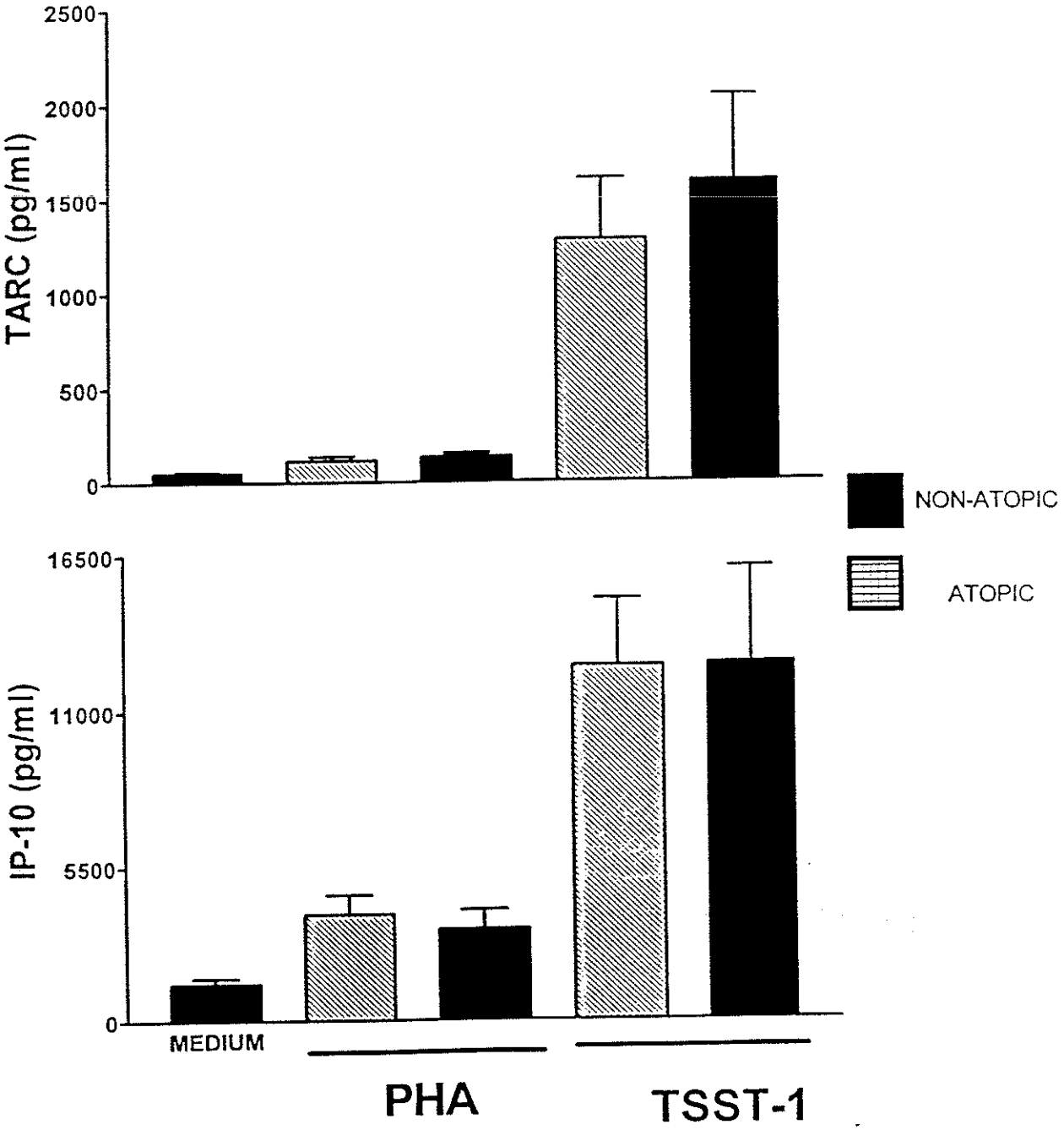

Figure 10: Polyclonal stimulation does not result in chemokine production that differentiates atopic (n=20), non-atopic, (n=10) individuals. PBMC were isolated from fresh EDTA-treated whole blood and stimulated with PHA (2ug/ml) or TSST-1 (0.1ng/ml). Supernatants were collected three days after stimulation as well as day 1 and day 5 (data not shown). IP-10 and TARC levels were measured using chemokine specific sandwich ELISA. Statistics were performed using a Students T test.

10.8 Allergen-driven Chemokine Production differentiates between Atopic Status

The ability of the immune system to preferentially recruit polarized populations of T cells to the site of inflammation is crucial to the outcome of the immune response. In atopy it is detrimental to the host to preferentially recruit Th2 cells to the lung, nose, or skin in response to allergen. Information regarding the involvement of chemokines in Th2 cell recruitment has been limited to analysis of chemokine receptors. We hypothesize that PBMC from atopics produce significantly more type 2 chemokine compared to non-atopics in response to allergen thereby promoting Th2 cell recruitment. In addition, we speculate that allergen-driven type 1 chemokine production is relatively higher in non-atopics compared to atopics ensuring primarily Th1 cell recruitment in response to allergen.

We randomly recruited 10 non-atopic, 10 grass atopic and 10 grass atopic mild asthmatics for this study. We specifically included atopic asthmatics in the study to examine the possible range in intensity of chemokine production in response to allergen. Atopic asthmatics experience intense inflammatory responses to allergen, often greater than those seen in atopics. In addition, several other factors such as pre-existing airway hyperesposiveness in response to inhalent allergens may intensify an immune response. We were interested in examining whether atopic asthmatics manifested an amplified chemokine response due to heightened sensitivity towards allergen. We stimulated PBMC from all three groups with 3 different concentrations of grass pollen allergen beginning with the lowest previously tested concentration that

demonstrated significant allergen-driven IP-10 and TARC production (4ug/ml). We chose to utilize lower concentrations of grass pollen allergen (as well as other allergens examined) to test the relevance of 'physiological' concentrations of allergen. In addition, we examined grass pollen concentrations at 0.4 ug/ml and 0.04ug/ml. House dust mite is a perennial allergen as opposed to seasonal grass pollen which we tested at 100 AU/ml, 20 AU/ml and 5 AU/ml. Our representative recall antigen, streptokinase, was examined at 500 AU/ml, 50 AU/ml and 5 AU/ml as well as TSST-1 at 0.1 ng/ml.

All three subject groups produced both IP-10 and TARC in response to grass pollen, house dust mite (HDM), streptokinase (SK) and TSST-1. Focusing on our representative type 1 chemokine, IP-10, we demonstrate similar intensities in response to all concentrations examined for grass pollen (Figure 11a), house dust mite (data not shown) and streptokinase (Figure 11b) from all three subject groups. This implies that IP-10 production in response to allergen is not indicative of atopic status. We examined an alternative type 1 chemokine, Mig and also did not observe any differences between subject groups. This does not exclude the fact that non-atopics may have increased levels of the cognate receptor, CXCR3. Unfortunately we were not able to analyze CXCR3 expression concurrently during this study.

Focusing on our representative type 2 chemokine, TARC, we clearly show elevated TARC production by atopics and atopic asthmatics compared to non-atopics in response to grass pollen (Figure 11a). The ability of sensitized individuals to produce enhanced TARC levels was observed regardless of type of allergen we used (grass pollen and HDM) or concentration (4, 0.4, 0.04 ug/ml grass pollen and 100, 20, 5 AU/ml). Interestingly, a similar picture emerged when we examined TARC production in

response to streptokinase (Figure 11b). Streptokinase is an enzymatic component of extracellular type B streptococci and should therefore be recognized as structurally and functionally unique compared to allergens such as grass pollen or house dust mite. However, it appears that atopic and atopic asthmatic individuals produce significantly more TARC in response to SK compared to non-atopics. Streptococci are extracellular bacteria and therefore utilize similar pathways for antigen presentation compared to allergen. We speculate that elevated levels of TARC in response to SK is representative of a biased immune response to produce type 2 chemokines in response to extracellular antigen.

Interestingly we did not observe a difference between atopic and atopic asthmatic populations in TARC, IP-10 or Mig production in response to any of the antigens tested. Atopic asthmatics produced similar elevated levels of TARC compared to atopics demonstrating their bias towards type 2 immunity in response to allergen. This finding is important because it demonstrates a common elevated type 2 chemokine response in asthma and atopy hilighting the common immune dysregulation seen in both subject groups.

We observed no significant differences between the allergen concentrations tested (ie between grass pollen allergen at 4ug/ml, 0.4ug/ml or 0.04ug/ml). However, there was a higher median production of IP-10, TARC, and Mig when PBMC were stimulated with grass pollen allergen at 4ug/ml, HDM allergen at 100AU/ml and SK at 500AU/ml. Thus, we chose to use these concentrations of allergens for future analysis of chemokine production from PBMC *in vitro*.

Figure 11a Allergen driven TARC production, but not IP-10, is elevated in atopic subjects

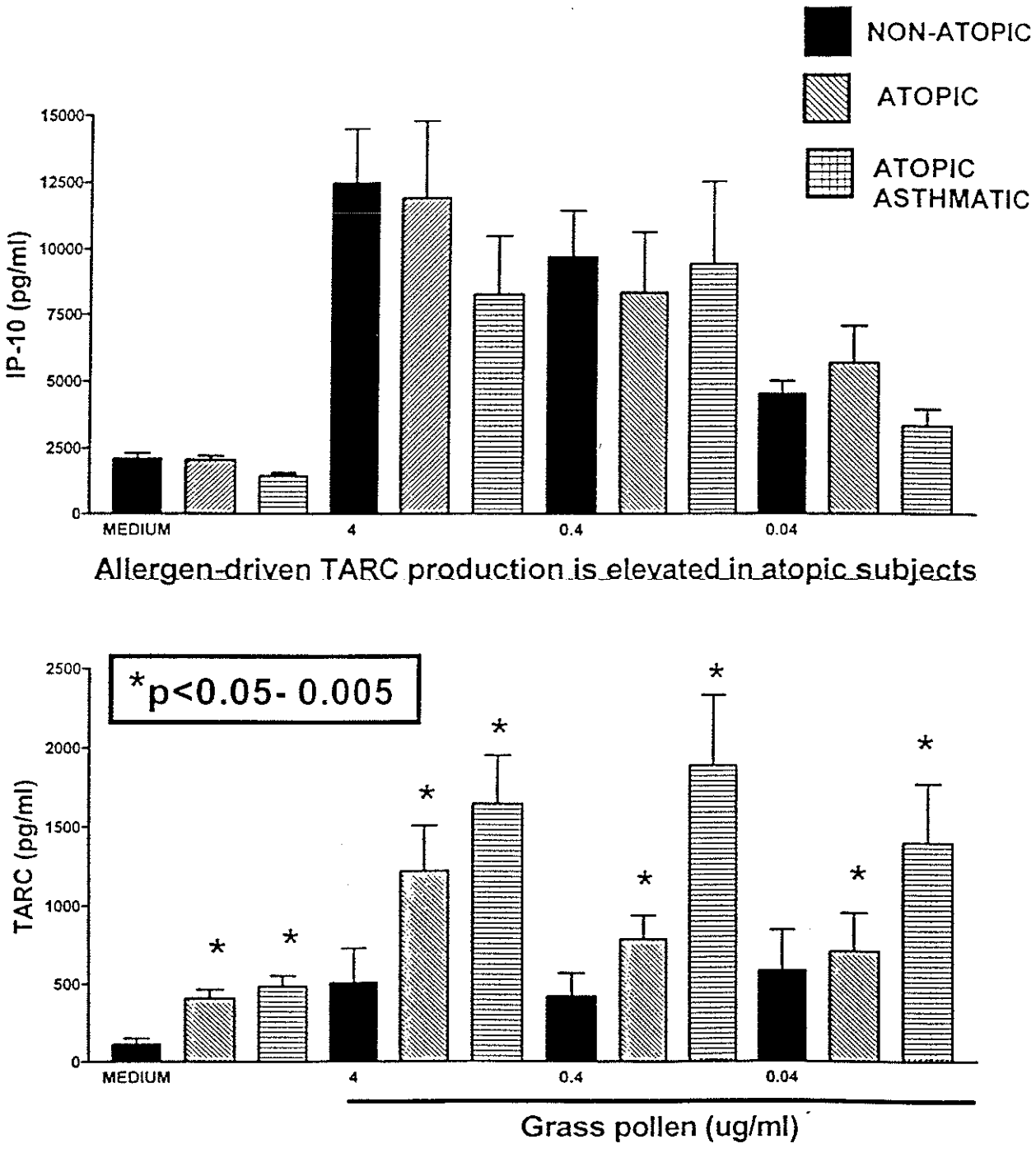

Figure 11a: A type 2 chemokine (TARC) response is significantly elevated in allergen driven and unstimulated PBMC from sensitized individuals compared to healthy, non-atopic controls. PBMC were isolated from fresh peripheral blood of grass atopic (n=10), grass atopic mild asthmatics (n=10), and normal healthy individuals (n=10). IP-10 and TARC were measured using a specific sandwich ELISAs. Data was analyzed using a Students T test.

Figure 11b Antigen (Streptokinase) driven TARC production, but not IP-10, is elevated in atopic subjects

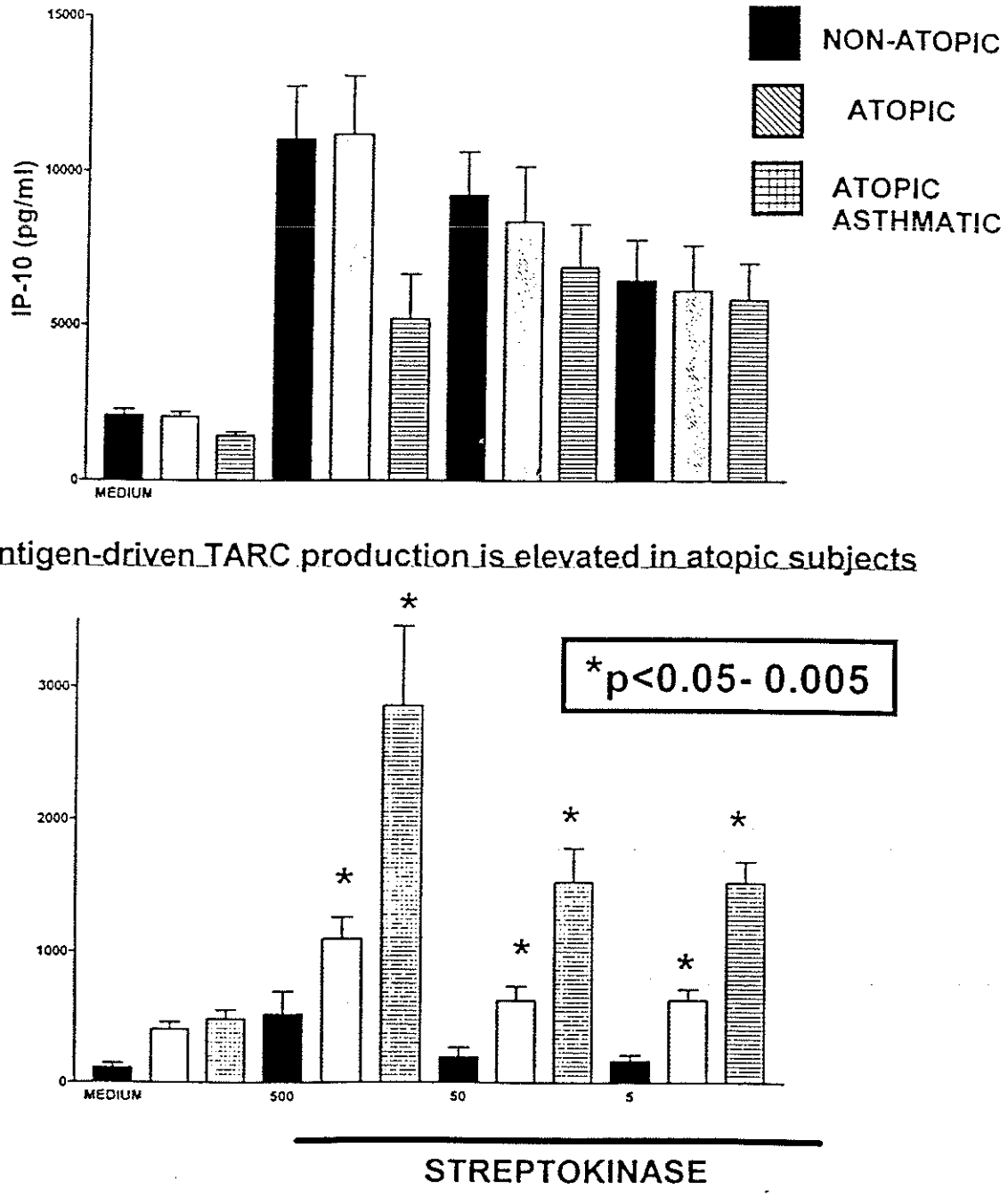

Figure 11b: A type 2 chemokine (TARC) response is significantly elevated in antigen driven and unstimulated PBMCfrom sensitized individuals compared to heatlhy, non-atopic controls. PBMC were isolated from fresh peripheral blood of grass atopic (n=10), grass atopic mild asthmatics (n=10) and normal healthy controls (n=10). Supernatents were collected and analyzed as in Figure 11a. Data was analyzed using a Students' T test.

The finding of enhanced TARC production by sensitized individuals represents a key stage in understanding how allergic inflammation is maintained. TARC binds exclusively to CCR4, which is primarily expressed on antigen-experienced Th2-like cells. Increased expression of TARC by atopic PBMC may be directly involved in maintaining allergic inflammation through the recruitment of Th2 cells to the site of allergen exposure. We argue that enhanced type 2 chemokine, TARC, production by atopic individuals represents the possibility of a positive feedback loop important for the maintenance of allergy. Analysis of CCR4 expression on atopic, and non-atopic Th cells would be required to firmly establish the possibility of a feedback loop in atopics but unfortunately specific antibodies for CCR4 are not available at this time.

10.9 Exogenous Cytokines Regulate the Intensity of Type 1 and Type 2 Chemokine Production

The cytokine milieu present during T cell recognition of antigen is extremely influential in promoting induction and maintenance of type 1 or type 2 immunity. Several studies have demonstrated the ability of cytokines to modulate chemokine production from cell lines or isolated cell populations. Specifically, IFNγ has been shown to enhance production of the type 1 chemokines IP-10 and Mig with or without polyclonal activation [296-298, 331]. Not surprisingly, the addition of the anti-inflammatory cytokine IL-10 to isolated neutrophils resulted in downregulation of IP-10, Mig and I-TAC. Unfortunately none of the aforementioned studies examined type 2 chemokines in concert, raising concern over the ability of IFNγ and IL-10 to regulate the intensity of type 2 as well as type 1 chemokines.

IL-4 has been demonstrated to inhibit IFNγ driven IP-10, Mig and I-TAC production in neutrophils, monocytes and macrophages [176, 296, 332]. In contrast, addition of exogenous type 2 cytokine, (IL-4) to isolated keratinocytes enhanced IFNγ or LPS driven IP-10, Mig and I-TAC production. These conflicting reports may represent some of the difficulties using isolated cell populations stimulated with polyclonal activators or may reflect the ability of exogenous cytokines to regulate the intensity of chemokine production. IL-13 and IL-4 have been shown to induce TARC production from bronchial epithelial cell lines. Surprisingly, optimal up-regulation of TARC required IFNγ and TNFα at severely elevated levels (300 U/ml of IFN(and 50ng/ml of TNFα). The inability of IL-13 and IL-4 to up-regulate TARC without the 'help' of a type 1 cytokine suggests further investigation of the role of IL-4 and IL-13 in promoting TARC production [265]. IL-4 present in the surrounding cytokine milieu is able to promote T cell commitment towards type 2 immunity in terms of cytokine production, even in the presence of substantial type 1 cytokine levels [140] demonstrating it's importance in driving the development of type 2 immunity. In the absence of sufficient levels of IL-4 during antigen-recognition IFN(is able to drive type 1 immunity.

We focused on the potential capacity of IL-4, IL-13, IL-10 and IFNγ to independently modulate the intensity of allergen-driven chemokine production. We hypothesized that allergen-driven type 1 and type 2 chemokine production would be selectively enhanced by the presence of exogenous type 1 and type 2 cytokines respectively. We examined representative type 1 (Mig) and type 2 (TARC) chemokines in non-atopic, atopic and atopic mild asthmatic subjects to determine any differences in the intensity of allergen driven chemokine production in response to exogenous cytokine. PBMC were

stimulated with grass pollen (4ug/ml) or streptokinase (500AU/ml) in the presence of exogenous cytokines to detect any differences between two different classes of antigen. To observe the ability of exogenous cytokines to modulate we cultured allergen-stimulated PBMC with 25 ng/ml of either IL-4, IL-13, IL-10 or IFNγ. 25ng/ml was chosen based upon several studies demonstrating the ability of IFNγ, IL-13, IL-10 and IL-4 to modulate chemokine responses at this concentration [209, 210, 265, 297, 298, 331].

We typically measure between 0-25 pg/ml of IL-4 in supernatants from PBMC stimulated with grass pollen. [326]. Similar culture conditions result in approximately 80pg/ml of IL-13, and 100pg/ml of IL-10 [330]. We were concerned that 25ng/ml (1000 fold higher) may not be representative of the microenvironment *in vivo* during allergen exposure. Thus, we examined the ability of cytokine to modulate allergen-driven chemokine responses at concentrations that we commonly detect from allergen stimulated PBMC in response to allergen (100-1000 pg/ml). Specifically we analyzed the ability of IL-4 at 100 pg/ml, and IL-13, IL-10 or IFNγ at 1000pg/ml to alter allergen-driven chemokine production. We observed the following:

1) Addition of exogenous IFNγ at 25 ng/ml selectively enhanced allergen driven Mig production and significantly inhibited TARC production. (Figure 13a and 12a repectively). Atopic, non-atopic and atopic asthmatic populations were equally responsive to exogenous IFNγ often with 3 to 4 fold increase or decrease in Mig (Figure 13b) or TARC (Figure 12b) production respectively. Similar results were seen when PBMC were stimulated with streptokinase (data not shown). Exogenous IFNγ at 1 ng/ml significantly decreased allergen-driven TARC production but did not alter allergen-driven Mig production. Differences between 25 ng/ml and 1ng/ml on

allergen-driven Mig production may indicate the ability of other cytokines to preferentially modulate the intensity of Mig production. Due to the involvement of IFNγ in allergen-driven chemokine production at 25 ng/ml, but more importantly at 1ng/ml (Figure 12d) we argue that IFNγ is important in shaping allergen-driven TARC and Mig responses.

2) Addition of exogenous IL-10 at 25ng/ml dramatically down-regulated both Mig and TARC production in response to allergen-driven and bacterial (SK) stimulation (Figure 12a, 13a). Non-atopic, atopic and atopic asthmatics were all inhibited to a similar degree (Figure 12c and figure 13c). The dramatic inhibition of both grass pollen and SK driven chemokine production demonstrates the global immunosuppressive ability of IL-10. Additionally, exogenous IL-10 at 1ng/ml was able to inhibit allergen-driven Mig and TARC production (Figure 12d), indicating the physiological relevance of IL-10 during grass pollen or SK driven chemokine production.

3) Addition of exogenous IL-4 and IL-13 (25ng/ml) preferentially up-regulated TARC production in response to allergen as well as SK. Non-atopic, atopic and atopic asthmatic populations all experienced significant up-regulation of TARC production (Figure 14a). Interestingly IL-4, but not IL-13, was able to inhibit allergen driven Mig production when added at 25 ng/ml (Figure 14b). This suggests allergen driven Mig production is dependent upon Th cell influences due to the inability of IL-13 to bind human Th cells [152, 159, 165]. Surprisingly when we examined a concentration of IL-4 commonly found in allergen stimulated culture, 100 pg/ml of IL-4; we did not observe any alteration in TARC or Mig production (data not shown). Similarly,

addition of 1ng/ml of exogenous IL-13, a commonly detected concentration in allergen stimulated culture of PBMC, we did not observe any alteration of TARC or Mig production (data not shown). These results stress the importance of the amount of cytokine used in vitro. Thus we are cautious in stating the ability of exogenous IL-4 and IL-13 to modulate the intensity of allergen-driven (or bacterial) Mig and TARC production. It is clear that at specific concentrations of IL-4 and IL-13 (25ng/ml) the intensity of allergen driven Mig and TARC production is significantly altered.

Table 7 Summary of the impact of exogenously added and endogenously removed type 1 and type 2 cytokines on allergen driven Mig and TARC production from non-atopics, atopics, and atopic asthmatics

STIMULUS	CONC.	Mig			TARC		
		NON-ATOPIC	ATOPIC	ATOPIC ASTHMATIC	NON-ATOPIC	ATOPIC	ATOPIC ASTHMATIC
IL-13	1 ng/ml	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ
IL-13	25 ng/ml	NO Δ	NO Δ	NO Δ	↑	↑	↑
αIL-13	2ug/ml	NO Δ	NO Δ	NO Δ	↓	↓	↓
IL-4	100 pg/ml	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ
IL-4	25 ng/ml	↓	↓	↓	↑	↑	↑
αIL-4	2ug/ml	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ	NO Δ
IL-10	1 ng/ml	↓	↓	↓	↓	↓	↓
IL-10	25 ng/ml	↓	↓	↓	↓	↓	↓
αIL-10	2ug/ml	NO Δ	NO Δ	NO Δ	↑	↑	↑
IFNγ	1 ng/ml	NO Δ	NO Δ	NO Δ	↓	↓	↓
IFNγ	25 ng/ml	↑	↑	↑	↓	↓	↓
αIFNγ	2ug/ml	NO Δ	NO Δ	NO Δ	↑	↑	NO Δ

Table7: The presence or absence of key T cell-derived cytokines modulates the intensity of allergen-driven TARC production. Non-atopic (n=10), atopic (n=10) and atopic asthmatic (n=10) subjects were stimulated with a previously optimized concentration of grass pollen allergen (4ug/ml) +/- **25ng/ml** or **0.1-1 ng/ml** of recombinant human IFNγ, IL-10, IL-13 or IL-4. In addition allergen-driven TARC and Mig production was in several cases dramatically altered by the addition of 2ug/ml of blocking antibody against IFNγ, IL-10, IL-13 or IL-4. In comparison to TARC/Mig production determined during allergen stimulation alone, TARC/Mig production was significantly increased (↑), decreased (↓) or no significant change (NO Δ) was observed.

Figure 12a The addition of exogenous T cell derived cytokines IFNγ and IL-10 markedly regulates the intensity of allergen-driven TARC production

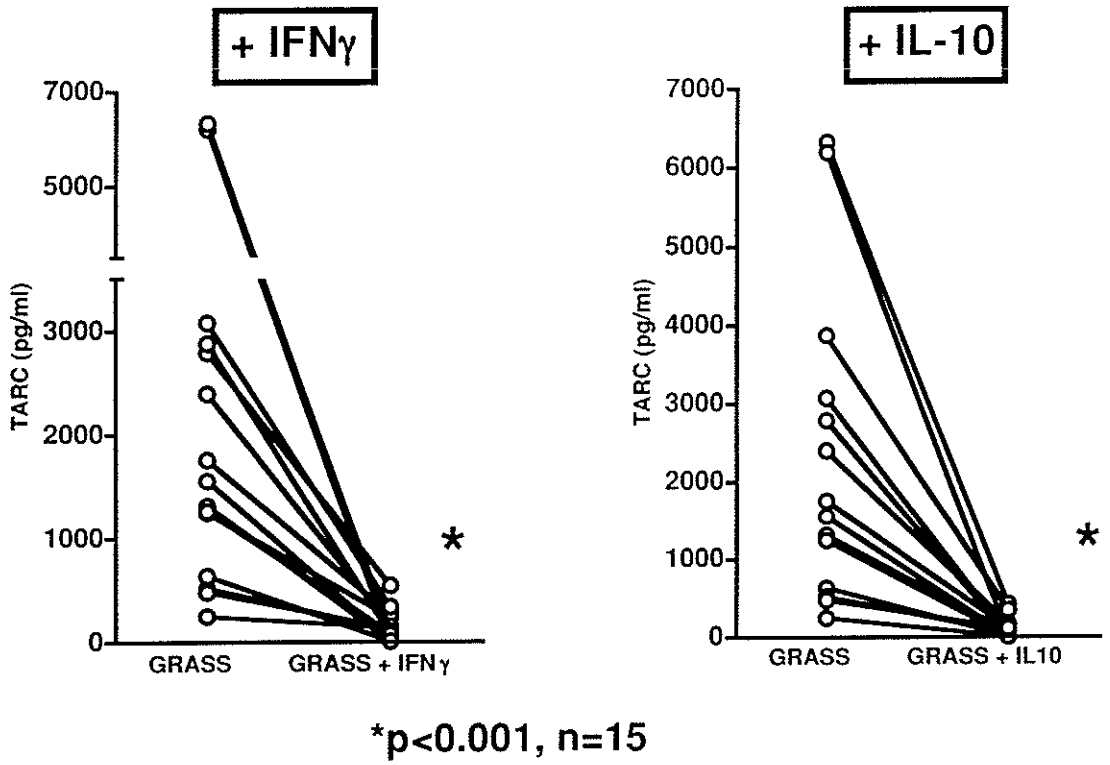

Figure 12a: The addition of exogenous T cell derived cytokines to allergen stimulated PBMC directly ex vivo markedly regulates the intensity of TARClevels. Non-atopic (n=5), grass atopic (n=5) and grass atopic mild asthmatic (n=5) PBMC were isolated from fresh EDTA-treated whole blood. PBMC were stimulated with grass pollen (4ug/ml) and either rhIFNγ, or rhIL-10 (25ng/ml). TARC levels were determined using TARC specific sandwich ELISA. Data were analyzed using a paired T test.

Figure 12b Addition of exogenous IFNγ dramatically reduces allergen-driven TARC production from non-atopic, atopic, and atopic asthmatic PBMC.

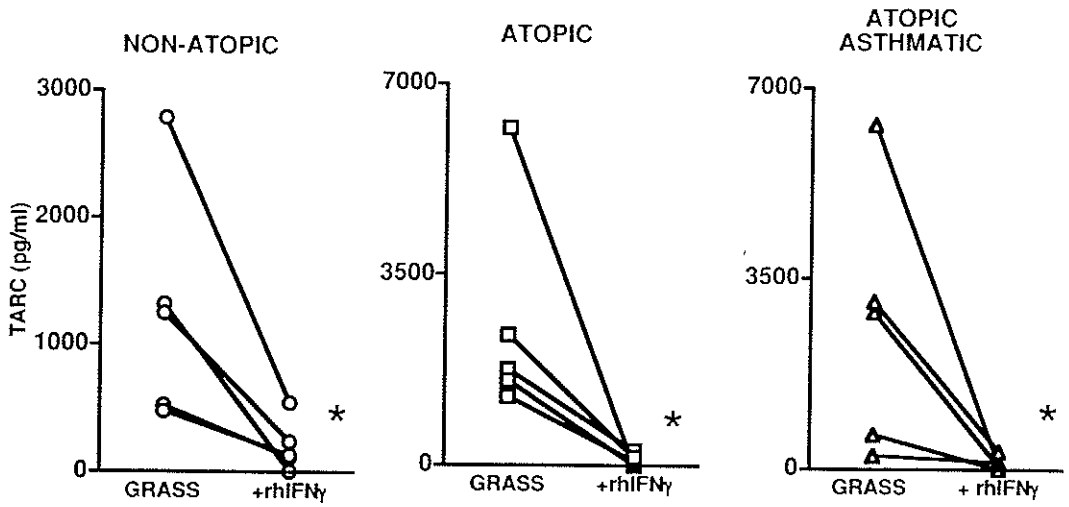

Figure 12b: Allergen-driven TARC production from non-atopic, grass atopic, and grass atopic, mild asthmatic PBMC is dramatically inhibited upon addition of exogenous rhIFNγ. PBMC are stimulated with a previously optimized concentration of grass pollen allergen (4ug/ml) +/- 25 ng/ml of rhIFNγ. TARC levels in the supernatents were measured 5 days after allergen +/- recombinant cytokine stimulation using a TARC specific sandwich ELISA. Non-atopic (circle, n=5), grass atopic (square, n=5) andgrass atopic, mild asthmatic(triangle, n=5) subjects were equally inhibited by addition of exogenous rhIFNγ, *$p<0.05$.

Figure 12c Addition of exogenous IL-10 dramatically reduces allergen-driven TARC production from non-atopic, atopic, and atopic asthmatic PBMC.

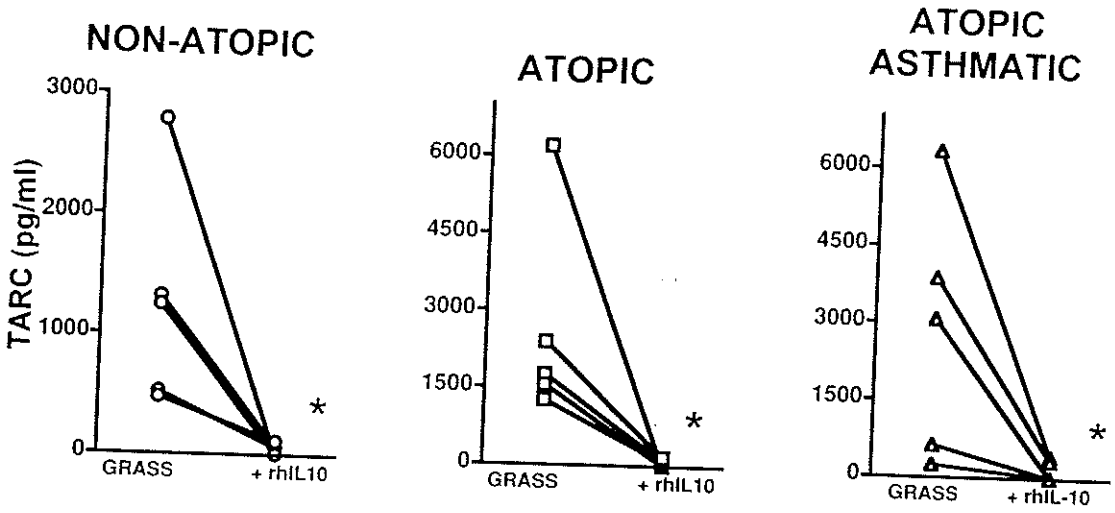

Figure 12c Allergen-driven TARC production from non-atopic, grass atopic, and grass atopic, mild asthmatic PBMC is dramatically inhibited upon addition of exogenous rhIL-10. PBMC are stimulated with a previously optimized concentration of grass pollen allergen (4ug/ml) +/- 25 ng/ml of rhIL-10. TARC levels in the supernatents were measured 5 days after allergen +/- recombinant cytokine stimulation using a TARC specific sandwich ELISA. Non-atopic (circle, n=5), grass atopic (square, n=5) andgrass atopic, mild asthmatic(triangle, n=5) subjects were equally inhibited by addition of exogenous rhIFNγ, *p<0.05.

Figure 12d Addition of exogenous rIFNγ and rIL-10 at concentrations that represent physiological levels found during allergen stimulation dramatically downregulate allergen driven TARC production.

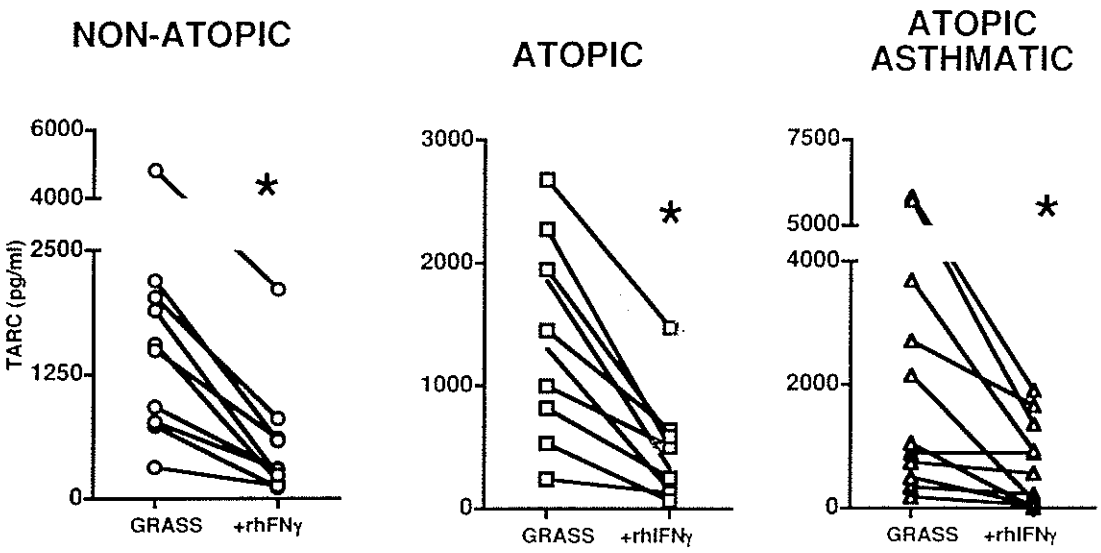

Figure 12d: Physiological levels of rhIFNγ significantly inhibit allergen-driven TARC production. Non-atopic (circles, n=10), grass atopics,squares, n=10) and grass atopic mild asthmatic (triangles, n=10) groups were stimulated with grass pollen allergen (4ug/ml) +/- rhIFNγ (1ng/ml). Supernatants were collected five days after stimulation and TARC levels were determined using a TARC specific sandwich ELISA. Statistics were determined using a paired T test. *p<0.005

Figure 13a The addition of exogenous T cell derived cytokines IL-10, but not IFNγ, markedly regulates allergen-driven Mig production

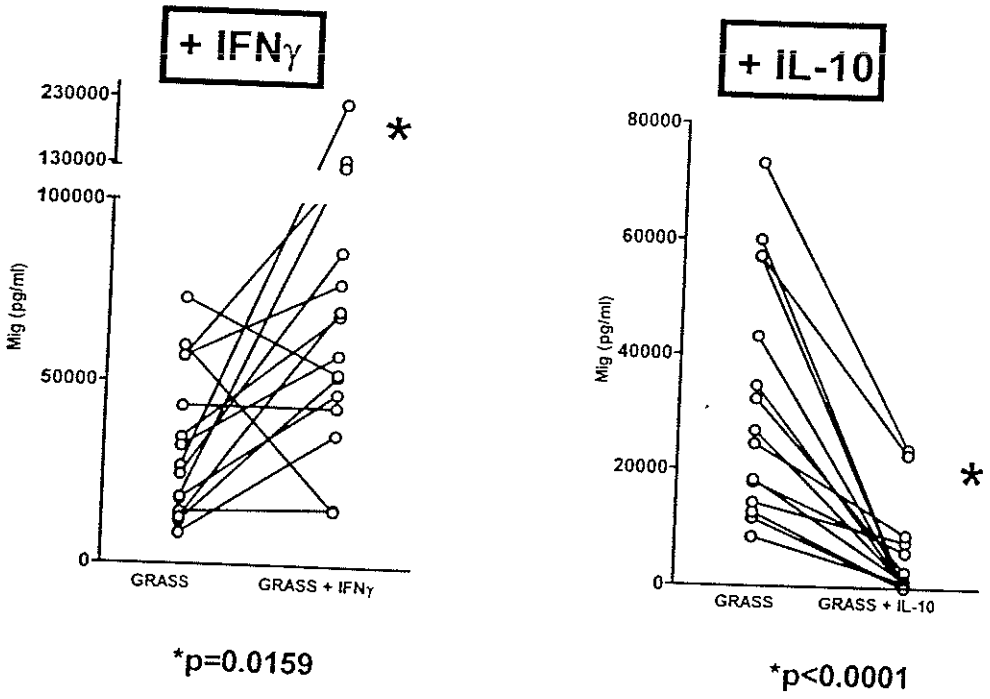

Figure 12b: The addition of exogenous T cell derived cytokines to allergen stimulaed PBMC markedly regulates the intensity of Mig production. Non-atopics (n=5), grass atopic (n=5), and grass atopic mild asthmatic (n=5) PBMC were isolated from fresh EDTA-treated whole blood. PBMC was stimulated with grass pollen (4ug/ml) and either rh IFNγ, or rhIL-10 (25 ng/ml). Mig levels were measured using a specific sandwich ELISA. Statistics were performed using a paired T test.

Figure 13b Addition of exogenous IFNγ dramatically reduces allergen-driven Mig production from non-atopic, atopic, and atopic asthmatic PBMC.

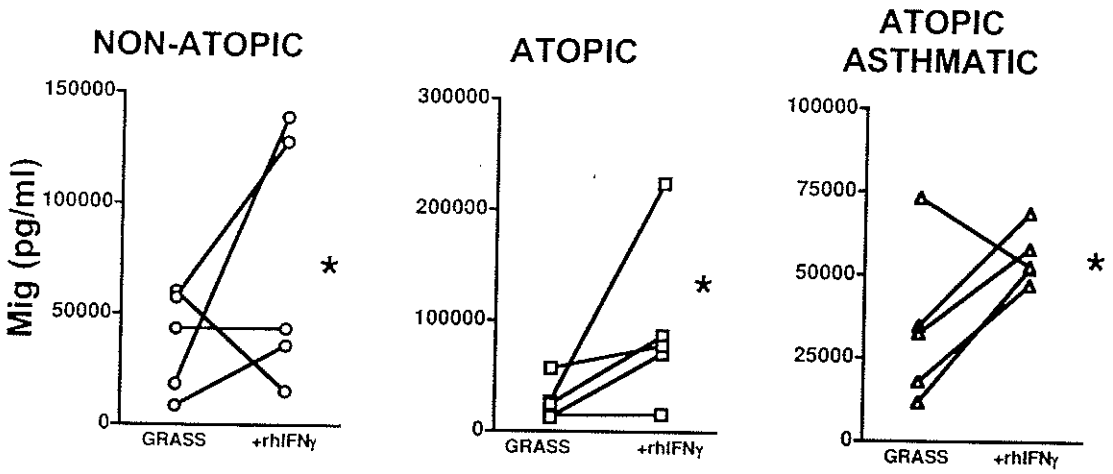

Figure 13b: Allergen-driven Mig production from human PBMC isenhanced by the addition of exogenous rhIFNγ. Non-atopic (n=5, circles), grass atopic (n=5, squares), and grass atopic, mild asthmatic (n=5, triangles) PBMC were stimulated with 4ug/ml ofgrass pollen +/- rhIFNγ at 25ng/ml. Mig levels in the supernatents were measured 5 days later using a Mig specific sandwich ELISA. *p<0.05.

Figure 13c Addition of exogenous IL-10 dramatically reduces allergen-driven Mig production from non-atopic, atopic, and atopic asthmatic PBMC.

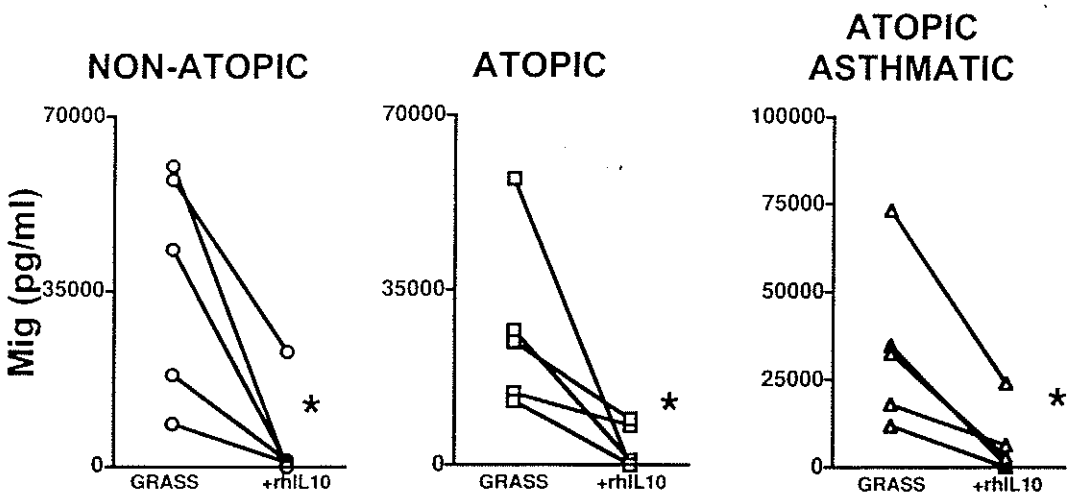

Figure 13c: Allergen-driven Mig production from human PBMC is inhibited by the addition of exogenous rhIL-10. Non-atopic (n=5, circles), grass atopic (n=5, squares), and grass atopic, mild asthmatic (n=5, triangles) PBMC were stimulated with 4ug/ml ofgrass pollen +/- rhIL-10 at 25ng/ml. Mig levels in the supernatents were measured 5 days later using a Mig specific sandwich ELISA. *p<0.05.

Figure 14a The addition of exogenous Th2 derived cytokines IL-4 and IL-13 dramatically upregulates allergen-driven TARC, a type 2 chemokine, production

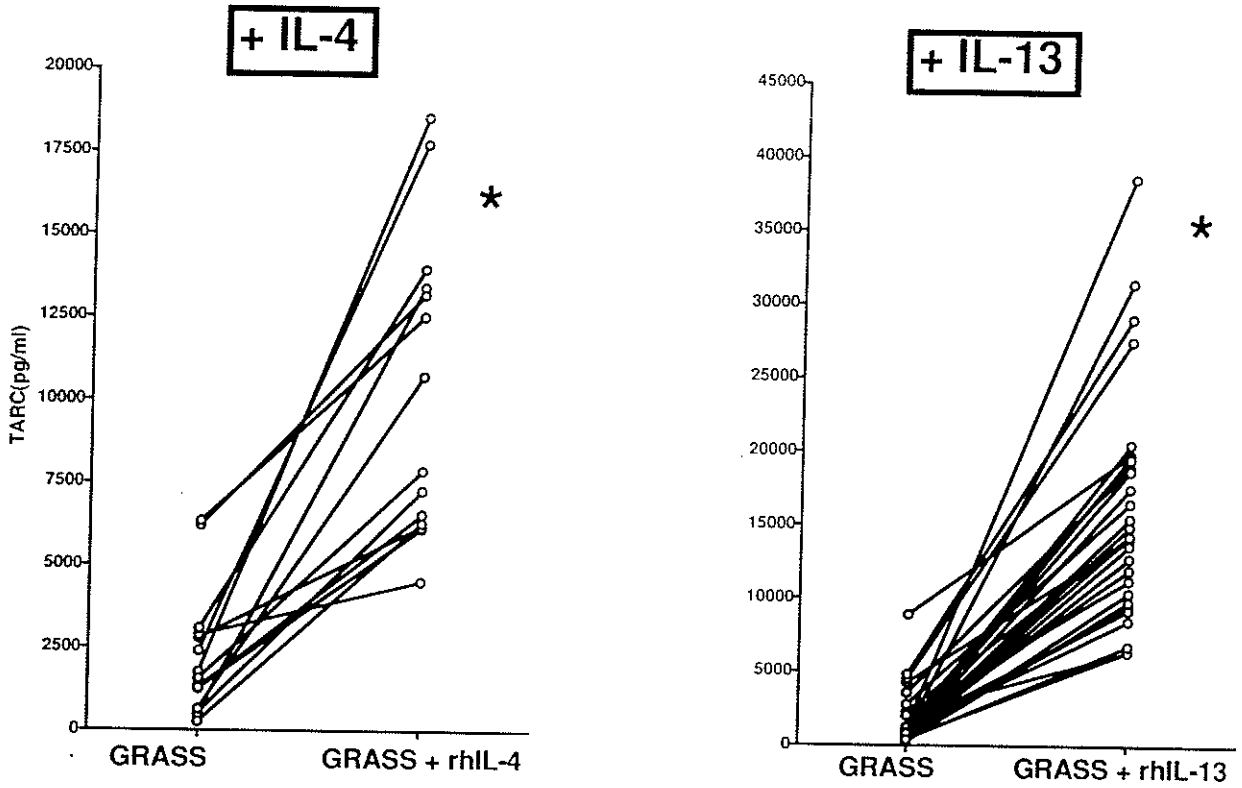

Figure 14a: The addition of exogenous T cell derived cytokines to antigen stimulaed PBMC markedly regulates the intensity of TARC production. Non-atopics (n=5), grass atopic (n=5), and grass atopic mild asthmatic (n=5) PBMC were isolated from fresh EDTA-treated whole blood. PBMC was stimulated with grass pollen (4ug/ml) and either rhIL-13 or rhIL-4(25 ng/ml). TARC levels were measured using a TARC specific sandwich ELISA. Statistics were performed using a paired T test.

Figure 14b The addition of exogenous Th2 derived cytokines IL-4, but not IL-13, markedly inhibits allergen-driven Mig production

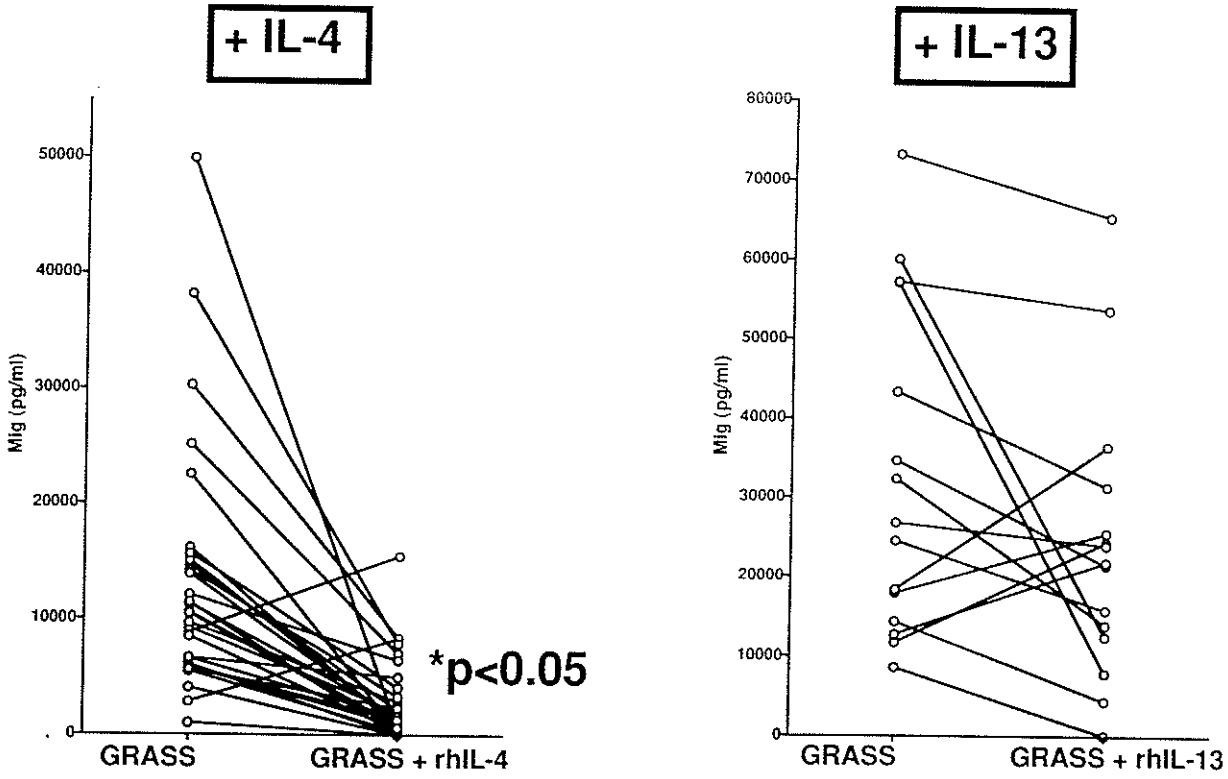

Figure 14b: The addition of exogenous T cell derived cytokines to antigen stimulaed PBMC markedly regulates the intensity of Mig production. Non-atopics (n=5), grass atopic (n=5), and grass atopic mild asthmatic (n=5) PBMC were isolated from fresh EDTA-treated whole blood. PBMC was stimulated with grass (4ug/ml) and either rhIL-13 or rhIL-4 (25 ng/ml). Mig levels were measured using a Mig specific sandwich ELISA.. Statistics were performed using a paired T test.

These results clearly demonstrate the ability of exogenous T cell derived cytokines to regulate the intensity of type 1 and type 2 chemokine production. This is important because it indicates the ability of allergen-driven cytokine production to regulate chemokine production. Chemokine production is a critical step in the development and maintenance of inflammation due to the ability of chemokines to preferentially recruit polarized T cell populations to the site of inflammation. The chemokines we measured, Mig and TARC, preferentially recruit polarized Th1 or Th2 cells respectively. The ability of type 1 and type 2 cytokines to modulate the intensity of chemokine production indicates the possibility of feedback loops responsible for the maintenance of clinical sensitivity or tolerance. For example, allergen-driven IFNγ production by Th cells in non-atopics preferentially promotes the up-regulation of Mig levels, resulting in preferential recruitment of CXCR3+ CD4/CD45RO+ Th cells to the site of inflammation. CXCR3+ cells primarily produce IFNγ upon restimulation with antigen thereby maintaining the presence of type 1 immunity in response to antigen. IL-4 and IL-13 driven TARC production may operate through a similar mechanism involving CCR4+ CD4/CD45RO+ Th cells.

To what degree IL-4 and IL-13 regulate allergen-driven chemokine production *in vivo* is questionable. The ability of IL-13 and IL-4 to enhance TARC production at 25 ng/ml is novel, but may not be relevant. The ability of exogenous IL-13 and IL-4 to enhance allergen-driven TARC production at levels commonly observed in similar culture conditions appears to be more relevant to the *in vivo* response. Thus, we are cautious in claiming the ability of IL-4 and IL-13 to modulate allergen-driven chemokine

production *in vivo* until further experiments are able to shed light into the relevance of these findings.

In conclusion, we argue that exogenous IFNγ, IL-10, IL-4 and IL-13 independently regulate the intensity of allergen-driven Mig and TARC production. The amount of exogenous cytokine added to allergen-stimulated PBMC resulted in varied responses with 25ng/ml demonstrating the strongest influence on the intensity of TARC and Mig production. The relevance of IL-4 and IL-13 on allergen-driven Mig and TARC production requires further examination to determine the relative importance of these two cytokines in modulating Mig and TARC responses. These findings are important because they demonstrate the ability of the cytokine milieu to regulate the intensity of allergen-driven type 1 and type 2 chemokine production. We argue that the link between Th1/Th2 cytokines that are tightly correlated with allergic inflammation and the ability of these cytokines to regulate the intensity of chemokine production indicates a possible role for chemokines in atopic disease.

10.10 Endogenous Cytokine Modulates the Intensity of Allergen-driven Chemokine Production

Addition of exogenous cytokines to allergen or bacterial (SK) stimulated culture has provided extremely useful information the ability of cytokines to modulate the intensity of chemokine production. Whether the basal levels of allergen-driven chemokine production we are measuring are currently under the influence of endogenous cytokines is currently undetermined. Previous work done by our lab has demonstrated that atopic PBMC produce elevated IL-13 in comparison to normal healthy controls in response to grass pollen [330]. Additional studies demonstrated higher frequencies of atopic individuals capable of exhibiting inhalant allergen-driven IL-4 responses in primary culture while displaying lower IFNγ responses [326, 333]. The ability of atopic PBMC to preferentially produce elevated levels of IL-4 and IL-13 may represent how allergen-driven TARC production is elevated in atopics. We speculated that removal of endogenous IL-4 or IL-13 present during allergen-driven chemokine production may clarify the relative involvement of IL-4 and IL-13 that we encountered (section 7.9). Advantages of this technique over the addition of exogenous cytokine lies in the physiological relevance of adding exogenous cytokine to culture. We argue that removal of endogenous cytokine allows for examination of the relative impact of cytokines present on the intensity of allergen-driven chemokine production. Thus we hypothesized that endogenous cytokines, IL-4 and IL-13 preferentially upregulate allergen-driven TARC (type 2) production while suppressing allergen driven Mig production (type 1). In addition, endogenous IFNγ is able to drive type 1 chemokine production and inhibit type 2 chemokine production.

To investigate the ability of endogenous cytokine to modulate chemokine production we utilized mAb to block IFNγ, IL-4, IL-13, as well as IL-10 during allergen-stimulation. We measured representative type 1 (Mig) and type 2 (TARC) chemokines in response to allergen co-cultured with blocking mAb to IFNγ, IL-4, IL-13, or IL-10. We observed striking results when each of the endogenous cytokines was blocked:

1) Blocking endogenous IFNγ significantly increased the amount of TARC produced by PBMC stimulated with grass pollen as well as streptokinase (Figure 15a). Atopic, and non-atopic subjects responded similarly to the removal of IFNγ often with responses as robust as 3 fold over allergen-driven alone. Interestingly, atopic asthmatics did not display a significant increase in allergen-driven TARC production in response to mAb αIFNγ treatment ($p=0.1230$). This may be a reflection of a lack of IFNγ production by PBMC in response to allergen, consistent with the presence of biased type 2 immunity in response to allergen.

   In contrast, blocking IFNγ did not significantly alter allergen-driven Mig production suggesting that endogenous IFNγ does not enhance allergen-driven Mig production. (Figure 15b) This is important because it demonstrates the ability of endogenous IFNγ to preferentially inhibit TARC production, but not Mig production. This is consistent with early results demonstrating the ability of 25ng/ml but not 1ng/ml to enhance Mig production. We believe this further reinforces the importance of using caution in interpreting the ability of exogenous cytokines to modulate chemokine responses *in vitro* to what normally occurs *in vivo*.

2) Blocking endogenously produced IL-13 significantly reduced allergen-driven TARC production but did not alter Mig production. (Figure 16a and 16b respectively) In

contrast blocking IL-4 did not significantly alter allergen-driven Mig or TARC production. Non-atopic, atopic and atopic asthmatic subjects all responded equally to treatment with mAb αIL-13 or mAb αIL-4 with median decreases in response to αIL-13 mAb treatment 3 fold below allergen stimulation alone. This finding demonstrates the ability of endogenous IL-13 to enhance allergen-driven TARC production.

Atopic subjects produce enhanced TARC and IL-13 levels in response to allergen. Combined with the finding that IL-13 at 25ng/ml but not 1ng/ml is able to enhance allergen-driven TARC production may reflect the requirement for higher levels of IL-13 to drive elevated TARC production in atopics. The ability of exogenous but not endogenous IL-4 to modulate the intensity of TARC and Mig production indicates that IL-4 may not be a critical for driving type 2 chemokine production *in vivo*. Specifically, the inability of 10 or 100 pg/ml of IL-4 to enhance allergen-driven TARC production supports this hypothesis because allergen-driven IL-4 production from PBMC is normally less than 100pg/ml and blocking endogenous IL-4 did not alter TARC production. The relevance of 25ng/ml or even 1ng/ml is questionable due to low levels of IL-4 normally produced *in vivo*.

3) Blocking IL-10 moderately enhanced TARC production in response to allergen but did not significantly alter Mig production. (Figure 15a and 15b respectively) On average there was a 31% increase in allergen-driven TARC production when endogenous IL-10 was blocked. Although blocking endogenous IL-10 statistically did increase the amount of allergen driven TARC production, the effects were modest. We did not observe any differences between non-atopic, atopic and atopic

asthmatics suggesting that a lack of endogenous IL-10 in atopic subjects is not inherently responsible for the enhanced allergen-driven TARC production. This work supports the current hypothesis regarding the ability of IL-10 to down-regulate immune responses. The inability of endogenous IL-10 to inhibit allergen-driven Mig production may be a result of insufficient levels of endogenous IL-10 suggesting different requirements for down-regulation of type 1 (Mig) and type 2 (TARC) chemokine responses.

Taken together, our system clearly demonstrates the ability of type 1 and type 2 cytokines to directly regulate the intensity of type 1 and type 2 allergen-driven chemokine production. This work is important because it demonstrates the ability of endogenously produced type 1 and type 2 cytokines to influence type 1 and type 2 chemokine production. We speculate the existence of type 1 and type 2 positive feedback loops involving cytokines, chemokines, and chemokine receptors to maintain clinical tolerance or sensitivity to allergen. IFNγ, IL-4 and IL-13 are directly involved in the development of type 1 and type 2 immunity strongly implicating their role in atopic disease. The ability of endogenously produced IFNγ, IL-4 and IL-13 to regulate the intensity of Mig and TARC combined with CXCR3 and CCR4 expression exclusively on Th1 and Th2 clones implicates these two chemokines in the maintenance of atopic disease.

Figure 15a Blocking key T cell derived cytokines IFNγ or IL-10 significantly enhances allergen-driven TARC production

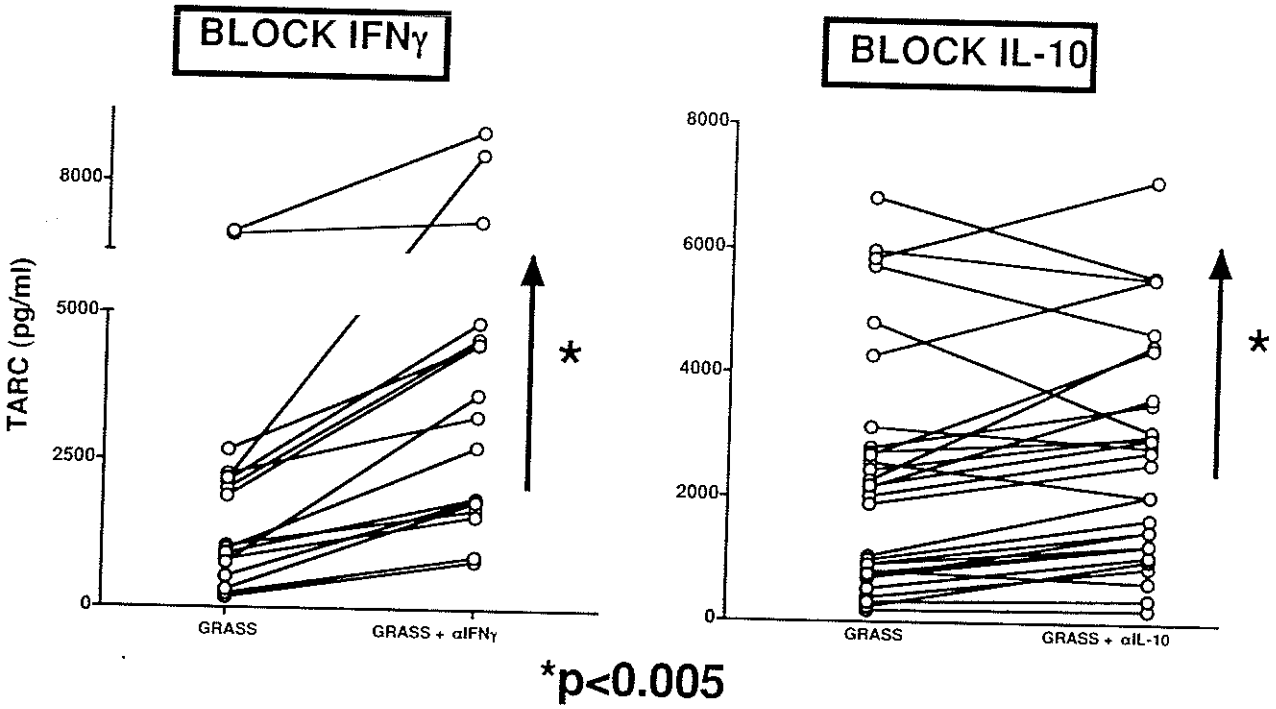

Figure 15a: Neutralisation of key T cell derived cytokines significantly alters allergen driven TARC production. PBMC from non-atopic (n=10), grass atopic (n=10) and grass atopic mild asthmatic (n=10) were isolated from fresh EDTA-treated whole blood. PBMC were placed into short term culture (five days) with grass pollen allergen (4ug/ml) +/- monoclonal mouse anti-human blocking antibodies to IL-10 or IFNγ (2ug/ml). Isotype-matched control (mouse mAb-IgG1) did not alter allergen-driven TARC or Mig production (data not shown). TARC levels were determined using a specific sandwich ELISA. Statistics were performed using a paired T test, *p<0.005, n=30.

Figure 15b Blocking key T cell derived cytokines IFNγ or IL-10 did not dramatically alter allergen-driven Mig production

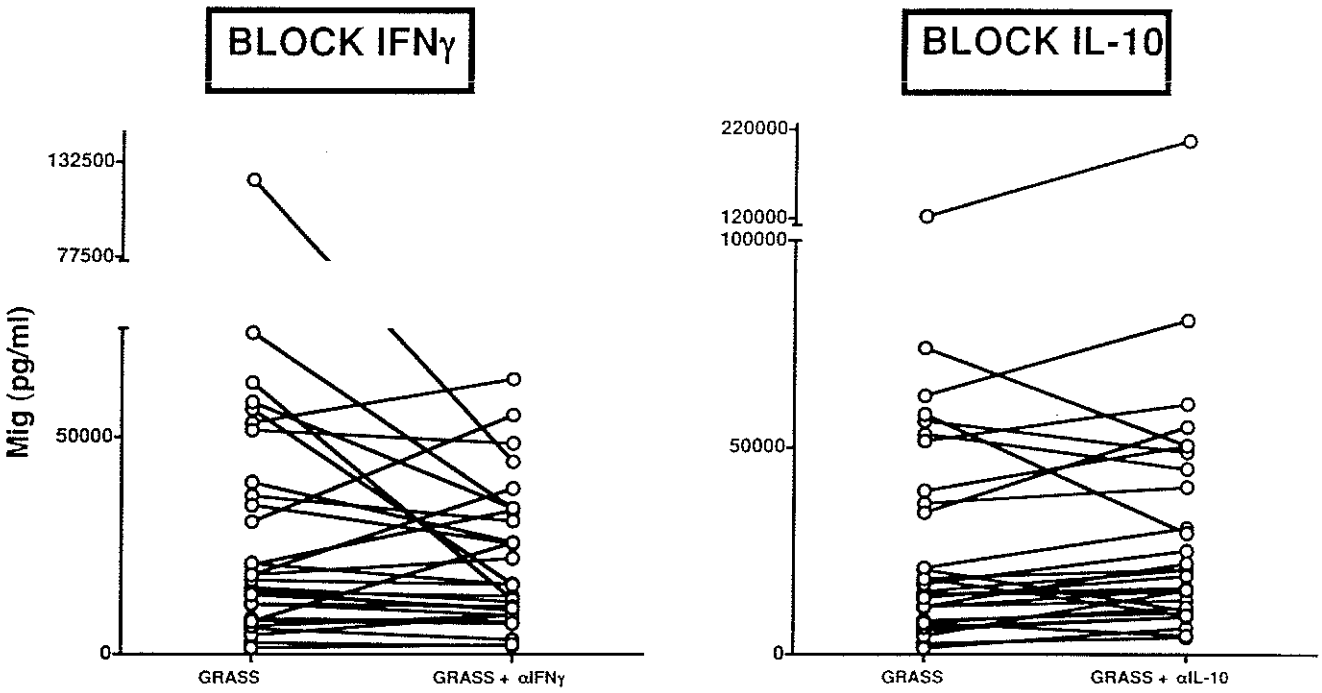

Figure 15b: Neutralisation of key T cell derived cytokines does not alter allergen driven Mig production. PBMC from non-atopic (n=10), grass atopic (n=10) and grass atopic mild asthmatic (n=10) were isolated from fresh EDTA-treated whole blood. PBMC were placed into short term culture with grass pollen allergen (4ug/ml) plus monoclonal mouse anti-human blocking antibodies to IL-10 or IFNγ (2ug/ml). Mig levels were determined using a specific sandwich ELISA. Statistics were performed using a paired T test, *p<0.005, n=30.

Figure 16a Blocking Th2 derived cytokine IL-13, but not IL-4, markedly inhibits allergen-driven TARC production

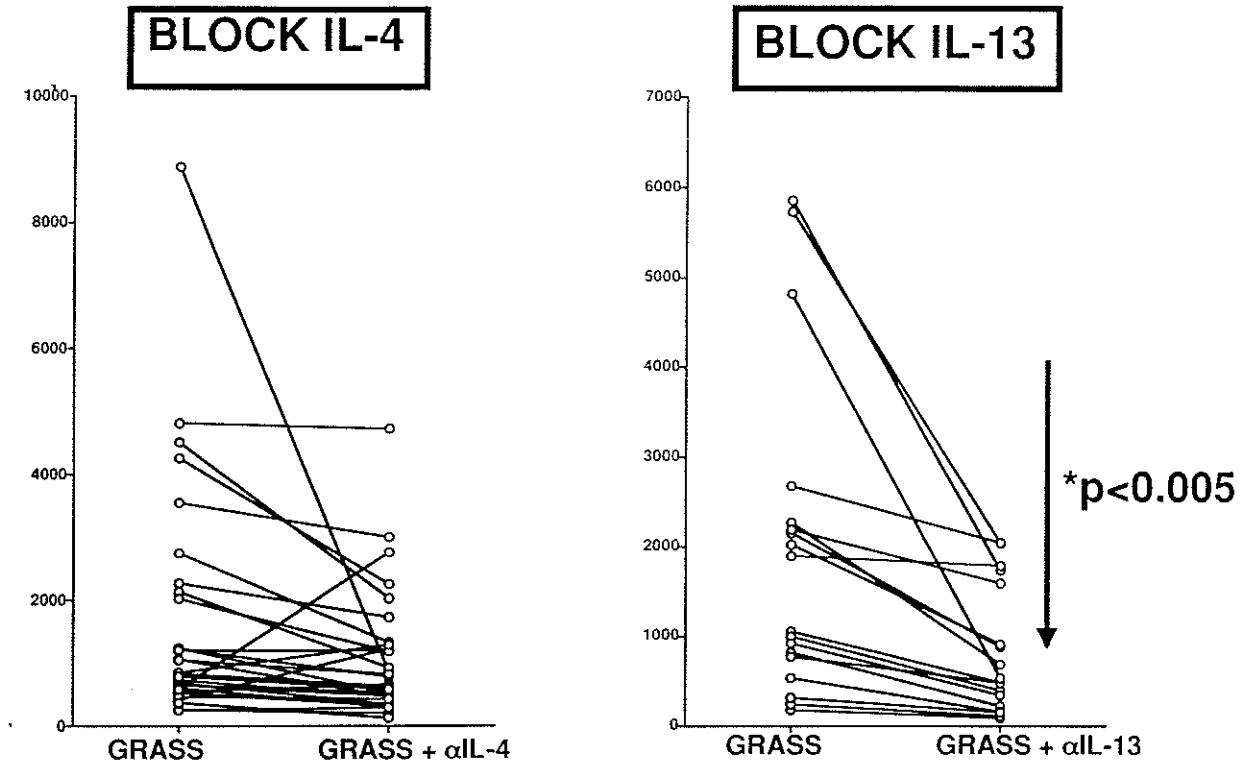

Figure 16a: Neutralisation of key T cell derived cytokines significantly alters allergen driven TARC production. PBMC from non-atopic (n=10), grass atopic (n=10) and grass atopic mild asthmatic (n=10) were isolated from fresh EDTA-treated whole blood. PBMC were placed into short term culture (five days) with grass pollen allergen (4ug/ml) +/- monoclonal mouse anti-human blocking antibodies to IL-13, IL-4 (2ug/ml). Isotype-matched control (mouse mAb-IgG1) did not alter allergen-driven TARC or Mig production (data not shown). TARC levels were measured using a specific sandwich ELISA. Statistics were performed using a paired T test, *p<0.005, n=30.

Figure 16b Blocking Th2 derived cytokine IL-13 or IL-4 did not significantly alter allergen-driven Mig production

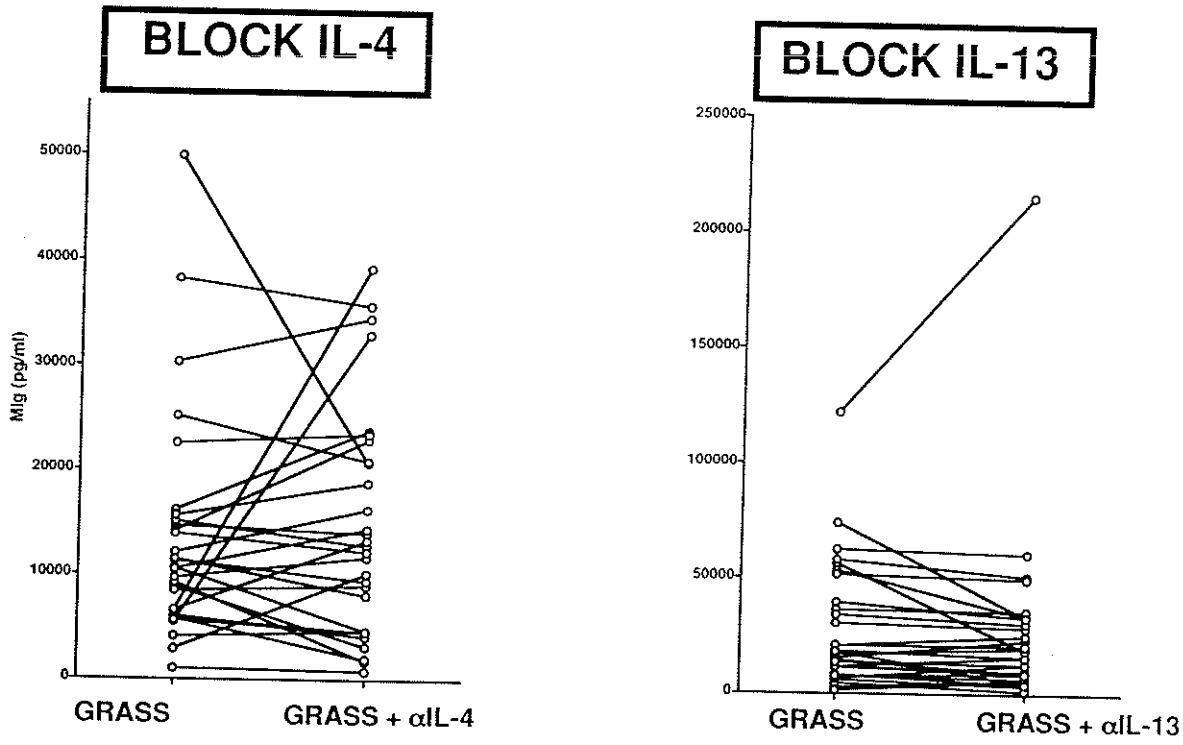

Figure 16b: Neutralisation of key T cell derived cytokines significantly alters allergen driven Mig production. PBMC from non-atopic (n=10), grass atopic (n=10) and grass atopic mild asthmatic (n=10) were isolated from fresh EDTA-treated whole blood. PBMC were placed into short term culture (five days) with grass pollen allergen (4ug/ml) +/- monoclonal mouse anti-human blocking antibodies to IL-13 or IL-4 (2ug/ml) Mig levels were quantified using a specific sandwich ELISA. Statistics were performed using a paired T test, n=30.

10.11 Allergen-driven TARC and Mig production is MHC II and CD4 dependent

Chemokine production has primarily been demonstrated using cell lines or isolated cell populations stimulated with LPS, PHA as well as other polyclonal activators making it difficult to assess the requirement of antigen presentation for chemokine production. In addition, many chemokines are constitutively produced and may be induced under 'appropriate stimuli' making it difficult to determine whether chemokine detected is due to the stimuli or not. A study by Herold et al demonstrated the dependence of isolated murine T cells for stimulation through both αCD3 and αCD28 for MIP-1α production. They were able to show the requirement for CD28 binding because chemokine production could not be replaced through IL-2 administration, indicating the requirement for intracellular signaling elicited during antigen recognition. In addition, MIP-1α production from bulk populations of murine spleen cells stimulated with αCD3 was subsequently inhibited by co-culture with anti-B7 mAbs, indicating that full production of the chemokines was dependent upon interactions with B7 ligands [334]. MIP-1α is produced by T cells, monocytes, as well as many other cell types. Regardless of the cell type producing MIP-1α production was abrogated if TCR signaling could not properly occur, suggesting that T cell recognition of peptide is required for MIP-1α production. To date, no studies examining the activation requirements type 1 and type 2 chemokine production from human T cells or APC exists. In our system, Mig and TARC are primarily produced by APC but they are regulated by T cell derived cytokines and bind to receptors found primarily on Th1 or Th2 clones respectively. We were

interested in determining whether T cell activation was a prerequisite for primarily APC driven chemokine production.

To test our hypothesis we co-cultured freshly isolated PBMC stimulated with grass pollen allergen at the previously optimized concentration (4ug/ml) and blocking antibodies to the dominant MHC II haplotypes HLA-DR. We examined both non-atopic and atopic individuals to address whether the presence of atopy to a particular antigen results in reduced or functionally different requirements for chemokine production from APC. Optimal concentration of αHLA-DR was determined in a previous experiment to be 5ug/ml (data not shown).

In addition we considered the importance of CD4 for formation of a stable TCR-peptide-MHC class II in chemokine production. In the case of exogenous antigens such as allergens, CD4 co-receptors are critical for the formation of the TCR-peptide-MHC class II trimer. We hypothesized that CD4 co-receptor was critical for trimer formation resulting in T cell activation leading to allergen-driven chemokine production. To test our hypotheses we analyzed allergen-driven Mig and TARC production from atopic and non-atopic PBMC co-cultured with blocking antibodies to CD4.

We observed a complete inhibition of allergen driven Mig and TARC production by treatment with αHLA-DR or αCD4. (Figure 17) This is contrary to many studies that have demonstrated the ability of isolated cell populations such as neutrophils stimulated with cytokine alone or polyclonal activator to be capable of producing vast amounts of Mig [296]. In addition, TARC production by isolated macrophage derived dendritic cells was induced by LPS stimulation alone [209, 232].

This is important because it demonstrates the dependence upon T cell recognition of allergen before chemokine production is induced from the APC. We did not observe any differences between atopic and non-atopic subjects in terms of inhibition of chemokine production. Therefore, we argue that production of TARC and Mig is dependent upon T cell recognition of allergen in the context of MHC class II. This does not dispute the fact that structural cells are able to produce chemokine in response to inflammatory stimuli such as cytokines or LPS but rather suggests an additional level of regulation within antigen-presenting cells in the circulation to present antigen to the T cell before chemokine production may occur. We demonstrated earlier the ability of T cell derived exogenous cytokines to shape the intensity of chemokine production in response to allergen. Taken together this data demonstrates the ability of T cells regulate the initiation and intensity of allergen-driven chemokine production.

Figure 17 Allergen-driven Mig and TARC production is MHC class II and CD4 dependent

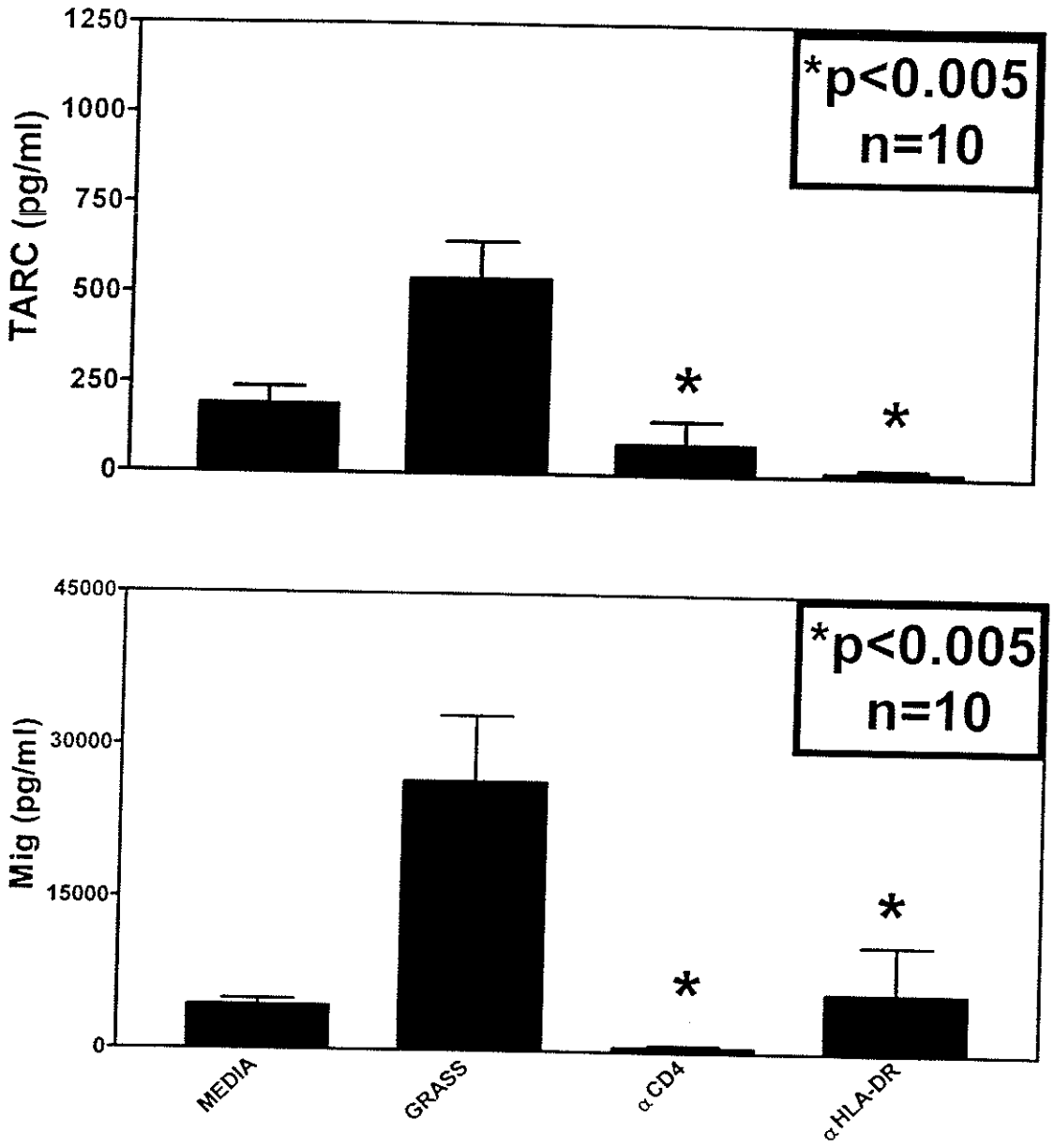

Figure 1 . Allergen-driven Mig and TARC production is dependent upon T cell recognition of allergen in the context of MHC class II and CD4. Non-atopic (n=5), and atopic (n=5) PBMC were stimulated directly ex vivo with grass pollen (4ug/ml) +/- blocking antibodies to CD4 or HLA-DR. (5ug/ml). Mig and TARC levels were measured using a specific ELISA. Statistics were performed using a Paired T test.

10.12 Allergen-driven TARC and Mig production is dependent upon co-stimulatory molecules involved in antigen presentation

MHC class II restriction of allergen-driven TARC and Mig production from primarily APC indicated the requirement of stimulation from allergen-specific T cells to activate the APC. A physical interaction resulting from TCR recognition of allergen in the context of MHC class II reveals itself as the most obvious type of signal. MHC class II molecules bind TCR-CD3 via hydrophobic and hydrophilic interactions. Co-stimulatory molecules present on APC specifically bind to cognate receptors tightly linked to T cell activation. Co-stimulatory molecules that have been demonstrated to be important in activation include B7-1 and B7-1 (CD80 and CD86 respectively) found on the APC that selectively bind to CD28 on the Th cell initially. This is important for T cell activation. CTLA-4 is then upregulated on the surface of Th cells and competes for binding to B7-1 and B7-2. CTLA-4 binding results in downregulation of Th cell activation, presumably as a mechanism to ensure immune response are not uncontrolled. There is much interest in blocking B7-1 and/or B7-2 molecules as a possible treatment of inappropriate immune responses such as transplants and atopy. Several studies have demonstrated the inhibition of cytokine production from Th cells by blocking either B7-1 or B7-2. Interestingly, there is a controversy over whether B7-1 or B7-2 is responsible for the activation of cytokine production, specifically type 2 cytokines. Some reports suggest that B7-2 (CD86), but not B7-1 (CD80), co-stimulation can preferentially activate Th2 cytokine expression and Th2 development. [133, 135, 136, 335]. Other studies suggest that although human PBMC memory responses, including those to allergen,

are dependent upon B7-2 (CD86) co-stimulation, recently activated T cells and human Th clones are not susceptible to inhibition by anti-B7-2 Abs. [137, 336, 337] There are relatively few if any studies to our knowledge that have examined the impact of blocking B7-1 or B7-2 co-stimulatory molecules on allergen driven chemokine responses in non-atopic, atopic, or mild asthmatic subjects.

We hypothesized that allergen-driven TARC production was dependent upon co-stimulatory molecule B7-2 and to a lesser degree B7-1. Allergen-driven Mig production would be equally dependent upon both B7-1 and B7-2 co-stimulatory molecules. Finally, we speculated that all three subject groups would experience a range of inhibition due to the blocking B7-1 and B7-2 mAbs together, with the non-atopics experiencing the strongest inhibition and the atopic asthmatics experiencing the weakest inhibition. To test this hypothesis, we examined fresh PBMC directly ex vivo from 10 non-atopic, 10 grass atopic, and 10 grass atopic mild asthmatic stimulated with grass pollen allergen +/- αB7-1, αB7-2, αB7-1 + αB7-2, or CTLA-4-Ig. CTLA-4-Ig binds to both B7-1 and B7-2 with equal affinity preventing binding of CD28, and has been demonstrated to prevent allergen-driven proliferation of PBMC. [133]

In Figure 18 we can see that allergen-driven TARC and Mig production was reduced to varying degrees following the addition of blocking antibodies to B7-1, B7-2, B7-1 and B7-2 as well as treatment with CTLA-4-Ig. When we examined the subject groups separately TARC production from the atopic asthmatic and atopic populations are not significantly reduced by treatment with B7-1 treatment whereas the non-atopics were (Figure 19a). Specifically, blocking with B7-1 or B7-2 resulted in a similar reduction in TARC production from non-atopic PBMC. This is in agreement with previous studies

demonstrating the greater importance of B7-2 in Th2 cytokine production [133, 135, 136, 335].

In contrast allergen-driven Mig production was significantly reduced by B7-1 in atopic and atopic asthmatics but not non-atopics. The requirement for both B7-1 and B7-2 signaling in non-atopics for TARC production may be under more complex regulation compared to atopics (Figure 19b). The same could be hypothesized for allergen-driven Mig production from atopics compared to non-atopics.

We observed an additive effect when both B7-1 and B7-2 blocking mAb were used as well as CTLA-4-Ig suggesting that both B7-1 and B7-2 are important in delivering the signals required for a robust chemokine response. TARC and Mig production from all three subject groups were equally reduced by blocking B7-2 alone suggesting that regardless of atopic status signaling through B7-2 is critical for allergen driven Mig and TARC production from APC. This is in agreement with previous studies that demonstrate the importance of B7-2 in allergen-driven cytokine production. However, allergen-driven chemokine production is regulated by B7-1, depending on the chemokine examined as well as the atopic status. This is important because it demonstrates the importance of signaling events that occur after the trimer formation for a great deal of allergen-driven chemokine production.

Figure 18 Allergen-driven TARC and Mig production is dependent upon signaling through B7-1 and B7-2

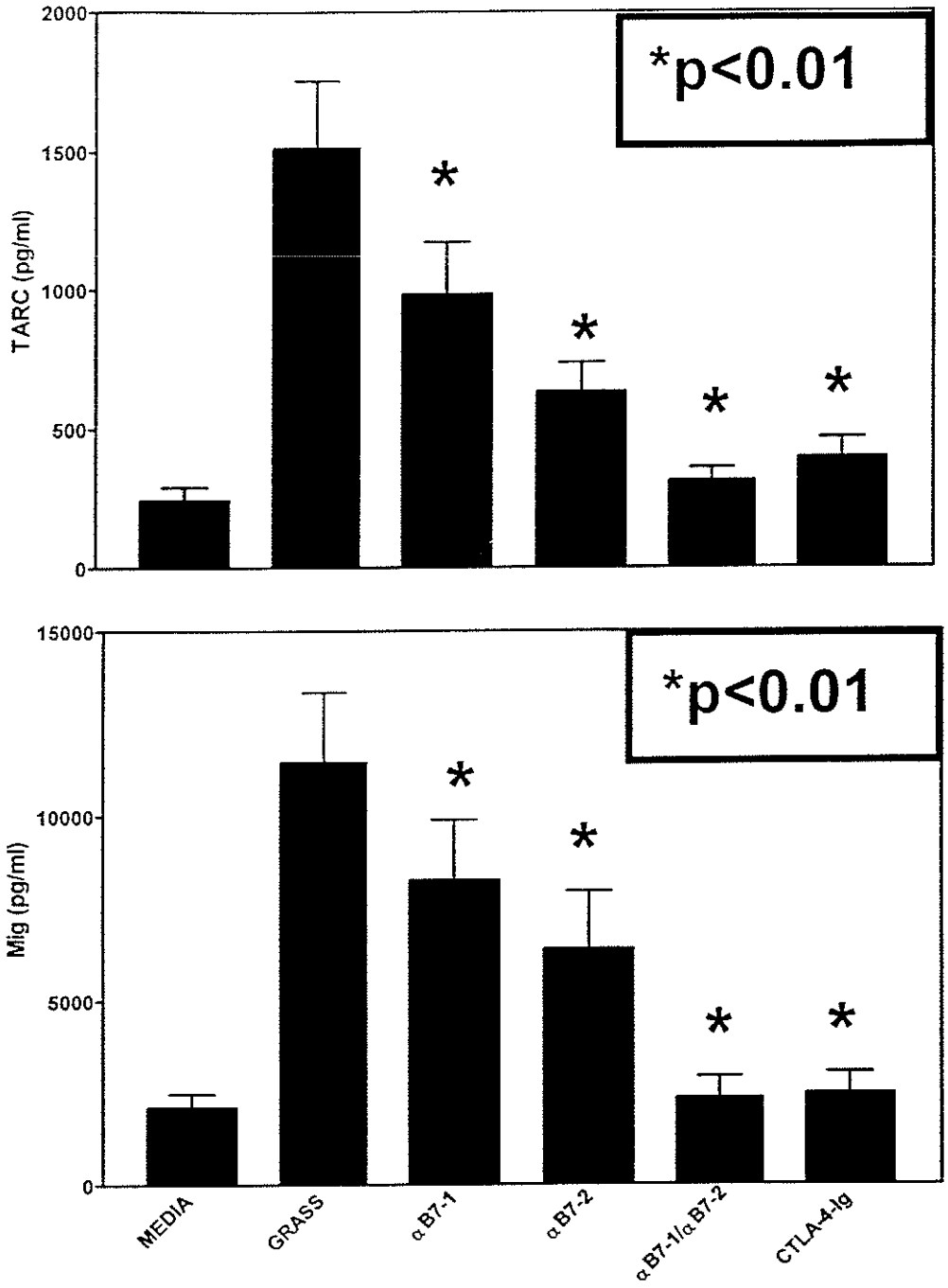

Figure1 : Allergen driven TARC and Mig production is dependent upon co-stimulatory molecules B7-1 and B7-2. Non-atopic (10), atopic (10) and atopic asthmatic (10) PBMC was placed into short term primary culture with grass pollen allergen +/- mAb against B7-1 (5ug/ml), B7-2 (5ug/ml) or both or a CTLA-4-Ig (20 ug/ml). Mig and TARC levels were measured using a specific sanwhich ELISA.

Figure 19a Co-stimulation through B7-2, and to a lesser extent B7-1, regulates allergen-driven TARC production from non-atopics, atopics and atopic asthmatics.

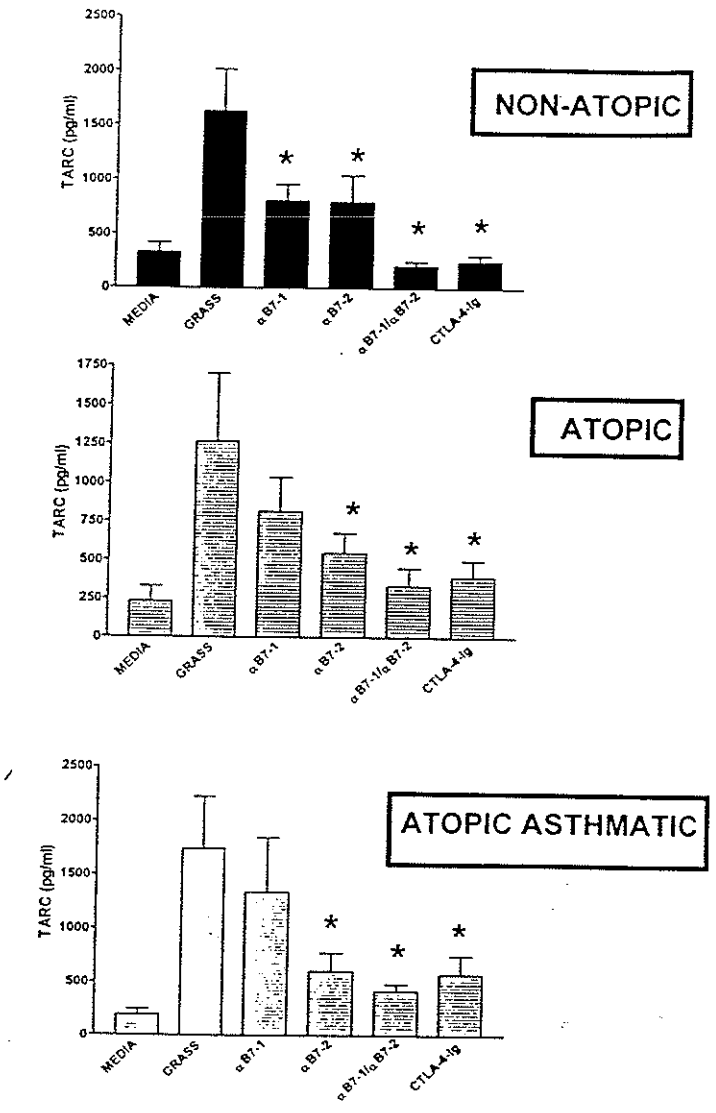

Figure 19a: Non-atopic, atopic and atopic asthmatics produce significantly less allergen-driven TARC when B7-2, and to a less extent B7-1, is blocked. Non-atopic (n=10), atopic (n=10) and atopic mild asthmatic (n=10) PBMC were stimulated with previously optimized concentration of grass allergen (4ug/ml) combined with either B7-1 (5ug/ml), B7-2 (5ug/ml), B7-1+ B7-2 (5ug/ml) or CTLA-4-Ig (20ug/ml). TARC and Mig levels were measured using specific sandwich ELISA. Statistics were determined using a Paired T test (*$p<0.01$).

Figure 19b Co-stimulation through B7-2, and to a lesser extent B7-1, regulates allergen-driven Mig production from non-atopics, atopics and atopic asthmatics.

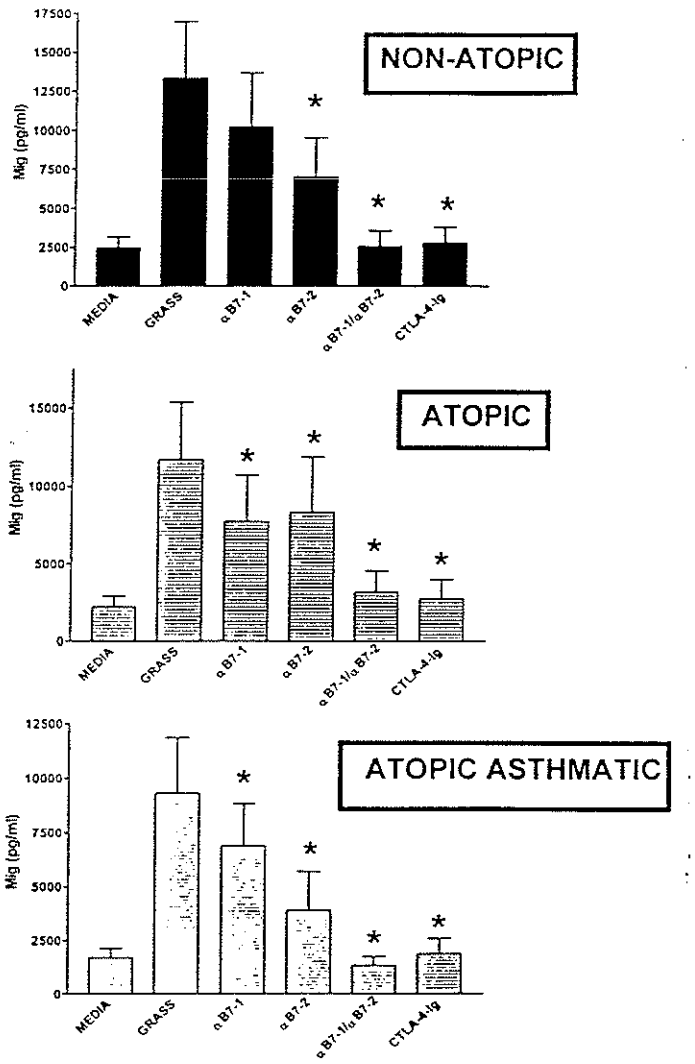

Figure 19b: Non-atopic, atopic and atopic produce significantly less allergen-driven Mig when B7-2, and to a lesser extent B7-1, is blocked. Non-atopic (n=10), atopic (n=10) and atopic mild asthmatic (n=10) PBMC were as indicated in Figure 18a. TARC and Mig levels were measured using specific sandwich ELISA. Statistics were deteremined using a Paired T test (*p<0.01).

10.13 Immunohistochemistry identification of Mig from allergen-stimulated PBMC

In this system, we demonstrated the ability of T cells to regulate the initiation and intensity of allergen-driven Mig production. Cell populations within PBMC include T and B lymphocytes, monocytes, macrophages, and a low number of dendritic cells. We hypothesized that APC were the primary producers of allergen-driven Mig production in our system. To test our hypothesis we stimulated fresh PBMC from normal healthy donors with grass pollen in short term culture. Cultured cells were fixed to glass slides using Poly-L-lysine. To maintain the integrity of the plasma membrane paraformaldehyde was used to preserve the intact cell membrane. Extracellular staining of CD3 molecules using FITC (Green) was used as a marker for T cells. Permeabilization allowed for intracellular and extracellular staining of Mig using Cy3 (Red). There was quantitatively less (Table 6) and qualitatively less intense Mig staining observed in cells that were not stimulated, a greater amount seen in the grass pollen stimulated as well as qualitatively observed extremely high amounts in the TSST-1 stimulated cultures reflecting what was observed by ELISA (Figure 20). We observed nearly half of the Mig+ cells to be CD3+(Figure 21). This is not consistent with the frequency of allergen-specific T cells in the peripheral blood (~0.1%). Interestingly, the staining of the CD3+/Mig+ cells was confined to primarily the edges of the cell similar to that seen with the CD3 staining suggesting the presence of Mig bound to CXCR3 (found on CD4/CD45RO Th cells). We could not confirm the binding of Mig to CXCR3 but were interested in the reasoning for the great majority of double positive cells within our

system. The observed staining of Mig on both CD3 + and CD3 – cell populations indicates one of the following:

1) Allergen-driven Mig production is from CD3+ and CD3- cell populations, presumably representing T cells and APC.

2) Allergen-driven Mig production is a result of T cell recognition of allergen and subsequent activation of APC to produce Mig. Mig released from APC is then able to bind to CXCR3 present on ~50% CD4/CD45RO+ T cells.

There have been studies demonstrating the downregulation of CXCR3 expression following Th cell activation followed by a slow recovery period [228] where CXCR3 expression is mainly restricted to Th1-like CD4+/CD45RO+ cells.

In an attempt to determine whether CD3+/ Mig+ cells represent a population of CD4+ T cells expressing CXCR3+ with bound Mig we examined the frequency of CXCR3+ expression by flow cytometry on fresh and allergen-stimulated PBMC. PBMC from two healthy non-atopic donors were examined directly ex vivo via flow cytometry (Fresh). In addition, we cultured isolated PBMC in either media alone or grass pollen allergen for five days and the harvested the cells, washed them and examined CXCR3 expression on both the T cell and B cell population.

We observed a dramatic downregulation in the frequency of CXCR3+ cells after 5 days of culture (~40% vs ~10%). This is important because it argues that the Mig staining seen on CD3+ cells is probably not due to Mig binding receptor alone and is possibly due to the ability of T cells to produce Mig. Unfortunately, at this time we cannot conclude that allergen-driven T cells can produce Mig because we cannot confirm that

CD3+/Mig+ cells are not representative of CD3+/CXCR3+ that have bound Mig produced from other cells. We argue that regardless of the ability of T cells to produce Mig, allergen-presentation is a pre-requisite for Mig production (Figure 17 and 18) and that immunohistochemically we can observe CD3- cells either producing Mig or have Mig bound via CXCR3.

Figure 20 Immunohistochemical analysis of Mig from fresh human PBMC

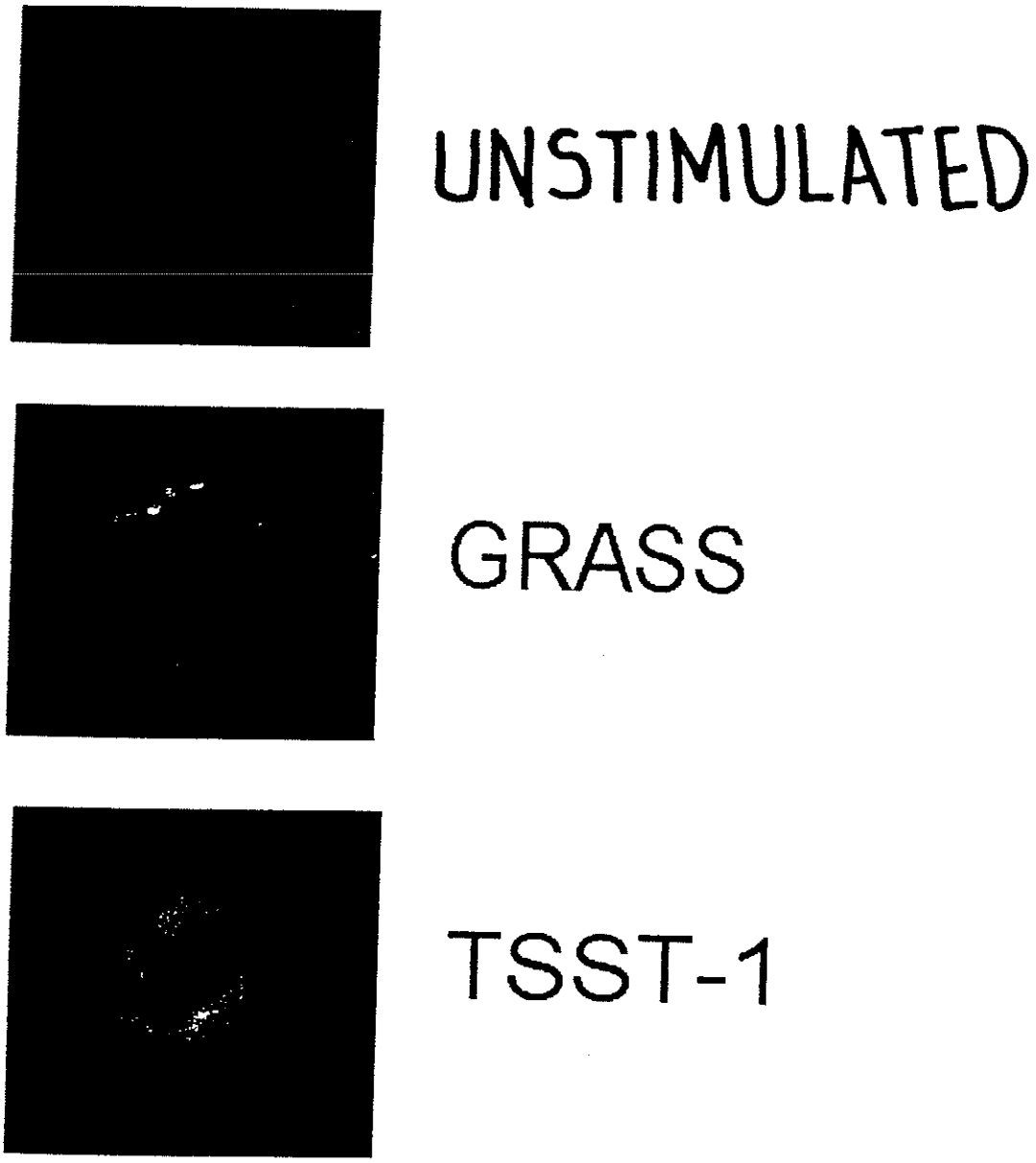

Figure 20: Fresh human PBMC stain for Mig with varying intensities depending upon stimulus. Human PBMC (n=10) were stimulated in culture with either grass pollen (4ug/ml) or TSST-1 (0.1ng/ml). Cells were then harvested and placed onto slides and stained with antibodies described in Materials and Methods. RED staining represents Mig.

Figure 21 Immunocytochemical analysis of CD3+/ Mig + and CD3-/ Mig + fresh PBMC

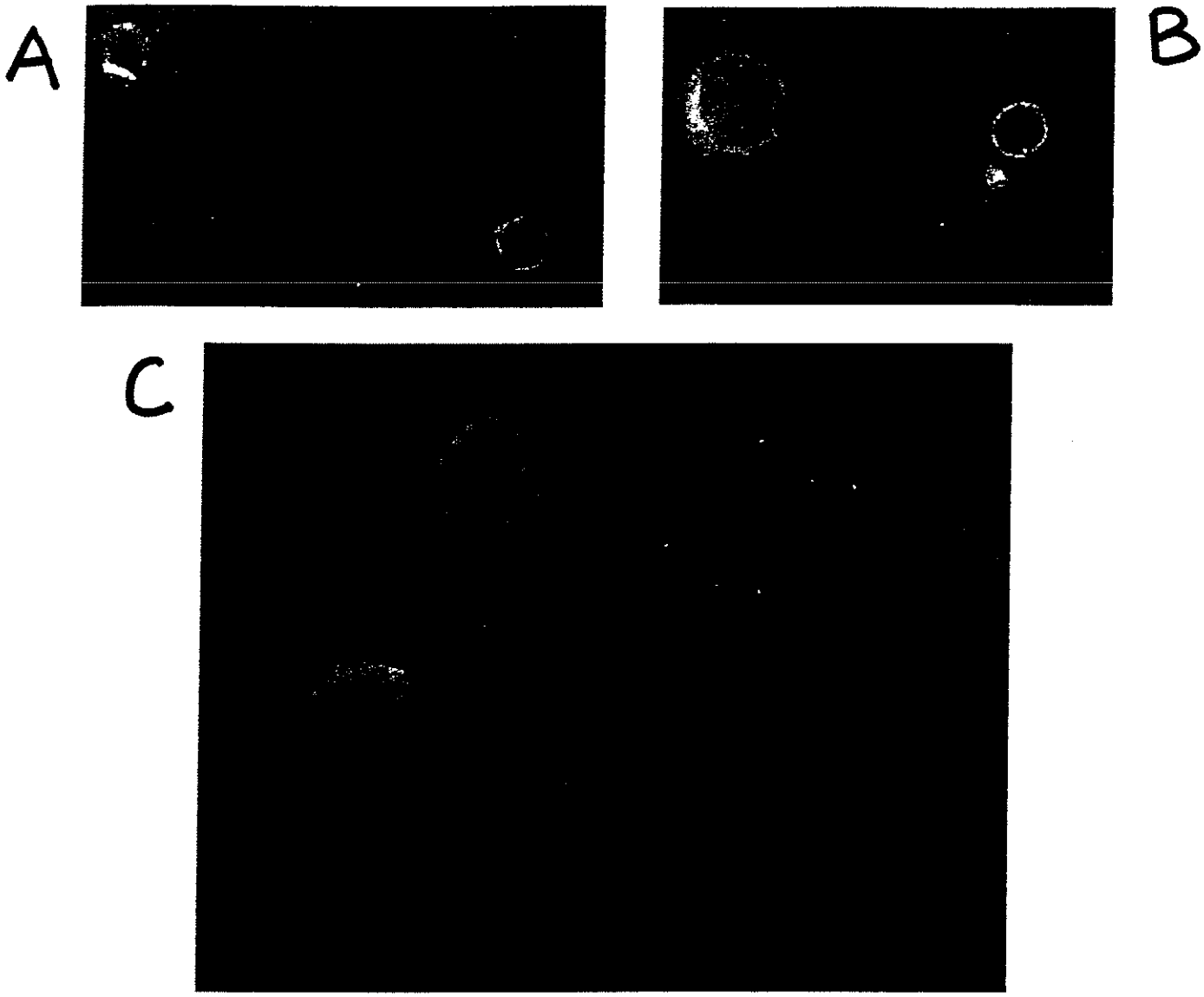

Figure 21: Fresh human PBMC stimulated with grass pollen produces two different populations based upon CD3 expression. There exists CD3+ (GREEN)/ Mig + (RED) double positives and CD3-/Mig+ PBMC. Human PBMC (n=10) were stimulated in culture with grass pollen (4ug/ml). Cells were then harvested and placed onto slides and stained with antibodies described in Materials and Methods. Panel A demonstrates two single postive leukocytes. Panel B demonstrates polarized staining of Mig (RED) on a CD3+ (GREEN) cell. Panel C demonstrates varying degrees of Mig and CD3+ double staining.

Table 8 Quantitative analysis of Mig and CD3 immunocytostaining of human PBMC stimulated with grass pollen allergen, or TSST-1

SUBJECT	STIMULUS	MIG +	CD3+	MIG+/CD3+
KTH	MEDIA	2	106	0
	GRASS	52	92	22
	TSST-1	70	115	44
HSK	MEDIA	0	84	0
	GRASS	61	125	38
	TSST-1	90	144	50

Table 9 Flow cytometry analysis of fresh and allergen-driven CXCR3 expression on human PBMC

Subject	Condition	Day	CXCR3
004 PLJ	Not Applicable	Fresh	38.41
	Media	Day 5	4.57
	Grass (4ug/ml)	Day 5	11.89
007 RDL	Not Applicable	Fresh	46.34
	Media	Day 5	5.24
	Grass (4ug/ml)	Day 5	12.53

11 Discussion

Atopic diseases are the result of intense inflammatory responses to normally innocuous environmental antigens. They are characterized by preferential recruitment and activation of basophils, eosinophils and Th2-like cells to the site of allergen exposure (eg. nose, lung). We examined the role of type 1 and type 2 chemokines in the maintenance of allergic inflammation. Specifically, we sought to establish potential differences in allergen-driven chemokine production in atopic disease versus healthy controls exposed to the same allergens. We went on to begin to characterize the mechanisms responsible for the activation and regulation of type 1 and type 2 chemokine synthesis in allergen specific immune responses.

Thus, the major goals of this study were to investigate a possible association between chemokine production and clinical allergy *in vivo* as well as *in vitro*. To accomplish this goal we took several independent approaches. (1) Plasma isolated from atopic and non-atopic donors allowed *in vivo* examination of relationships between systemic levels of type 1 and type 2 chemokines and clinical status. (2) Utilizing fresh PBMC taken directly *ex vivo* we examined allergen-driven type 1 and type 2 chemokine production in non-atopic, atopic and atopic asthmatic populations to assess how allergen-specific chemokine production differed in these populations. (3) The ability of exogenously added and endogenously produced T cell derived cytokines to regulate the intensity of allergen-driven chemokine production. (4) Detailed analysis of the regulatory

mechanisms involved in the initiation of representative type 1(Mig) and type 2(TARC) chemokine production by PBMC.

11.1 Technical Considerations

Subject Selection

Our objective was to study the intensity and regulation of type 1 and type 2 chemokines in a range of disease severity present within allergy. Therefore, we required examination of non-atopic, atopic as well as atopic mild to moderately asthmatic individuals. Severely asthmatic subjects were not included because of the requirement of anti-inflammatory and/or immunosuppressive medications for maintenance of the disease. These medications would potentially interfere with analysis of allergen-driven chemokine responses.

To objectively measure the clinical sensitivity of the allergic subjects we examined the size of the wheal response elicited during a skin test to a panel of 14 common allergens (grass, house dust mite, cat, etc...) as well as taking a detailed clinical history. Incorporating both measures into clinical diagnosis is important because it ensures that the possibility of a false positive skin test result, or a positive skin test result with no clinical history, is not misinterpreted as established clinical sensitivity. A positive skin test result with no clinical history may well indicate the future development of allergy but without previous clinical manifestations, diagnosis of established allergic rhinitis is not possible. Medications taken by the subjects enrolled in the study were examined in detail to ensure that they would not interfere with the skin test results. For example, any individual currently taking anti-histamines would be excluded from the study at that time

due to the possible interference the medication may have on the skin test. Together with clinical history and skin test results we were able to clearly classify the severity of allergic disease and therefore allow for examination of allergen-driven chemokine production in non-atopic versus atopic populations

We focused our study on allergic rhinitis, a widespread form of allergy found within southern Manitoba. Exposure to grass pollen begins in the springtime (May/June) peaking in summer (July/August) and tapers off into the fall (September) (Lesley ShyJak/ Lee-Ann Graham, Aerobiology Research laboratories Pollen and Spore Report 1999, 2000). During this time frame, grass atopic individuals often experience intense inflammatory responses ranging from itchy eyes and sneezing to congestion and impaired breathing. Subjects recruited for this study were diagnosed based upon skin tests to a panel of allergens as well as clinical history. A positive skin test was used to allocate a score for each subject based upon the size of the wheal response during a skin test. All of the atopic individuals enrolled displayed wheal responses equal to or above 2+ with the majority of the recruited subjects displaying a response equal to 4+. Atopic subjects recruited for this study were representative of a population specifically sensitized to grass pollen.

We wanted to specifically examine the more severe form of allergic rhinitis, which has progressed to grass atopic, mild to moderate asthma [338]. This group provides us with a population that experiences a more intense inflammatory response to grass pollen resulting in intense wheezing and bronchoconstriction of the airways. The severity of atopic asthma is often greater than that seen in the atopic population alone. The diagnosis of asthma is often parallel by the presence of airway remodeling. At what

stage this occurs is not known, therefore we chose to examine mild to moderately asthmatic patients in an attempt to avoid the majority of complications associated with airway remodeling. In addition, the usage of anti-inflammatory and immunosuppressive medications tends to be higher in the severe asthmatics therefore eliminating their usage in our study. The mild to moderately asthmatic individuals enrolled in this study represent a population most likely to experience the most intense, hence readily quantified differences in immune response to allergen.

In summary, analysis of allergen-driven type 1 and type 2 chemokine production by non-atopic, atopic, and atopic asthmatic populations provides a unique perspective for the analysis of the role(s) of chemokines in allergy. We reason that through analysis of non-atopic, atopic and atopic asthmatics we have the opportunity to sample a range of severity of allergy that will enable us to begin to characterize the involvement of key chemokines in the maintenance of allergic inflammation.

Chemokine Specific Sandwich ELISA

To date, the great majority of publications examining chemokine production in humans have relied upon mRNA analysis (in situ hybridization, RT-PCR) or immunohistochemical staining of tissue sections (ie at inflammatory loci). We required a method of detecting and quantifying allergen-driven chemokine production at concentrations as low as 10 pg/ml due to the low number of antigen-specific T cells present. Additionally, we wished to compare allergen and polyclonal stimuli in terms of chemokine production, indicating the requirement for accurate measurement of concentrations as high as 100 ng/ml. Thus, we developed chemokine-specific ELISAs capable of accurately quantifying the representative type 1 (IP-10, Mig) and type 2

(TARC, Eotaxin) chemokines we planned to examine over a range of concentration from 10pg/ml to 100ng/ml. To accomplish this we performed extensive optimization to yield assays that utilize very little culture supernatant (10-25ul / assay) and provide the ability to analyze a range of *in vitro* culture conditions (ie. Allergen vs polyclonal) as well as the ability to detect <10 pg/ml of systemic chemokine *in vivo*. Multiple dilutions of the same sample as well as the sensitivity of the assay allowed rigorous testing of samples with a high level of confidence in the intra-assay and inter-assay variation. Intra- and Inter-assay determination demonstrated a coefficient of variation ~ 0.080.

Fresh PBMC vs Long term clones or isolated cell populations

A second technical consideration we dealt with at the initiation of this project concerned the nature of the experiemental system we wished to use. We chose to analyze allergen-driven chemokine production from atopic and non-atopics using a mixed population of APC and antigen-specific lymphocytes as opposed to clones or isolated cell populations. This technique allows us to study how the cells directly involved in responding to an immune response initiated by an allergen regulate chemokine production *in vivo*. Therefore, we isolated fresh PBMC directly *ex vivo* from atopic, atopic asthmatic and non-atopic individuals and placed them into short term primary culture (1-5 days) to examine allergen-driven chemokine production. We demonstrated allergen-specific chemokine production from PBMC in all subject populations. This technique allows the investigator greater confidence in extrapolating the *in vitro* findings to the *in vivo* response because of the physiological relevance of using an unbiased cell populations and a naturally occurring allergen.

Use of T cell clones as a tool to quantitatively infer chemokine production *in vivo*, a widely used alternative strategy that we did not pursue, has the advantages of: (1) An entire population of T cells with the same antigen specificity, (2) Experimental variation is greatly reduced when the same cell is utilized during each experiment. However, it also has several limitations such as the number of extrinsic factors the clones are subjected which strongly influences the number and function of the clones obtained (Paroncchi 1992, Romagnani1993). Moreover, clones (even exceptional cases where ~10 clones are obtained from one individual) represent only a very small proportion of the total allergen-specific T cell repertoire. Therefore, results obtained from these studies may not be reflective of *in vivo* responses. Recognizing these limitations, we argue that using freshly isolated PBMC directly *ex vivo* is a strategy that we believe is more representative of how chemokine production and regulation occurs in response to allergen normally *in vivo* and provides critical insight into the role of chemokines in allergy.

Examination of allergen-driven chemokine production from BAL leukocytes is another possible technique that we did not pursue. Critical analysis of allergen-driven chemokine production from BAL of non-atopic, atopic and atopic asthmatics has the distinct advantage of examining a select population of specifically recruited cells to the site of allergen exposure. However, the major disadvantage of this technique is number of leukocytes obtained from bronchoalveolar lavage (BAL) ranging from 22 to 60 x 10^6 cells (median 37.6) for every 250 ml PBS washing [339].

We obtained roughly 60 to 120 x 10^6 cells for every 50ml of peripheral blood (Materials & Methods). In addition, there is a considerable amount of discomfort experienced by

most individuals during a BAL, therefore making recruitment of a sufficient population size difficult. We argue that analysis of allergen-driven type 1 and type 2 chemokine responses from PBMC provides useful information into the chemokine profiles of allergen-specific lymphocytes in response to allergen, and that this may be representative of what may occur at the site of inflammation.

Allergen driven vs Polyclonal driven Chemokine production

A third major strategic issue we needed to address was the mode of activation we wished to use. We wanted to compare the differences between allergen and polyclonal-driven chemokine production in vitro. Specifically, we examined both type 1 and type 2 chemokines to identify potential differences in terms of production in response to allergen versus polyclonal stimuli. Polyclonal driven responses are advantageous because: 1) they produce large amounts of mediators, making analysis easier, and (2) they require smaller numbers of lymphocytes because allergen-specificity is not required. The disadvantages with polyclonal driven responses lie within the nature of the stimulus. Polyclonal activators stimulate through different receptors compared to allergens thereby possibly activating different intracellular pathways required for production of mediators such as chemokines. In addition, the lack of antigenic specificity may result in activation of cells that would not normally be activated in vivo. These qualitative and quantitative disadvantages disqualifies the usage of polyclonal activators if the goal of the study is to examine chemokine production by two subject populations characterized by their immune responses to allergen.

In contrast, use of allergens *in vitro* allows the investigator to identify potential differences in type 1 and type 2 chemokine production from atopic and non-atopics.

Specifically, use of allergens is advantageous because of the relevance to the *in vivo* immune response. The intracellular pathways and the frequency of cells stimulated by allergens are comparable to those seen *in vivo*. A major advantage of allergen-driven stimulation in comparison to polyclonal activation is that the allergen specifically activates the cells of interest. Cells specific for allergens other than the one being examined are not targeted, thereby dramatically increasing the signal to noise ratio for the allergen-specific response. Thus, we chose to examine allergen-driven chemokine production to identify potential differences between non-atopic and atopic chemokine (type 1 and type 2) synthesis and regulation. In cultures that were set up with the same subjects on the same days we compared type 1 and type 2 chemokine production by polyclonal activators such as PHA or TSST-1 to chemokines responses to allergen stimulation.

Therefore, we argue that analysis of allergen-driven chemokine production represents a method by which key differences present between atopic and non-atopic individuals may be elucidated that would not otherwise be obvious if polyclonal activators were used.

To analyze allergen-driven chemokine production we faced the task of choosing a purified natural allergen extract or recombinant proteins. We wanted to examine the role of chemokines in allergy to grass pollen (allergic rhinitis). Skin tests in combination with clinical history allowed for classification of our subjects based upon atopy to grass pollen. Therefore, we wanted to examine the chemokine response of these subject groups to stimulation with grass pollen in short-term primary culture. The natural extract (as opposed to recombinant proteins) chosen represents the majority of grass species

found within Manitoba. The complex natural extract was deliberately chosen because a vast majority of atopic individuals exhibit hypersensitivity to multiple entities. Thus, use of a purified allergen would sample only a small proportion of the grass pollen specific response. Examination of chemokine production by atopic and non-atopics in response to this mixture has an inherent advantage in reflecting the broader picture of chemokine production induced by component(s) of a heterogenous grass pollen mixture to which an individual is customarily exposed.

The limitations of this approach include the possibility that polyclonal contaminants may be present in the allergen mixture (eg LPS). We believe that the chemokine responses measured reflect antigen-driven rather than polyclonal activation for several reasons. These include, (1) the intensity and the kinetics of the allergen induced responses fall into the same range as elicited by antigen rather than that induced by known polyclonal activators such as TSST-1. (2) Blocking MHC II presentation (αHLA-DR or αCD4) of allergen to the T cell resulted in abrogation of chemokine production. (3) Blocking co-stimulatory molecules B7-1 and B7-2 dramatically inhibited chemokine production. These results suggest that classical presentation of allergen must occur for chemokine production to occur. If polyclonal contaminants were present within in the mixture blocking MHC II as well as co-stimulatory molecules would not alter polyclonal driven chemokine production. Therefore, we are confident that the natural extract of grass pollen chosen for this study elicits chemokine production based upon immune responsiveness the grass pollen mixture, not through unforeseen contamination with polyclonal stimulators (eg bacterial-LPS).

In summary, we developed an allergen-driven primary culture system to characterize type 1 and type 2 chemokine production and regulation by non-atopic, atopic and atopic moderate asthmatic PBMC.

11.2 Systemic levels of type 1 and type 2 chemokines and atopic disease

We wanted to identify type 1 and type 2 chemokines in the plasma of atopic and non-atopics, potentially for use as a clinical marker of the severity of allergic disease. In doing so, we examined several possible regulators of plasma chemokine levels including atopy, seasonal exposure to elevated levels of grass pollen and the relative expression of a potential systemic chemokine sink, the Duffy antigen for chemokine receptors (DARC) expressed on erythrocytes.

Cross-sectional Analysis of Plasma Chemokines

In the first section of this research, we wanted to determine the relative levels of type 1 and type 2 plasma chemokines as a possible clinical marker. Plasma was freshly isolated from atopic and non-atopic subjects and used to identify the presence of representative type 1 (IP-10, Mig) and type 2 (Eotaxin, TARC) chemokines. To begin with, we determined the stability of type 1 and type 2 chemokine expression in plasma as well as chemokine receptor expression on CD4+/CD45RO+ T cells. We determined that within any given individual there exists less than 5% variability in both plasma chemokine and chemokine receptor expression over a three week period. We were then able to perform a cross-sectional analysis of type 1 as well as type 2 plasma chemokines (ie comparing non-atopic and atopic plasma chemokines at any given single time point). Interestingly, we did not observe significant differences in the median

intensity levels of any of the chemokines examined as both non-atopic and atopic subjects displayed a similar range in plasma chemokine levels. Within each population, we observed a 4-5 log range from 4pg/ml to >20ng/ml in each of the chemokines examined, an observation indicating active and individualized regulation of systemic levels of chemokines. However, there is no evidence that allergic disease is responsible for this wide range of concentrations as it is seen equally in atopic and non-atopics.

This finding is important because it argues against the ability of IP-10, Mig, Eotaxin and TARC, at least at the plasma/serum level, to be useful markers of allergic rhinitis or the severity of the disease. In contrast to many chemokines which bind multiple receptors, (ie. MIP-1∀: CCR1 and CCR5) each chemokine selected for examination in this study has been shown to exclusively bind one receptor (ie IP-10 and Mig bind CXCR3, Eotaxin binds CCR3, and TARC binds CCR4). These receptors are present on Th1 or Th2 polarized cell populations, as well as other cell types, suggesting their involvement in type 1 and type 2 biased immunity. [194, 232]

The candidates chosen have strong connections with type 1 and type 2 disorders. Eotaxin levels were elevated in asthmatics compared to normal controls [318]. In addition, elevated TARC and MDC levels have been documented in serum from atopic dermatitis patients [266, 268]. In comparing these studies with our own it is important to note that both of these studies examined plasma or serum during an acute exacerbation of disease (ie Hospital room visits). In addition, Kakinuman et al showed the greatest differences between serum TARC levels from atopic dermatitis patients compared to controls only if the most severe forms of atopic dermatitis were examined. The

possibility that systemic levels of chemokines are useful markers of atopic disorders may be limited to examination during or immediately after an intense clinical exacerbation. Our subjects were analyzed over two grass pollen seasons to ensure that examination of only one weak or one strong grass pollen season did not promote biased results. Our study does not exclude the possibility that there exist other inflammatory chemokines that may provide useful information as surrogate markers of stable atopic disease. However, the current data argue that systemic (constitutive . plasma) levels of the type 2 chemokines, Eotaxin and TARC, which are strongly associated with allergy, are not reliable markers of clinical status in immediate hypersensitivity disorders such as allergic rhinitis.

Longitudinal Analysis of Plasma Chemokines and Chemokine Receptors

Having seen no significant differences in type 1 and type 2 chemokine plasma levels between atopic and non-atopics we sought to determine if longitudinal examination of type 1 and type 2 chemokines would provide a more sensitive indicator of seasonal responses to allergen. Thus, we examined the ability of extended exposure to elevated levels of grass pollen (Winter vs. Summer)to modulate type 1 and type 2 plasma chemokines in atopic and non-atopic individuals. Longitudinally, we observed an increase in plasma IP-10 levels in both subject groups upon seasonal allergen exposure ($p<0.05$, n=34 non-atopics, n=53 atopics). Non-atopics exhibited a significant increase ($p=0.0103$, n=34) in systemic levels of eotaxin whereas the atopics did not. This is important because it indicates the ability of allergen exposure to alter systemic levels of chemokines.

The finding that systemic levels of both IP-10 (atopic and non-atopic) and eotaxin (non-atopic) were elevated in response to natural allergen exposure prompted us to examine the cognate receptor expression on circulating antigen-experienced T cells. We conducted a parallel analysis of CXCR3 and CCR3 expression on CD4/CD45RO+ cells from these subjects. We hypothesized that any changes in chemokine receptor expression may provide insight into a mechanism through which systemic chemokines are regulated in atopy. We examined expression of CXCR3 and CCR3 on CD4+/CD45RO+ T cells taken from the same sample the plasma was isolated from.

We did not observe any alterations in CCR3 expression most likely a result of the low percentage of CD4/CD45RO+ cells expressing CCR3 (~5 %) (Campbell JD, submitted Sept 2001). We speculate that up-regulation of plasma levels of eotaxin in non-atopics may represent chemokine that was not able to bind CCR3 due to the lack of eosinophils, basophils or Th2 cells present during allergen exposure in non-atopics.

Analysis of CXCR3 expression revealed a substantial increase in non-atopic subjects only. This is important because it indicates a possible mechanism through which clinical tolerance is mediated in non-atopics. Specifically, up-regulation of CXCR3 expression on antigen experienced cells could allow for selective T cell recruitment to sites of elevated IP-10, Mig, or I-TAC expression and increased type 1 cytokine production [220]. CXCR3 is almost exclusively expressed on polarized Th1, IFN(-secreting clones [232] suggesting that preferential recruitment of CXCR3+ cells would result in more type 1 dominated immunity. Recently, chemokine receptor expression profiles for Th1 and Th2 clones have provided evidence for preferential involvement of specific chemokines in the recruitment and possibly activation of polarized T cell

populations. To date information detailing the ability of fresh T cells and/or APC directly *ex vivo* to produce type 1 and type 2 chemokines in response to allergen remains unknown, most likely as a result of an inability of most investigators to measure the low levels of chemokine produced.

Collectively, we identified that seasonal exposure to grass pollen results in up-regulation of systemic IP-10 levels in both atopic and non-atopic individuals. CXCR3 expression is up-regulated in non-atopics during the grass pollen season only, suggesting that clinical tolerance may be manifested through enhanced recruitment of CXCR3+ cells to the site of allergen exposure promoting type 1 immunity and inhibiting induction or expression of type 2 allegen specific responses.

Correlation Within and Between Type 1 and Type 2 Plasma Chemokines

We next examined potential correlations between plasma expression of the two representative type 1 chemokines, IP-10 and Mig, in atopic and non-atopic individuals. A similar analysis was performed examining the two representative type 2 chemokines studied, Eotaxin and TARC. A strong positive correlation was demonstrated between IP-10 and Mig ($p<0.001$, $r=0.493$). Similarly, Eotaxin and TARC were highly correlated ($p<0.001$, $r=0.689$), a finding equally evident in both atopic and non-atopic populations. This is consistent with cytokine data demonstrating enhanced production of several type 1 or type 2 cytokines are concurrently upregulated during an immune response to antigen. Hence, elevated IL-4 is commonly accompanied by elevated IL-5 and IL-13. We conclude that the positive correlation between IP-10 and Mig, as well as Eotaxin and TARC demonstrates that type 1 and type 2 systemic chemokines are coordinately regulated.

Having established that expression of type 1 chemokines is highly coordinated (as is expression of type 2) we sought to determine if there was a negative correlation between type 1 and type 2 plasma chemokines, as is seen for cytokine production. Thus, type 1 and type 2 cytokines are often negatively correlated in both atopic and non-atopic individuals. Specifically, Imada et al showed that allergen-driven PBMC production of IFNγ and IL-4 resulted in a negative correlation. [333] In atopics, IL-4 levels were elevated and IFNγ levels were relatively reduced compared to non-atopics. We analyzed systemic type 1 and type 2 chemokines in atopic and non-atopic populations. Surprisingly, a strong positive correlation was evident between type 1 and type 2 plasma chemokines. This relationship was evident in both subject groups. A strong positive correlation within both subject groups suggests that regulation of systemic chemokine levels differs from cytokine regulation and further is not correlated with atopic disease. This finding is contrary to what is observed in previously reported cytokine data.

DARC, Plasma chemokines and Allergy

We observed a 4-5 log range in plasma chemokine levels in both populations. We hypothesized that differential expression of the Duffy antigen receptor for chemokines (DARC) found on RBC was a possible candidate due to its promiscuous ability to bind both CXC and CC chemokines. We identified DARC on atopic and non-atopic human RBC at a strikingly high frequency (~97%) within both populations, a finding consistent with the literature. Interestingly, no correlation was evident between either the frequency or intensity of DARC expression on RBC and systemic type 1 and type 2 chemokines. We did observe a range in the intensity of DARC expression (10-50 MFI)

but there was no correlation between DARC expression and plasma chemokine levels. In addition, the intensity of DARC expression did not correlate with clinical status.

This finding is important because of studies that suggest DARC is a sink for systemic chemokines to prevent unnecessary cellular recruitment. DARC binds both CXC and CC chemokines making it the most promiscuous chemokine receptor to date. Nickel et al suggested a link between the incidence of asthma and the absence of DARC on RBC in >70% of African Americans [197]. We argue that the intensity of DARC expression on RBC does not correlate with systemic type 1 or type 2 chemokines in our (primarily caucasian and oriental) population. In addition, there is no correlation between the intensity of DARC expression and clinical sensitivity suggesting that differences in DARC expression may not be predictive of atopic disorders. We conclude that the range of systemic chemokine observed in atopic and non-atopics is not a result of relative differences in the intensity DARC expression.

11.3 Allergen-driven type 2 chemokine (TARC) levels are elevated in atopics

In an independent approach to examining chemokine expression and regulation we developed allergen-driven *in vitro* culture systems. To test the hypothesis that Th2-like cytokine and clinical sensitivity responses are associated with dominant type 2 chemokine production. Similarly we hypothesized an association between enhanced type 1 chemokine production (*in vitro* in response to acute Ag mediated restimulation) and clinical tolerance in contrast to data obtained with plasma chemokine analysis. This experimental approach demonstrated that patients with seasonal allergic rhinitis exhibit

a clear imbalance in chemokine synthesis profile compared to that of non-atopic individuals upon exposure to common environmental allergen.

Specifically, we observed that atopic individuals produce significantly elevated levels of TARC, a type 2 chemokine, in response to grass pollen compared to the responses of non-atopics. Elevated TARC levels were also observed in response other allergens such as HDM and interestingly, to SK. This suggests a global tendency to respond to extracellular pathogens via elevated type 2 chemokine production.

In contrast to the enhanced TARC responses seen in atopic subjects upon allergen-specific restimulation in short term culture, no differences were evident in allergen-driven Mig production (a representative type 1 chemokine) when comparing between subject groups. This is important because it suggests that atopic subjects are not deficient in their ability to produce type 1 chemokines to environmental allergens, but rather that they exhibit an elevated type 2 chemokine profile. Whether this difference promotes the development of type 2 immunity or is a reflection of an established allergic state would require long term prospective studies.

To our knowledge, the ability of atopic PBMC to preferentially produce elevated levels of allergen-specific TARC compared to non-atopics is the first study demonstrating differential regulation of allergen-driven type 2 chemokine production between atopic and non-atopics. The presence of TARC or its receptor, CCR4, in cell lines or tissue biopsies from inflammatory loci has hinted at the possible involvement of TARC in type 2 disorders such as atopic dermatitis as well as asthma. Sallusto et al demonstrated a bias in CCR4 expression on Th2 clones [232]. In addition elevated CCR4 levels on CD4+/CD45RO+ cells from atopic dermatitis patients compared to controls [266].

However, MDC binds to CCR4 as well, thereby implicating either MDC or TARC or both in this type 2 disorder. Recently, TARC expression by human bronchial epithelial cell lines combined with intense staining of TARC on bronchial mucosa biopsies from asthmatic donors has suggested a possible link between TARC and allergic airway inflammation [265]. These studies were not able to demonstrate up-regulation of TARC production in response to allergen, nor were they able to determine whether TARC production was elevated in atopics compared to normal controls. Usage of cell lines and/or polyclonal activators may have prevented observation of elevated allergen-driven TARC production from atopics compared to controls.

The observation that atopic individuals produce enhanced TARC levels compared to non-atopics in response to allergen is important because it suggests that maintenance or exacerbation of allergic inflammation may be a result of preferential recruitment of type 2 cells expressing CCR4 to the site of inflammation.

11.4 Exogenous and endogenous T cell derived cytokines regulate the intensity of TARC and Mig production.

In light of the ability of atopics to produce enhanced type 2 chemokine in response to allergen compared to non-atopics we sought to determine whether exogenously added or endogenously produced type 1 or type 2 cytokines were able to regulate the intensity of allergen-driven type 1 (Mig) and type 2 (TARC) chemokine production. To accomplish this we independently examined the ability of exogenously added or endogenously produced IFNγ, IL-4, IL-13 or IL-10 to regulate the intensity of allergen-driven type 1 and type 2 chemokine production.

Exogenously Added IFNγ, IL-4, IL-13 or IL-10: Impact on recall chemokine responses

To investigate whether allergen-driven type 1 (Mig) and type 2 (TARC) chemokine production is regulated by the cytokine milieu present during allergen exposure we stimulated non-atopic, atopic, and atopic asthmatic PBMC with either grass pollen or SK +/- 25ng/ml or 100-1000 pg/ml of recombinant IFNγ, IL-4, IL-13, or IL-10. Interestingly, we found that commonly used levels of recombinant cytokines, 25ng/ml, modulated Mig and TARC production dramatically compared to exogenous cytokines added at levels commonly seen produced by PBMC in response to allergen (1-1000pg/ml depending on cytokine in question).

We demonstrated the ability of exogenous type 1 cytokine, IFNγ, to significantly inhibit allergen-driven TARC production regardless of the concentration of cytokine we tested. In contrast, exogenous levels of IFNγ enhanced allergen-driven Mig production but only if IFNγ was added at a concentration several orders of magnitude above (25ng/ml) what is normally detected in allergen-driven culture. This is important because it demonstrates the ability of low levels of IFNγ to suppress allergen-driven TARC production. Previous studies in our lab have demonstrated relatively low IFNγ levels from allergen-stimulated atopic PBMC compared to non-atopics [333]. We speculate that elevated TARC levels from atopic and atopic asthmatic PBMC reflect this lack of IFN(present during allergen-driven responses. In contrast the inability of 1ng/ml to enhance allergen-driven Mig production suggests that IFNγ may not be a critical mediator regulating type 1 chemokine (Mig) production *in vivo*.

Similar results were seen using a non-allergenic antigen, streptokinase, suggesting the ability of exogenous cytokines to regulate the intensity of chemokine production is not dependent on the allergenic specificity of an antigen. Non-atopic, atopic and atopic mild asthmatics responded similarly, suggesting the ability of exogenous cytokines to regulate the intensity of type 1 and type 2 chemokine production regardless of atopic status.

The addition of exogenous type 2 cytokines, IL-4 and IL-13, resulted in a significant up-regulation in allergen-driven TARC production but only at levels well above those normally detected in culture (25ng/ml). IL-4, but not IL-13, was able to inhibit allergen-driven Mig production. Similarly, commonly detected levels of IL-4 (100pg/ml) in allergen-driven culture did not significantly alter allergen-driven Mig production. These data suggest that exogenous levels of IL-4 and IL-13 may not play a significant role in allergen-driven TARC or Mig production unless they are dramatically upregulated. We argue that the relevance of adding elevated levels (25ng/ml) of exogenous IL-4 and IL-13 to what normally occurs *in vivo* is questionable due to the discrepancies between 25ng/ml and 100pg/ml (IL-4) or 1000pg/ml (IL-13) on TARC and Mig production.

Lastly, we examined the ability of IL-10, a potent anti-inflammatory cytokine, to downregulate allergen-driven TARC and Mig production. We observed dramatic inhibition of allergen-driven TARC and Mig production in response to exogenous IL-10 (25ng/ml). In contrast, levels of IL-10 that are commonly found in culture (1ng/ml) did not significantly alter Mig. TARC was equally sensitive to both concentrations of exogenous IL-10. We conclude that allergen driven TARC production is sensitive to

inhibition by exogenous IL-10, but that allergen-driven Mig production requires dramatically higher levels of IL-10 to be significantly inhibited.

We are cautioned by the discrepancies seen between two different concentrations of cytokine. We argue that the commonly detected levels of exogenous cytokine used *in vitro* are more physiologically relevant to what occurs naturally *in vivo* than the high levels (25-100 ng/ml) used in many experimental systems. Due to the range of IL-13 and IL-4 levels measured from allergen-stimulated PBMC [189, 330, 333] it is difficult to determine the optimal concentration of IL-4 and IL13 required to significantly regulate the intensity of type 1 and type 2 chemokine production. We speculate that experimental removal of endogenously produced IL-4 and IL-13 would allow for confirmation of the ability of these cytokines to regulate allergen-driven chemokine production.

In summary these data demonstrate the ability of elevated levels of exogenous cytokine to regulate the intensity of TARC and Mig production. Similar results were seen whether grass pollen or SK was used as a stimulus, suggesting the ability of cytokines to globally regulate allergen and bacterial driven chemokine production. In addition, non-atopic, atopic and atopic asthmatic subjects responded similarly to treatment with exogenous cytokine demonstrating the ability of these cytokines to regulate the intensity of chemokine production regardless of atopic status. This is important because it demonstrates the ability of chemokines to be regulated by type 1 and type 2 cytokines involved in the development of type 1 and type 2 immunity. Chemokines are able to traffic specific cell populations (Th1-CXCR3, Th2-CCR4) to the site of inflammation. Thus, combined with the ability of exogenous cytokines to regulate the intensity of type

1 and type 2 chemokine production, we speculate that chemokines are important mediators in type 2 disorders such as atopy.

Removal of endogenous IFNγ, IL-4, IL-13, and IL-10

In light of the conflicting results obtained from the addition of two different concentrations of exogenous cytokine we sought to examine the ability of endogenous cytokines, IFNγ, IL-4, IL-13, and IL-10 to modulate the intensity of allergen-driven type 1 (Mig) and type 2 (TARC) production. To investigate this hypothesis we stimulated non-atopic, atopic and atopic asthmatic subjects with grass pollen or SK +/- the addition of blocking antibodies to IFNγ, IL-4, IL-13, and IL-10.

Allergen-driven TARC production from non-atopic and atopic PBMC was dramatically up-regulated by the removal of endogenous IFNγ. Responses of atopic mild asthmatics were not significantly altered by the removal of endogenous IFNγ, suggesting that there may be a lack of endogenous IFNγ present or that atopic mild asthmatic PBMC are insensitive to endogenous IFNγ (ie. No IFNγ receptor). Blocking endogenous IFNγ did not significantly alter allergen-driven Mig production, suggesting that additional type 1 cytokines may be regulating the intensity of Mig production (eg IL-12, IL-18). The ability of endogenous IFNγ to inhibit TARC production is important because it demonstrates a possible mechanism through which allergen-driven type 2 chemokine (TARC) is inhibited in non-atopics. Type 1 immunity is characterized by elevated IFNγ levels in non-atopics. We speculate that enhanced allergen-driven TARC production by atopics and atopic mild asthmatics is a result of depressed IFNγ levels present endogenously.

Allergen-driven TARC production was inhibited by the removal of IL-13 but not IL-4 suggesting that IL-13 the importance of IL-13 in driving TARC production in vitro. Removal of these type 2 cytokines did not significantly alter Mig production. This is important because it clarifies the involvement of these two cytokines in allergen-driven chemokine production. IL-13 and IL-4 are upregulated upon secondary exposure to allergen in atopics. IL-13 remains at elevated levels for a prolonged period (3 days) whereas IL-4 levels are often quickly downregulated. Combining the fact that Th2 clones express CCR4 with the continued up-regulation of IL-13 in atopics suggests the presence of a positive feedback loop involving cytokines and chemokines in type 2 immunity.

The removal of endogenous IL-10 from allergen-driven culture dramatically down-regulated TARC production but did not alter Mig production. This is important because is suggests the existence of different requirements for the down-regulation of type 2 chemokine production compared to type 1 chemokine production.

Taken together we conclude that endogenous IFNγ and IL-10 actively inhibit allergen-driven TARC production. Additionally, endogenous IL-13, but not IL-4, selectively enhances allergen driven type 2 chemokine (TARC) production. The inability of endogenous IFNγ, IL-10, IL-4 or IL-13 to regulate the intensity of Mig production may be a result of a relatively weak cytokine response to allergen or a lower sensitivity of this chemokine to such regulation than TARC. The ability of IFNγ at greatly elevated levels to enhance allergen-driven Mig production suggests that an intense inflammatory response may produce levels of cytokine capable of enhancing Mig production. We

speculate that allergen-driven Mig production may also be regulated by other cytokines present in produced endogenously such as IL-12, or IL-18.

These results are important because they indicate the importance of type 1 and type 2 cytokines in regulating type 2 chemokine (TARC) production. We speculate that allergen-driven TARC production is enhanced in atopic and atopic mild asthmatics due to endogenous levels of IL-13 and a relative lack of IFNγ. We argue that the type 2 chemokine, TARC, is a critical component of a positive feedback loop in atopy. We propose that elevated type 2 cytokine, IL-13, at the site of inflammation is able to enhance allergen-driven TARC production, which may then work to recruit Th2 cells to the site of inflammation via CCR4 thereby maintaining or exacerbating allergic inflammation.

11.5 Allergen-driven TARC and Mig production is MHC class II dependent

Exogenously added and endogenously produced cytokines from T cells are able to regulate the intensity of type 1 (Mig) and type 2 (TARC) chemokine production. We speculated that activated T cells in response to allergen produced these cytokines. Based upon these findings we sought to determine whether Mig and TARC production was dependent upon allergen recognition by Th cells. To accomplish this we stimulated non-atopic, atopic and atopic mild asthmatic PBMC with grass pollen +/- neutralizing antibodies to HLA-DR, to block the vast majority of MHC II presentation to Th cells. In addition we blocked the co-receptor molecule CD4 found on T cells that specifically binds to MHC class II helping to form a stable trimer.

Interestingly, neutralizing HLA-DR completely abrogated allergen-driven TARC and Mig production from PBMC. This is important because it demonstrates a requirement for T cell recognition of allergen in the context of MHC class II to induce type 1 (Mig) and type 2 (TARC) production.

Blocking CD4 using a mAb also resulted in complete abrogation of allergen-driven Mig and TARC production, confirming the requirement for the formation of a stable TCR-peptide-MHC class II trimer in allergen-driven chemokine production.

We did not observe any differences between non-atopic, atopic and atopic mild asthmatics, suggesting the importance of allergen recognition by Th cells before chemokine production occurs. We argue that previous studies utilizing isolated cell populations (epithelial cells, dendritic cells) stimulated with polyclonal activators have ignored the importance of allergen-recognition by Th cells for chemokine production. This is the first documentation of the requirement for Th cell recognition of allergen in the context of MHC class II for Mig and TARC production. We argue that atopic and atopic mild asthmatic subjects display enhanced allergen-driven TARC production due to an expanded Th2-like repertoire of allergen specific cells capable of producing elevated levels of IL-13.

11.6 Allergen-driven TARC and Mig production is markedly inhibited by blocking key molecules involved in antigen presentation

Due to the finding that allergen-driven Mig and TARC production is dependent upon the abililty of Th cells to recognize allergen in the context of MHC class II, we were interested in determining the co-stimulatory signals responsible for driving recall

chemokine production. We examined the ability of the co-stimulatory molecules B7-1 and B7-2 found on the APC to induce allergen-driven MHC class II dependent type 1 (Mig) and type 2 (TARC) chemokine production. To accomplish this we blocked using mAb against B7-1, or B7-2 and then combined the blocking antibodies (αB7-1 and αB7-2) to determine if the combination of the two molecules produced an additive or synergistic response. In addition to using αB7-1 and αB7-2 mAb we utilized the CTLA-4-Ig fusion protein that competes for binding to B7-1 and B7-2 preventing signaling through CD28. CTLA-4-Ig fusion protein binds B7-1 and B7-2 equally well and should result in a comparable response seen using αB7-1 and αB7-2.

Blocking B7-1 significantly reduced allergen-driven TARC production in non-atopics but not atopic or atopic mild asthmatics. In contrast, allergen-driven Mig production was significantly inhibited through blocking B7-1 in atopic and atopic mild asthmatics but not non-atopics. However, both studies show that all three groups displayed a trend towards downregulation of TARC and Mig, albeit to different degrees, when both B7-1 and B7-2 were blocked individually. Regardless of the intensity of downregulation demonstrated in the three subjects groups tested, the dependence upon B7-1 and B7-2 for allergen-driven chemokine production may indicate key molecules required for the production of allergen-driven type 1 and type 2 chemokines.

Blocking B7-2 resulted in significant inhibition of allergen-driven TARC production from all of the subject groups tested. Non-atopics, atopics, and atopic mild asthmatic individuals were inhibited by similar amounts (~50% reduction). This implies that signaling through B7-2 is relatively more important than through B7-1 because of the significant reduction in Mig and TARC regardless of atopic status. Kay et al

demonstrated the dependence of IL-5 (type 2 cytokine) production and T cell proliferation upon signaling through B7-2 but not B7-1 [133]. In addition, several groups have speculated that IL-4 production requires signaling through B7-2. However, Kuchroo et al demonstrated the importance of B7-1 but not B7-2 in IL-4 production [135]. We conclude that allergen-driven type 1 (Mig) and type 2 (TARC) chemokine production is dependent upon B7-2 but is differentially dependent upon signaling through B7-1 depending on atopic status.

Blocking both B7-1 and B7-2 using either multiple blocking antibodies or CTLA-4-Ig fusion protein resulted in complete abrogation of allergen-driven TARC and Mig production. Because atopic and non-atopics were differentially producing Mig and TARC upon neutralization of B7-1 we argue that the combination of neutralizing B7-1 and B7-2 results in synergistic inhibition of allergen-driven Mig and TARC production. This is important because it indicates the differential involvement of B7-1 depending upon the presence of functional B7-2. We conclude that the great majority of allergen-driven MHC class II dependent Mig and TARC production is stimulated through signaling of both co-stimulatory molecules B7-1 and B7-2.

11.7 Immunocytochemical visualization of allergen-driven Mig production

In light of the finding that allergen-driven chemokine production is dependent upon T cell recognition of allergen in the context of MHC class II and the sufficient signaling through B7-1 and B7-2 we wished to identify immunocytochemically the source of Mig production by PBMC. Staining freshly isolated and cultured allergen-stimulated PBMC we were able to identify Mig staining on PBMC. The staining was the most intense and

frequent on polyclonal stimulated PBMC (i.e. TSST-1) and weakest on unstimulated PBMC with grass pollen stimulation demonstrating moderate staining. Using two color staining we determined Mig staining was not restricted to CD3+ cells. Both CD3+ and CD3- cells stained positively for the presence of Mig. Approximately 50% of Mig+ cells were CD3+ indicating that CD3+ cells (T cells) are able to produce Mig as well as CD3- cells (APC). This is in contradiction of the findings demonstrating Mig production from non-CD3 cells exclusively as well as the extreme unlikelihood that there exists such a high frequency of grass pollen specific CD3+ T cells. A possible explanation for this finding may lie in the receptor for Mig, CXCR3, on Th CD4/CD45RO+ cells. We have identified previously approximately ~40-60% of CD4+/CD45RO+ T cells express CXCR3 when freshly isolated from normal healthy and atopic donors. We measured the frequency (percentage) of CXCR3 positive cells after 5 days stimulation with grass pollen (4ug/ml) to determine the possible number of Th cells expressing CXCR3 capable of binding Mig and resulting in double-positive cells. We found a significant down-regulation of CXCR3 expression on CD4/CD45RO+ cells from ~50 % down to ~10 % suggesting that the source of the high percentage of double positive cells is not a result of antibody recognition of Mig bound to CXCR3 on Th cells.

We conclude that allergen-driven Mig production is dependent upon MHC class II recognition of allergen and is stimulated by sufficient signaling through B7-1 and B7-2 resulting in Mig production by CD3+ and CD3- negative cells. We argue that the majority of Mig staining on CD3+ cells cannot be attributed to binding to surface CXCR3 and therefore are possibly due to the ability of CD3+ cells to produce Mig. To confirm this finding further investigation is required to confirm the ability of CD3+ cells to

produce Mig in response to allergen stimulation through MHC class II with sufficient stimulation through CD28 (cognate receptor for B7-1 and B7-2).

11.8 Global Summary

To investigate the regulation of type 1 and type 2 chemokines in allergic disease we took two separate approaches. Firstly, we examined systemic levels of chemokines isolated from a range of clinically sensitive individuals (non-atopic, atopic, atopic asthmatic) to determine whether systemic chemokine levels were useful markers of allergy. Secondly, we examined fresh human PBMC directly *ex vivo*, stimulated with allergen as opposed to polyclonal activators, to identify key differences between allergen-driven chemokine production in atopic and atopic mild asthmatics compared to controls. Combining these two approaches (*in vivo* and *in vitro*), with chemokine specific sandwich ELISAs capable of detecting concentrations as low as 4pg/ml we were able to demonstrate the following:

1) Systemic levels of type 1 (IP-10, Mig) and type 2 (Eotaxin, TARC) chemokines are present at a wide range of concentrations within both atopic and non-atopic populations (4pg/ml to 20ng/ml). The intensity of DARC expression is not correlated with plasma chemokine levels in atopic and non-atopic groups.

2) In cross-sectional analysis, these representative type 1 and type 2 chemokines are not useful markers of allergic rhinitis. Longitudinally, IP-10 and CXCR3 levels were significantly elevated in the non-atopics as a consequence of seasonal allergen exposure. This may indicate a potential mechanism driving or enhancing clinical tolerance versus sensitivity to environmental allergens.

3) *In vitro*, we demonstrated significantly elevated allergen-driven, and identical polyclonally driven, TARC production by atopic and atopic mild asthmatic individuals

when compared to non-atopics. Representative type 1 chemokines did not differ between subject groups, suggesting preferential production of TARC, a type 2 chemokine, in response to allergen. This is important because of the ability of TARC to recruit CCR4 bearing Th2 cells to the site of inflammation.

4) *In vitro*, we demonstrated the ability of exogenously added or endogenously produced IFNγ to regulate the intensity of allergen and antigen (SK) driven TARC, but not Mig, production. Together with the finding that inhibition of endogenous IL-13 dramatically reduced allergen-driven TARC production we speculate that TARC may be a key molecule in atopics to maintain allergic inflammation through recruitment and activation of type 2 cells.

5) Exogenously added IL-10 dramatically reduced allergen-driven TARC and Mig production. This is important because it demonstrates the ability of TARC and Mig to be down-regulated in response to an anti-inflammatory cytokine.

6) We demonstrated that MHC class II and CD4 are critical for allergen-driven type 1 (Mig) and type 2 (TARC) chemokine production to occur. This is important because it shows a direct link between Th cell recognition of allergen and type1/type 2 chemokine production, confirming the importance of chemokines in allergen-driven responses.

7) We characterized the requirements of co-stimulation through B7-1 and B7-2 to initiate allergen-driven chemokine production. We demonstrate that there exist differential requirements for allergen-driven Mig and TARC production in non-atopic, atopic and atopic asthmatic individuals. This is important because it demonstrates the importance of B71 and B7-2 in allergen-driven chemokine production and

suggests an additional regulatory difference between type 1 and type 2 chemokine production based upon atopic status.

In summary, we argue that these data strongly indicate a role for type 1 and type 2 chemokines in allergy. We suggest that systemic levels of type 1 and type 2 chemokines are not indicative of clinical sensitivity *in vivo* but provide essential information when examined *in vitro* using fresh PBMC stimulated with allergen. Specifically, we provided evidence for a potential mechanism through which TARC, a type 2 chemokine, may be able to drive the maintenance of allergy through a positive feedback loop. The ability of type 1 and type 2 cytokines to regulate the intensity of type 1 and type 2 chemokines, together with the requirement for MHC II recognition of allergen for chemokine production indicates the interconnected regulatory relationship between these families in allergic disease. Together these findings indicate a strong association with allergen-driven chemokine production and allergic rhinitis and mild asthma.

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