CYTOKINE REGULATION OF IMMUNE RESPONSES IN THE RESPIRATORY MUCOSA

By

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ABSTRACT

Atopic asthma is an immunologically-driven condition characterized by reversible bronchoconstriction and Th2-polarized eosinophilic airways inflammation. As such, the development of Th2 responses is a key step in the pathogenesis of the disease. We utilised a murine model of respiratory mucosal sensitization to examine the roles of different cytokines in the generation of polarized immune-inflammatory responses in the airways. Mice were sensitized to ovalbumin (OVA) by daily aerosol exposure in the context of airway expression of GM-CSF, resulting in a Th2-polarized eosinophilic inflammatory response in the airways, reminiscent of asthma; the addition of IL-12 to the airway microenvironment deviated this response toward the Th1 phenotype. We analysed expression of key Th1- and Th2- associated genes in the lymph nodes during Th1- and Th2-polarized sensitization, and showed that cytokine expression compartmentalized to the respiratory tract can have a profound impact on the nature of developing immune responses in the draining lymph nodes. Next, we investigated the necessity for IL-4 during GM-CSF-driven respiratory mucosal sensitization. We analysed a variety of Th2-associated factors, including transcription factors, cytokines, chemokine receptors, and cell surface markers, and found that IL-4 was not necessary for Th2 polarization in this model. Finally, we examined whether IL-10 played a role in mediating Th2 polarization, and observed that mice treated with anti-IL-10 antibodies or genetically deficient in IL-10 showed impaired development of Th2-polarized immune-inflammatory responses. This research highlights the importance of the cytokine microenvironment of the airways in determining the nature of the ensuing immune-inflammatory response, and defines some of the molecular requirements for the polarization of Th responses during respiratory mucosal sensitization *in vivo*.

PREFACE & ACKNOWLEDGEMENTS

Each of the 4 scientific manuscripts included as part of this thesis are multiply-authored; as such, McMaster University's Guidelines for the Preparation of Theses dictate that I must clearly document my contribution to each of these works, and indicate when the work was conducted.

Please note that all of the manuscripts contained in this thesis are being or have been peer-reviewed prior to publication.

❶ Ritz, Stacey A, Meghan J Cundall, Beata U Gajewska, Filip K Swirski, Ryan E Wiley, David Alvarez, Anthony J Coyle, Martin R Stämpfli, and Manel Jordana. The lung cytokine microenvironment fundamentally alters molecular events in the lymph nodes during Th1 and Th2 respiratory mucosal sensitization to antigen *in vivo*. Prepared for submission to Clinical & Experimental Immunology.

This work was conducted over the period May 2000-July 2003. My contributions to this work included:

- equal partner (with MJC, BUG, FKS, REW, and DA) in the design and execution of the *in vivo* portions of the originating experiment in Summer 2000
- equal partner (with MJC) in the preparation and analysis of the original RNA samples by real-time quantitative PCR (TaqMan) at Millennium in September 2000; these preliminary data are *not* included in the manuscript, but formed the basis for our future analyses conducted at McMaster
- equal partner (with MJC) in the development of TaqMan techniques here at McMaster, during the period September 2000-January 2001
- sole responsibility for the design, execution, analysis, and interpretation of all experiments and all TaqMan analyses conducted since 2001, which encompasses all of the TaqMan data actually presented in the manuscript; BUG provided the flow cytometric analysis data
- sole responsibility for the writing and preparation of the submitted manuscript

❷ Ritz, Stacey A, Meghan J Cundall, Beata U Gajewska, David Alvarez, Jose-Carlos Gutierrez-Ramos, Anthony J Coyle, Andrew N McKenzie, Martin R Stämpfli, and Manel Jordana. Granulocyte/macrophage colony-stimulating factor-driven respiratory mucosal sensitization induces Th2 differentiation and function independently of IL-4. American Journal of Respiratory Cell and Molecular Biology 27:428 2002.

This work was conducted over the period September 1998-October 2002. My contributions to this work included:

- sole responsibility for the design, analysis, and interpretation of all experiments
- sole responsibility for the execution of all experiments (with the exception of 1, the first of the IL-4/IL-13 double-knock-out experiments, which was conducted by

BUG and DA during my comprehensive examinations, hence their inclusion as co-authors on the manuscript)
- sole responsibility for the writing and preparation of the published manuscript
- primary responsibility for responding to comments from peer-reviewers

❸Ritz, Stacey A, Nicolas W Lukacs, Andrea Keane-Myers and Manel Jordana. IL-10 contributes to Th2 polarization during GM-CSF-driven respiratory mucosal sensitization in mice. Prepared for submission to Journal.

This work was conducted over the period April 2002-April 2003. My contributions to this work included:
- sole responsibility for the design, execution, analysis, and interpretation of all experiments
- sole responsibility for the writing and preparation of the submitted manuscript

❹ Ritz, Stacey A, Martin R Stämpfli, Donna E Davies, Steven T Holgate, and Manel Jordana. On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends in Immunology, 23:396 2002.

This paper was researched and written over the period October 2001-August 2002. My contributions to this work included:
- extensively reviewing and evaluating the published literature
- devising a structure and hypothesis for the article
- preparing the proposal for submission to Trends in Immunology
- primary responsibility for the writing and preparation of the manuscript, in collaboration with MRS, STH, and MJ
- design and preparation of original figures
- primary responsibility for responding to the comments from peer-reviewers, along with MJ

Scientific research is almost invariably the result of the efforts of an entire team, not a single individual, and the work presented in this thesis is no exception. By highlighting my own contributions to this work in this preface, I do not mean to disregard the myriad contributions of others. Over the course of my time in Manel Jordana's lab, I have been assisted, learned from, received reagents from, or been supervised by many colleagues (all at McMaster unless otherwise indicated), including: David Alvarez, Elizabeth Cates, Duncan Chong, Gerry Cox, Tony Coyle (Millennium), Meghan Cundall, Monika Cwiartka, Joanna DeJong, Ramzi Fattouh, Xueya Feng, Beata Gajewska, Jack Gauldie, Susanna Goncharova, Mark Inman, Jill Johnson, Andrea Keane-Myers (NIH), Christine Kerr, Mary Kiriakopoulos, Xue-Feng Lei, Nick Lukacs (University of Michigan), Steve Manning (Millennium), Claudio Mastruzzo, Andrew

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My appreciation also goes to my partner, Scott Neigh. For his encouragement and support, his love and affection, and for being a model of perseverance and discipline, I thank him heartily. I think we have an exciting and interesting future ahead of us.

Finally, I would like to thank Liam Neigh, for timing his arrival so perfectly – 16 hours after I typed the final words of this thesis.

– SAR
October 2003

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CHAPTER 1: INTRODUCTION

In the past 50 years, scientific thinking about asthma has undergone a considerable paradigm shift. Although it used to be regarded primarily as a disease originating in dysfunctional airway physiology, our current view of asthma (at least, *atopic* asthma) is that it is principally an immunologic disorder which has pathophysiological consequences in the airways (Lacy 2001). As a result, our understanding of the immunological origins of asthma have expanded rapidly. Because of the advent of murine models of asthma in the early 1990s, the past 15 years have been a particularly fruitful period for asthma research.

It has become clear that T helper cells are crucial to the pathogenesis of atopic asthma. It has long been known that T helper cells were prominent in the airways of human patients with asthma, although the significance of their presence was not fully appreciated. Further investigation demonstrated that the pathophysiological hallmarks of atopic asthma appear to derive largely from the cytokine products of Th2-polarized Th cells, most notably IL-4, IL-5 and IL-13. As such, in order to understand the origins of atopic asthma it is imperative that we understand the genesis of Th2 cells.

The research I have engaged in during my PhD investigates cytokine regulation of T helper cell differentiation during allergic sensitization in the respiratory tract. This thesis endeavours to define the molecular events associated with Th1- and Th2-polarization *in vivo* during respiratory mucosal sensitization, and particularly, to investigate the contribution of key cytokines to the development of Th2 responses *in vivo*.

CURRENT UNDERSTANDING OF ASTHMA

The prevalence of asthma, and of allergic diseases more generally, has risen

dramatically over the past 3 decades. One of the most highly-cited studies of the change in asthma prevalence administered the same questionnaire to schoolchildren in Aberdeen, Scotland, and found that whereas the prevalence of asthma was 4.1% in 1964, this had increased to 10.2% in 1989, and further to 19.6% in 1994 (Omran 1996). In the United States, asthma prevalence was found to be 3.1% in 1980, increasing to 5.4% in 1994, with much higher prevalence among impoverished inner city children (Sly 1999). It has been suggested that perhaps the incidence of asthma *per se* has not increased, but rather that the medical establishment diagnoses the disease more readily; however, several studies using objective measures of airway dysfunction and allergic sensitization suggest that this is not the case (Ulrik 1996)(Ciprandi 1996)(Ulrik 2000).

Currently, it is estimated that asthma may affect as much as 5-15% of the population in developed countries. The economic costs of asthma in Canada alone in 1990 were estimated to be $500-650 million per year, and given the current morbidity and mortality trends, this is likely to have increased (Krahn 1996). Although current pharmacotherapy for asthma is very effective for the vast majority of patients, the rapid rise in asthma and the costs associated with it continue to motivate a massive research effort into the origins of the disease and into the development of novel treatment strategies.

Clinically, allergic asthma is recognized to be a heterogeneous syndrome (Bonini 2001) involving 3 main features: the presence of antigen-specific IgE; airway inflammation characterized by eosinophilia and the presence of Th2 cells; and airway dysfunction, manifesting as intermittent reversible airway obstruction. The reason for the physiological dysfunction is not entirely clear, but is probably due to a complex interaction of acute and chronic factors, including cytokines and lipid mediators acting on airway smooth muscle, the release of cytotoxic proteins by activated eosinophils and neutrophils, and permanent changes in the airway structure due to chronic inflammation. In the past 15 years, human and animal studies have attempted to unravel

the contributions of these various factors to the development of the asthma phenotype.

The presence of antigen-specific IgE is a defining feature of any allergic disease. It is not merely a marker of allergic disease, but can also play a pathogenic role directly. IgE binds to its high-affinity receptor expressed on mast cells, which reside primarily at subepithelial sites, including the respiratory tract. Upon antigen exposure, IgE is cross-linked by the multi-valent antigens for which it is specific, thereby activating the mast cell. Such activation causes the release of the mast cell granules, and the rapid synthesis of lipid and cytokine mediators. These substances, particularly histamine, leukotrienes, and TNFα, initiate a local inflammatory response, causing an influx of other leukocytes into the tissue. Clinically, this often leads to a rapid decrease in airflow due to the effects of these mediators on bronchial smooth muscle. Although IgE and mast cells can assuredly contribute to the development of eosinophilic inflammation and airway hyperreactivity (Maezawa 2003), numerous studies in mouse models have demonstrated that it is possible for these features of asthma to occur in the complete absence of IgE, B cells, and mast cells (Hamelmann 1999)(MacLean 1999)(Korsgren 1997). It is important to note, however, that these studies were all in acute models of allergic airway inflammation, whereas there is evidence that mast cells (and hence IgE, indirectly) can contribute to airway remodelling under chronic antigen exposure (Masuda 2003), which is one mechanism that may contribute to airway hyperreactivity.

The development of an eosinophilic inflammatory infiltrate into the airways and lung tissue is a prominent feature of asthma, but is a terminal event in a complex set of processes (Stampfli 1998b). Eosinophils are normally present in very low numbers in the peripheral blood, so in order to generate a substantive airway eosinophilia there must be eosinopoietic events in the bone marrow (mediated largely by IL-5 and GM-CSF) as well as recruitment of eosinophils from the blood into the airways (Stampfli 1998b). Eosinophils present at the site of inflammation produce a variety of substances including a variety of cytokines and chemokines, lipid mediators, reactive oxygen species

and proteases, all of which contribute to the airway dysfunction of asthma, either through direct effects on smooth muscle, or by causing injury to pulmonary tissues leading to long-term structural changes in the lung (Fireman 2003)(Lacy 2001). Although the majority of the evidence linking eosinophilia and airway dysfunction has been correlative, a recent study used adoptive transfer of eosinophils and demonstrated the induction of mucous production and airway hyperreactivity (Shen 2003), indicating that eosinophils are not simply bystanders or markers of asthma, but actively participate in the induction of the asthma phenotype. However, there have been numerous studies in mouse models demonstrating that eosinophilic inflammation and airway hyperreactivity can be dissociated from one another (Koarai 2003)(Tournoy 2001)(Mathur 1999)(Coyle 1998)(Corry 1996); thus there must be other, eosinophil-independent, mechanisms that can trigger airway dysfunction.

The role of T helper cells in asthma cannot be underestimated. The cytokine products of Th2 cells have been shown to be critical mediators of many aspects of the asthmatic phenotype. IL-4 is important for isotype switching to IgE (Finkelman 1988), expression of VCAM-1 and other molecules important for leukocyte recruitment (Kotowicz 2000)(Hickey 1999)(Fukuda 1996), and has also recently been implicated in airway remodelling (Liu 2003)(Bergeron 2003). IL-5 mediates eosinopoiesis in the bone marrow (Cyr 2001)(Roboz 1999) and promotes eosinophil recruitment, activation, degranulation and survival at sites of inflammation (Weltman 1998). IL-13 has been shown to be important for mucous production, airway hyperreactivity, and eotaxin expression (Wills-Karp 1998)(Grunig 1998)(Matsukura 2001)(Wills-Karp 2003). In combination, these 3 cytokines can account for most of the major properties of asthma (Foster 2002). Although these cytokines can be produced by other cell types, the asthma phenotype cannot be generated in mice in the absence of $CD4^+$ T helper cells (Garlisi 1995)(Gonzalo 1996), and it has become widely accepted that Th2 cells are crucial mediators of asthma in humans (Romagnani 2001)(Foster 2002)(Larche 2003).

As such, elucidating the development of the Th2 subset is a crucial element in understanding the origins of asthma.

Aeroallergens are breathable entities, and enter the respiratory tract along with a myriad of other particles, gases, and pathogens. Epithelial cells lining the airways are the first to confront this mixture, and complex interactions occur between these entities, the epithelial cells themselves, and the underlying mesenchymal cells. The response of this 'epithelial-mesenchymal trophic unit' (Holgate & Davies 2000) to such challenges may involve the production of cytokines, growth factors, and other molecules which can influence the resident antigen-presenting cells of the lung. Whether or not these APCs become activated, and in what context, likely determines the immunological ramifications of aeroallergen exposure. From this perspective it is evident that it is important to understand the events which occur at the site of initial contact between host and environment in order to get to the origins of the disease. Studying these events in humans during the natural course of antigen exposure would be virtually impossible; hence, animal models of allergic sensitization and asthma are particularly useful in this enterprise.

MURINE MODEL OF RESPIRATORY MUCOSAL SENSITIZATION

By far, the most commonly utilized murine model for human asthma is what I will refer to as the 'conventional' model. In general, mice are sensitized by one or more intraperitoneal injections of a model antigen (usually ovalbumin, or OVA) along with an adjuvant (most commonly aluminum hydroxide); this causes the induction of OVA-specific IgE, a transient surge in serum Th2 cytokines, the establishment of OVA-specific Th2 memory systemically, and transitory bone marrow and blood eosinophilia, but has no discernable effects on the airway. In order to induce an asthma-like phenotype, OVA is introduced into the respiratory tract, which elicits eosinophilic airway inflammation, local Th2 activation and cytokine production, goblet cell

hyperplasia, and physiological dysfunction of the airways leading to hyperreactivity. Although all conventional models follow this general schema, there are many variations in timing and dosage in use in different laboratories.

Like any experimental model, the conventional model of asthma has both strengths and limitations that must be understood by the investigators using it. Probably its greatest strength is that it reproducibly elicits a consistent and predictable inflammatory response. As such it is a very serviceable model for studying the generation of the secondary immune response in the lungs, and for investigating the effects of different intervention strategies that might be used in established disease. However, the route and method of allergic sensitization is completely unlike the natural mode of allergic sensitization to airborne antigens in humans, which most likely occurs through the respiratory tract, and in the absence of chemical adjuvants. If we are interested in understanding the requirements and mechanisms for the establishment of allergic sensitization *per se*, conventional models are unlikely to be very informative. Thus, we must look to other models in order to investigate these processes.

In 1997, our lab began to pursue the development of alternative models of allergic airways inflammation that more closely mimicked allergic sensitization in humans. The original approach was to expose mice to aerosolized OVA daily for 10 days, which had been described by Hamelmann *et al* as leading to allergic sensitization (1997). However, we and others were unable to reproduce their findings; in contrast, such a protocol did not lead to allergic sensitization in our hands, and further experimentation revealed that in fact such exposure led to the development of *inhalation tolerance*. This is not merely ignorance of the antigen's presence, but an active process of immunological regulation which prevents the generation of potentially harmful immune-inflammatory responses to ubiquitous non-pathogenic airborne antigens. This phenomenon has been investigated extensively by our group (Swirski 2002) and by others (Holt & Leivers 1982)(McMenamin 1995)(Tsitoura 2000).

In a series of experiments, the ability of various cytokines to initiate allergic sensitization via the airways was investigated by the intranasal administration of adenoviral vectors encoding cytokines of interest; of the panel tested, only GM-CSF was able to elicit antigen-specific immune-inflammatory responses, and these were characterized by eosinophilic airways inflammation, OVA-specific IgE production, and Th2-polarized cytokine responses (Stampfli 1998)(Ritz 2000). This model of respiratory mucosal sensitization was extensively characterized (Stampfli 1998). Moreover, the ability of other cytokines to modulate this process was investigated. Co-administration of IL-10 along with GM-CSF was found to inhibit the development of these responses (Stampfli 1999a), whereas IL-12 apparently deviated the responses from a Th2- to a Th1-polarized phenotype (Stampfli 1999b). These models have been used extensively in the work which constitutes this thesis.

There are at least 2 major advantages to the use of this model for the investigation of allergic sensitization. First, it reproduces the route through which humans would normally be exposed to airborne antigens. This means that the processes of antigen capture, processing, and presentation, and the trafficking of cells from the lungs to the draining lymph nodes, are likely to be much more similar to those occurring during human allergic sensitization than is the case using conventional models. Second, it is able to elicit allergic sensitization without the use of non-physiological adjuvants such as alum. Although it does employ GM-CSF, we have argued that GM-CSF is physiologically relevant to the induction of allergic sensitization (Ritz 2002a); moreover, recent studies from our lab indicate that endogenously produced GM-CSF can indeed facilitate allergic sensitization to true allergens (Cates 2003a)(Cates 2003b).

THE CYTOKINES OF INTEREST

Cytokines are a diverse group of soluble proteins which regulate immune-inflammatory responses by mediating communication between cells. Signalling

primarily through highly specific cell surface receptors, cytokines typically act in an autocrine or paracrine fashion, although they can have systemic effects when present in the circulation at sufficiently high levels.

The research of this thesis has concerned itself primarily with the cytokine regulation of T helper cell responses in the respiratory tract. Thus, I will take some space here to give some general information about the known characteristics and functions of the cytokines of greatest relevance to this work: GM-CSF, IL-4, IL-10, and IL-12.

• *GM-CSF*

GM-CSF is a 23 kDa monomeric glycoprotein of the hematopoietin family (Ruef 1990). It shares a common receptor subunit (β_c) with IL-3 and IL-5, but will bind and initiate intracellular signalling only when it binds to the GM-CSF-specific receptor (GM-CSFRα) in conjunction with the β_c chain. GM-CSF can be produced by a wide variety of different cell types, but its primary producers are epithelial cells, macrophages, and activated T cells. The receptor is expressed on myelo-monocyte precursors, monocytes, neutrophils, eosinophils, and endothelial cells.

GM-CSF is a highly pleiotropic molecule. The earliest recognized function of GM-CSF, as it eponymously implies, is to elicit the production of granulocytes and macrophages from myeloid precursors in the bone marrow (Ruef 1990). It can also promote the proliferation and maturation of DCs and other APCs, enhancing antigen processing, presentation by MHC molecules, and expression of costimulatory molecules, thereby indirectly facilitating the activation of naive T cells (Christensen 1995)(McKenna 2001)(Heystek 2000). This may be of particular importance in the lung, where GM-CSF treatment inhibits the immunosuppressive activity of resident pulmonary alveolar macrophages (Bilyk 1993). Injection of GM-CSF into mice elicits the expansion of the myeloid DC subset (Wang 2000)(Daro 2000)(Pulendran 1999),

which are characterized by the expression of CD11b, CD11c, F4/80, and the absence of CD8α (De Smedt 2001). In addition, eosinophil survival at sites of inflammation is significantly prolonged by the presence of GM-CSF (Adachi 1995)(Saitou 1997)(Esnault 2001).

The GM-CSF knock-out mouse reveals the homeostatic role played by GM-CSF *in vivo*. Although they do not demonstrate any overt perturbations of hematopoiesis, the animals are unable to maintain pulmonary surfactant homeostasis or clear proteinaceous secretions in the lung efficiently, and develop severe alveolar proteinosis as a result (Dranoff 1994)(Stanley 1994)(Huffman 1996). This disorder resembles human pulmonary alveolar proteinosis, which as also recently been associated with defects in GM-CSF or GM-CSF-receptor expression (Dirksen 1997)(Tchou-Wong 1997).

• *IL-4*

IL-4 is a 129 amino acid monomeric protein, binding to the IL-4R chain in the presence of the γ_c chain (Janeway 2001). The only cells known to be capable of producing IL-4 are T cells, mast cells, and basophils, and possibly eosinophils (Nelms 1999). The IL-4R is expressed on mature B and T cells, endothelial, epithelial, and muscle cells, fibroblasts, hepatocytes, and on hematopoietic precursor cells in the bone marrow (Nelms 1999). Binding the receptor complex causes activation of the Stat6 signalling pathway, leading to the transcription of IL-4 responsive genes (Nelms 1999).

IL-4 was identified because of its important effects on B cell activation and essential for their differentiation into IgE-producing plasma cells (Foster 2002); as such its original name was 'B Cell Stimulatory Factor (BSF)'. IL-4 is thought to be an important autocrine factor for T helper cell differentiation, and widely thought to be important in Th2 polarization (Foster 2002). Other functions of IL-4 include promoting mast cell development in the tissues (Foster 2002), expression of adhesion molecules such as VCAM-1 (Kotowicz 2000)(Hickey 1999)(Fukuda 1996), and

regulating collagen turnover and tissue remodelling (Liu 2003)(Bergeron 2003).

IL-4 knock-out mice are characterized primarily by the complete absence of IgE. A number of experimental models indicate that these mice are also unable to mount effective Th2-polarized immune responses (Hogan 1998)(Hogan 1997)(Kips 1995)(Brusselle 1994)(Coyle 1995)(Herrick 2000)(Kopf 1993); however, as will be discussed in detail later, other data indicate that this is not the case (Ritz 2002b)(Herrick 2000)(Noben-Trauth 1997)(Hogarth 1998)(Kopf 1996)(Brewer 1999).

• *IL-10*

IL-10 is a 17-18 kDa polypeptide, depending on glycosylation, and forms non-covalent homodimers which bind with high affinity to the IL-10 receptor (Moore 2001). It can be expressed by a variety of cell types, including T cells, monocytes, and macrophages (Moore 2001). Several other molecules have demonstrated weak homology to IL-10 (<30%), but are clearly structurally related to IL-10; however, the biological functions of these proteins are largely unknown (Moore 2001). In addition, there are known viral homologues of IL-10, which likely act to downregulate immune responses upon infection with agents such as Epstein-Barr virus and human cytomegaloviruses (Moore 2001). The IL-10R is constitutively expressed by most bone marrow-derived cells, and is inducibly expressed on a variety of non-hematopoietic cells as well including fibroblasts, epidermal cells, placental cytotrophoblast, and colonic epithelium (Moore 2001).

IL-10 is a regulatory cytokine, and was first described as 'cytokine synthesis inhibitory factor (CSIF),' as it potently inhibits the production of IL-1, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, PAF, as well as CC and CXC chemokines and lipid mediators by activated macrophages (Moore 2001). The ability of IL-10 to downregulate IL-12 and IFNγ production significantly inhibits the activation and function of NK cells and CTLs (Moore 2001). IL-10 also acts to inhibit immune

responses through its ability to hamper antigen-presentation by macrophages and dendritic cells via downregulation of MHC class I and II (Koppelman 1997) (Chang 1994) and the inhibition of the expression of costimulatory and adhesion molecules such as B7 and ICAM (Chang 1994) (Willems 1994)(Moore 2001). Such effects on APC function profoundly inhibit proliferation and cytokine production by CD4+ T cells, and may induce T cell anergy that is refractory to reversal by IL-2 or stimulation through CD3 and CD28 (Moore 2001). Although its immunosuppressive roles are most frequently recognized it can also play immunostimulatory roles, depending on the biological context, particularly with respect to CD8+ T cells (Groux 1999)(Moore 2001).

The constitutive presence of IL-10 appears to be important for preventing certain harmful inflammatory responses, since IL-10 KO mice spontaneously develop inflammatory bowel disease by 3 months of age; IL-10 also appears to be beneficial in limiting the development of experimental autoimmune encephalomyelitis, the NOD mouse model of type 1 diabetes, and animal models of rheumatoid arthritis (Moore 2001). In humans, higher expression of IL-10 has been associated with better outcomes after allogeneic transplantation (Asderakis 2001)(Baker 1999). It may also play a role in maintaining the fœtus during pregnancy (Piccinni 2002), and in the immunomodulatory effects of allogeneic blood transfusion (Claas 2001). However, high levels of IL-10 are not necessarily universally protective against immune hyperresponsiveness, as high expression of IL-10 has been associated with susceptibility to systemic lupus erythematosis (Llorente 1995)(Grondal 1999).

• *IL-12*

IL-12 is a heterodimeric protein comprised of a 35 kDa and a 40 kDa subunit. The p35/p40 heterodimer is biologically active as IL-12, whereas the p40 subunit is capable of homodimerizing and binding the IL-12 receptor, thereby blocking the

activity of the heterodimer, at least in mice (Trinchieri 2003). Phagocytes and dendritic cells are the most important producers of IL-12, and the receptor is expressed primarily on activated T cells, NK cells, dendritic cells, and some B cell lines (Trinchieri 2003).

IL-12 is an important immunomodulatory cytokine which is of particular importance for the generation of antigen-specific cytotoxic T cell responses (Trinchieri 2003). It is a potent stimulus for NK cell activation and IFNγ production, and stimulates proliferation of activated T cells and NK cells (Trinchieri 2003). Of particular relevance to this thesis is that it is probably the most soluble important factor for the differentiation of Th1 cells. Th1 cells, in turn, are critical for the efficient development of effective $CD8^+$ cytotoxic T lymphocyte responses for the clearance of intracellular pathogens. As a result, IL-12 knock-out mice are particularly susceptible to opportunistic infections.

THE TH1/TH2 PARADIGM

Th2-polarized responses have been repeatedly implicated in the pathogenesis of asthma and other allergic diseases. The work of this thesis has focussed on the development of such responses in the airways during sensitization to antigen, and hence I will invest some space here in elaborating upon the current state of the Th1/Th2 paradigm.

In the late 1980s Mosmann and Coffman described 2 distinct subsets of T helper cells which they named Th1 and Th2 cells, as defined by their pattern of cytokine secretion (1989). Th1 and Th2 cells were originally defined through *in vitro* experimentation in which naive T helper cells were activated in the presence of differing cytokine microenvironments. After several rounds of stimulation in culture conditions containing IL-12, the Th1 cells produce primarily IFNγ and TNFβ, but not IL-4 or IL-5 (Hsieh 1993)(Seder 1993). By contrast, when the culture conditions contained IL-4, the cytokine profile was reversed, with IL-4 and IL-5 predominating and diminished levels

of IFNγ and TNFβ (LeGros 1990)(Swain 1990). Th1 and Th2 cells are typically conceived of as being at opposite ends of a spectrum, with their cytokine products further consolidating the nature of the response and reciprocally inhibiting the other: IFNγ production by developing Th1 cells promotes further Th1 development and inhibits the development of Th2 cells, and conversely, IL-4 production by Th2 cells reinforces Th2 polarization while constraining the differentiation of Th1 cells. Since these original descriptions, the Th1/Th2 paradigm has been widely embraced in immunology because it provides a very useful general categorization of common immunological phenomena, and a great deal of research has gone into further investigating the generation, maintenance, and characteristics of Th1 and Th2 cells.

The role of cytokines in the polarization of T helper cells is popularly thought to be the most important in determining the outcome, with IL-12, IFNγ, and IL-18 promoting Th1 polarization, and IL-4 motivating Th2 differentiation (Glimcher & Murphy 2000). Although they are frequently overlooked, a variety of other factors are known to be influential as well. Other factors thought to promote Th1 responses include antigen presentation by $CD8\alpha^+$ 'lymphoid' dendritic cells, costimulation dominated by B7.1, high-affinity TCR-antigen-MHC interactions, and high antigen doses (Glimcher & Murphy 2000)(Constant & Bottomly 1997)(O'Garra 1998)(Tao 1997)(Jones 2001)(Jankovic 2001). Non-cytokine influences which may foster Th2 development include antigen presentation by $CD8\alpha^-$ 'myeloid' dendritic cells, costimulation by B7.2, OX40, ICOS, T1/ST2, lower-affinity TCR-antigen-MHC interactions, and low antigen doses (Glimcher & Murphy 2000)(Akiba 2000)(Tao 1997)(Lambrecht 2000)(Lambrecht 2001)(Jones 2001)(Jankovic 2001).

Since their original description, further research has demonstrated that Th1 and Th2 cells have other distinct hallmarks, aside from their characteristic cytokine secretion profiles. Surface markers associated with Th1 cells include the IFNγ receptor-β chain, IL-12 receptor-β chain, IL-18 receptor, PSGL-1, CXCR3, and CCR5 (Glimcher &

Murphy 2000)(Chiu 2002). In contrast, the chemokine receptors CCR3, CCR4, CCR8, and the enigmatic T1/ST2 (the ligand and function of which are not currently known) are surface markers purportedly associated with Th2 cells (Glimcher & Murphy 2000)(Chiu 2002). Differential expression of transcription factors has also been observed, with t-bet predominantly expressed in Th1 cells, and c-maf and GATA-3 in Th2 cells (Glimcher & Murphy 2000). In addition, the stability of Th1 and Th2 phenotypes is maintained in part by remodelling of the chromatin structures at the cytokine loci, with evidence of DNA demethylation in the relevant regions, with GATA-3 and t-bet implicated in mediating these changes (Glimcher & Murphy 2000)(Grogan 2001). This variety of cytokine and non-cytokine markers makes it possible to distinguish and identify Th1 and Th2 cells using a variety of experimental approaches.

Both Th1 and Th2 cells undergo certain changes in gene expression and chromatin structure such that they are largely refractory to re-polarization once the phenotype has been established (Glimcher & Murphy 2000). *In vitro* studies indicate that Th2 differentiation is accompanied by a rapid downregulation and loss of IL-12Rβ2 expression, rendering them insensitive to the effects of IL-12 (Guler 1996)(Szabo 1997). However, sustained stimulation of Th2 cells in the presence of high levels of IL-12 has been shown to reverse their phenotype (Smits 2001), and forced expression of t-bet in Th2 cells or GATA-3 in Th1 cells can cause the cells to switch to the alternate phenotype (Nawjin 2001). Such changes have only been observed in non-physiologic systems, however.

As previously noted, most characterization of Th1 and Th2 cells has been performed in *in vitro* culture systems, utilizing multiple rounds of T cell stimulation and relatively high concentrations of recombinant cytokines. Although the Th1/Th2 paradigm is very useful, it is problematic in that many in the research community appear to have forgotten that it is a conceptual construct, and have reified it, with the upshot

being that many people tend to take an overly simplistic view of immune responses characterized by Th1- or Th2-associated markers (Gor 2003). *In vivo* immune responses are rarely 'pure' Th1 or Th2 in nature, and clear-cut Th1 or Th2 cells are not often present under physiological immune-inflammatory conditions; as Gor *et al* point out, "these extremely polarized responses are usually observed under highly contrived immunization protocols" (2003).

IS IL-4 ACTUALLY REQUIRED FOR TH2 DIFFERENTIATION?

The standard protocol for induction of Th2 cells *in vitro* is to stimulate naive T helper cells in the presence of IL-4 and anti-IFNγ antibodies, whereas blocking antibodies against IL-4 tend to prevent Th2 polarization and promote Th1 development instead. Over the past 15 years, IL-4 has been widely promulgated as the principal factor for Th2 development. It is frequently implied that Th2 responses do not occur in the absence of IL-4, in spite of many reports in the literature to the contrary. If indeed IL-4 were required for Th2 polarization, then there would presumably have to be an early source of IL-4 that would kick-off the process, and there was a concerted effort, particularly in the late 1990s, to find the "original source" of IL-4 that mediated Th2 polarization *in vivo*. However, the known cellular sources of IL-4 are few, limited to activated mast cells, basophils, T helper cells, and a rare subset of lymphocytes which express both NK cell and T cell markers (NK T cells). Although some have been suggestive, there have been no studies thus far which have been able to identify which, if any, of these cells are the early source of IL-4 for Th2 polarization. The most recent candidate proposed to be the original source of IL-4 is the NK T cell. These rare lymphocytes express CD3, CD4, CD28, and other T cell surface markers including an invariant TCR$\alpha\beta$, but also typical natural killer cell markers. Support for this cell type as the early source of IL-4 comes largely from evidence that NK T cells stimulated with anti-NK1.1 antibodies can readily produce large amounts of IL-4 *in vitro* (Asea 1998);

however NK T cell-deficient mice have been shown to produce completely normal Th2 responses (Brown 1996). Others have suggested that classical T helper cells themselves could be the original source of IL-4 instructing Th2 development (Launois 1997).

The search for the "original source" of IL-4 is somewhat misguided, however. Recently it has been demonstrated that Th2 cells can be generated *in vitro* by repeated polyclonal stimulation of naive T cells in the absence of IL-4 signalling (Jankovic 2001). The *in vivo* situation is certainly more complex and nuanced than can be represented by *in vitro* culture systems, and accordingly the data from *in vivo* systems regarding the role of IL-4 in Th2 polarization is more difficult to interpret. It is clear, however, that IL-4-independent Th2 responses occur *in vivo* in a number of different experimental models.

The murine model of cutaneous leishmaniasis has been extensively used to examine Th polarization. Similar to leprosy, the clinical outcome of *Leishmania* infection is highly correlated with the nature of the Th response that is generated. Th1 responses tend to produce sterile immunity against the microbe, although not without attendant immunopathology. On the other hand, Th2 responses are generally ineffective, fail to clear the infection, and often result in systemic dissemination and death. C57BL/6 and Balb/c mice illustrate this dichotemy well, since C57BL/6 have an inherent tendency to mount Th1 responses to *Leishmania*, and do not succumb to infection, whereas Balb/c mice produce Th2 responses, and usually die. Numerous studies have been carried out in IL-4 KO, IL-4R KO, and Stat6 KO Balb/c mice to examine whether the absence of IL-4 signalling could transform the susceptible into a resistant phenotype. Although the results vary depending on the strain of *Leishmania* utilized, it is clear that the absence of IL-4 signalling was not always sufficient to inhibit Th2 development and confer resistance in Balb/c mice (Noben-Trauth 1999)(Noben-Trauth 2003)(Kropf 1999)(Mohrs 2000), suggesting that other factors can promote Th2 differentiation in the absence of IL-4.

Data from the early studies of the role of IL-4 in conventional models of murine

allergic airways inflammation generally tended to confirm the importance of IL-4 for the initiation of Th2-polarized responses. The description by Kips *et al* used anti-IL-4 blocking antibodies, and showed that when administered over the sensitization period, anti-IL-4 treatment prevented the subsequent development of eosinophilic airways inflammation after challenge, but that treatment only during the challenge period had no effect (1995); they concluded that IL-4 was necessary for the induction of Th2-polarized immune-inflammatory responses, but not for the effector phase of the response. Subsequently a number of studies affirmed these conclusions using IL-4 KO, IL-4R KO, and Stat6 KO mice (Hogan 1997)(Kips 1995)(Brusselle 1994)(Coyle 1995)(Kopf 1993)(Kuperman 1998). However, what was largely ignored in these studies was that although airway eosinophilia and other markers of Th2-polarized responses were markedly reduced (usually by 85% or more compared to the WT control), *they were not absent*, as one would predict if these responses depended wholly upon IL-4 for their initiation. A few later studies acknowledged the residual presence of Th2-associated phenomena in spite of the complete absence of IL-4 (Hogan 1998)(Herrick 2000).

Studies in other model systems also confirm that signalling via the IL-4 receptor is not required for Th2 responses. Using intracellular cytokine staining and flow cytometric analysis, Jankovic *et al* demonstrated that $CD4^+$ cells with a Th2 cytokine profile can indeed develop in Stat6 KO mice during helminth infection, albeit at a lower level than in WT controls (Jankovic 2001). Other examples of IL-4-independent Th2-polarized responses have been observed in other *in vivo* models as well (Brewer 1999)(Hogarth 1998)(Chensue 1997).

The numerous examples of IL-4-independent Th2 polarization inevitably raise the question of what other factor(s), aside from IL-4, can mediate Th2 differentiation. There has been some suggestion that Th2 differentiation does not require instructive signals at all, but rather arises as an intrinsic property of the cell in the absence of other factors (such as IL-12) which would otherwise drive Th1 responses, or the

downregulation of molecules (such as SHP-1) that would otherwise act as constitutive negative regulators of Th2 responses (Kamata 2003). Some suggest that this may be a genetically-determined property (Guery 1996)(Hsieh 1995), hence the ascribed tendency of Balb/c mice to produce Th2 responses. Others have suggested that the early events of Th polarization are a stochastic process (Kelso 1995), and that the role of exogenous factors is limited to the stabilization and consolidation of emerging populations of Th1 or Th2 cells (Coffman & Reiner 1999). Most pertinent to this thesis is evidence that adoptive transfer of dendritic cells of the 'myeloid' phenotype preferentially induce Th2 polarization *in vivo* (Pulendran 1999)(Lambrecht 2000)(Lambrecht 2001).

OBJECTIVES

The research described in this thesis was undertaken in an effort to define the molecular requirements for the polarization of Th responses during respiratory mucosal sensitization *in vivo*, and to describe and characterize notable hallmarks of these processes. In particular, we examined the impact of 4 cytokines on the development of respiratory mucosal sensitization to OVA: GM-CSF, IL-12, IL-4, and IL-10.

The first manuscript describes our investigation of Th1- and Th2-polarization in the lymph nodes during respiratory mucosal sensitization. Although the characteristics of Th1 and Th2 cells have been relatively well defined by others, these previous investigations have been carried out almost exclusively in *in vitro* systems after multiple rounds of stimulation, using either peptide-specific T cell clones or non-specific stimulation of T cells through the T cell receptor (Chtanova 2001). However, information gained through such an approach must be validated in more physiologically relevant systems before applying conclusions beyond the petri dish. Others have attempted such an investigation (Chiu 2002), however, this particular analysis is problematic because of the non-comparable nature of the 2 models used, making it difficult if not impossible to draw relevant comparisons between Th1 and Th2. In

addition, the analysis was carried out after *ex vivo* sorting of $CD4^+$ T cells, introducing the possibility of artefactual changes in gene expression generated during this procedure. An examination of gene expression in the lymph nodes during respiratory mucosal sensitization was carried out in our previously described Th1- and Th2-polarized models (Stampfli 1998a)(Stampfli 1999b). Our objectives were several-fold. First, we analysed whether the patterns of gene expression ascribed to Th1 and Th2 cells as generated *in vitro* held true in this *in vivo* system. Second, we established a time course of the observed changes in gene expression during the development of such polarized immune responses. Finally, we used this opportunity to understand the mechanism by which the local cytokine microenvironment in the lung affected the ensuing inflammatory response: directly through effects on T helper cell differentiation in the draining lymph nodes; or through some other mechanism, such as preferentially attracting and maintaining cells of particular phenotypes. To achieve these ends, we used real-time quantitative PCR (TaqMan™) to analyse the expression of a panel of purportedly Th1- and Th2-affiliated genes in the thoracic lymph nodes at various time points during the Th1- and Th2-polarized models of respiratory mucosal sensitization. Such an approach has been used by others previously to examine cellular and cytokine responses in mice in other models (Hempel 2002)(Stordeur 2002).

The second manuscript is an investigation of the requirement for IL-4 in Th2-polarization in our model of GM-CSF-driven respiratory mucosal sensitization (Ritz 2002b). The origins of this project were in two key observations made early during the development of these models. The first was that delivery of Ad/IL-4 alone intranasally prior to OVA exposure did not lead to antigen-specific immune memory, nor in responses that resembled allergic airway inflammation. The second was that co-infection with both Ad/GM-CSF and Ad/IL-4 intranasally prior to OVA exposure did not appreciably enhance the Th2-polarized response. Given the purportedly central role of IL-4 in the generation of Th2-polarized immune responses, we were surprised by

these findings. Since OVA exposure in the context of GM-CSF expression was sufficient for the generation of eosinophilic airway inflammation, and since IL-4 did not enhance this response, we set out to examine the necessity for IL-4 for the generation of Th2-polarized immune-inflammatory responses in this model. Our approach was to compare WT and IL-4 KO mice during GM-CSF-driven respiratory mucosal sensitization to OVA, and examine a variety of markers associated with Th2-polarized responses during this process. We utilized a number of different technologies in order to do this, including cytokine analysis by ELISA, flow cytometry, TaqMan™ , in addition to assessment of cellular inflammation in the BAL and peripheral blood.

The third manuscript in this thesis grew directly out of the observations we made in IL-4 KO mice in the GM/OVA model. Having shown that IL-4 was not necessary for Th2-polarized responses in this model, we sought to elaborate the mechanism through which IL-4-independent Th2 responses were generated. We hypothesized that since GM-CSF is the most potent factor for the development, proliferation, and activation of myeloid dendritic cells, and since myeloid dendritic cells have been shown by others to preferentially elicit Th2 responses (Pulendran 1999)(Lambrecht 2000) in an IL-4-independent IL-10-dependent fashion (Maldonado-Lopez 2001), that perhaps IL-10 was contributing to the IL-4-independent Th2 polarization we observed in this model of respiratory mucosal sensitization. We pursued this hypothesis by treating WT and IL-4 KO mice with anti-IL-10 antibodies during their exposure to OVA in the context of GM-CSF expression, and examining many of the same markers examined in the IL-4 KO study.

CHAPTER 2:
THE LUNG CYTOKINE MICROENVIRONMENT FUNDAMENTALLY ALTERS MOLECULAR EVENTS IN THE LYMPH NODES DURING TH1 AND TH2 RESPIRATORY MUCOSAL SENSITIZATION TO ANTIGEN IN VIVO

The following manuscript describes our investigation of molecular events taking place in the lymph nodes during Th1- and Th2-polarized respiratory mucosal sensitization. We used real-time quantitative PCR analysis to assess the expression of a variety of Th1- and Th2-associated genes in the thoracic lymph nodes at various time points after the initiation of respiratory exposure to OVA in the context of GM-CSF alone (Th2-polarized) or GM-CSF and IL-12 (Th1-polarized) in Balb/c mice.

From these studies, we concluded that: [*A*] the pulmonary cytokine microenvironment at the time of primary antigen exposure critically determines the immunological outcome of that exposure; [*B*] molecular events in the draining lymph nodes are profoundly affected by cytokines expressed at distant sites; and [*C*] that the pattern of gene expression *in vivo* during the development of Th1- and Th2-polarized immune-inflammatory responses is largely consistent with that predicted by *in vitro* experimentation.

The manuscript has been submitted to *Clinical & Experimental Immunology*, and is currently in the process of being submitted. I am responsible for the design, execution, and interpretation of the TaqMan experiments presented, as well as the writing of the manuscript. My co-authors aided in establishing TaqMan™ technology in our lab, conducting and analysing the flow cytometric data (BUG), contributing to the preliminary experimentation upon which the manuscript was based (the data from which does not appear in the manuscript), providing technical advice and reagents, and providing supervision and funding for the work (see the *Preface* for more details).

THE LUNG CYTOKINE MICROENVIRONMENT FUNDAMENTALLY ALTERS MOLECULAR EVENTS IN THE LYMPH NODES DURING TH1 AND TH2 RESPIRATORY MUCOSAL SENSITIZATION TO ANTIGEN IN VIVO

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ABSTRACT

Originally defined by their patterns of cytokine production, Th1 and Th2 cells have been more recently described to differentially express other genes as well, at least *in vitro*. In this study we compared the expression of allegedly Th1- and Th2-associated genes directly during *in vivo* sensitization to OVA in Th1- and Th2-polarized models of airways inflammation. Th1-polarized airway inflammation was achieved by the intranasal instillation of adenoviral vectors (Ad) encoding GM-CSF and IL-12, followed by daily aerosolizations of OVA; instillation of Ad/GM-CSF alone with OVA aerosolization led to Th2-polarized responses. Lymph nodes were obtained at various time points, RNA extracted, and analyzed by real-time quantitative PCR. Consistent with reports from *in vitro* and human studies, mice undergoing Th1-polarized inflammation showed preferential expression of the transcription factor t-bet, the chemokines IP-10 and MIP-1α, the chemokine receptor CCR5. In contrast, the transcription factor GATA-3, the chemokines I-309 and TARC, the chemokine receptors CCR3 and CCR4 were preferentially expressed in the Th2 model. Importantly, we also show that Ad/transgene expression remains compartmentalized to the lung after intranasal instillation. Flow cytometric analysis of lung myeloid dendritic cells indicated that B7.1 was more strongly expressed in the Th1 model than in the Th2 model. These studies provide a direct comparison of gene expression in *in vivo* Th1- and Th2-polarized models, and demonstrate that molecular events in the lymph nodes can be fundamentally altered by cytokine expression at distant mucosal sites.

INTRODUCTION

The molecular signals delivered to T helper cells during their primary activation determine their differentiation, effector, and directional activities, and profoundly affect the nature of the ensuing immune-inflammatory response. This has been convincingly demonstrated in *in vitro* systems, where Th cells stimulated in the presence of IL-12 and anti-IL-4 antibodies differentiate into Th1 cells (distinguished by their production of

IFNγ), while those stimulated with IL-4 and anti-IFNγ follow the Th2 differentiation pathway (distinguished by their production of IL-4, -5, and -13)[1,2]. This pattern of cytokine expression fundamentally affects the downstream nature of the immune-inflammatory response *in vivo*. Th1-polarized responses are associated with enhanced CTL killing, production of distinct immunoglobulin isotypes (IgG2a in mice), and mononuclear and neutrophilic inflammation. In contrast, Th2-polarized responses tend to result in the production of IgE and are typically characterized by eosinophilia in the blood and eosinophilic inflammation in the target organ.

Although Th1 and Th2 cells were originally defined by their profile of cytokine expression, analysis of such *in vitro* polarized Th1 and Th2 cells has revealed differential expression of a number of other molecules as well, including chemokines, chemokine receptors, and transcription factors [2-5]. However, whether the differential expression of these genes observed *in vitro* holds true during *in vivo* immune responses as well has not been carefully examined in systems where a *direct* comparison is possible. Although Chiu *et al* compared gene expression of chemokine receptors in $CD4^+$ T cells after *in vivo* exposure to Th1- or Th2-polarizing infections, the approach taken in that study does not allow us to be certain that the differential expression they observed after sorting and *in vitro* restimulation was actually present *in vivo* [6].

We have previously described that the transient transgenic expression of GM-CSF in mouse airways during exposure to aerosolized OVA leads to an inflammatory response reminiscent of asthma, which is characterized by the presence of Th2 cytokines in the BAL and eosinophils in the BAL and tissue [7]. When we concurrently expressed IL-12 with GM-CSF during OVA exposure, the airway inflammatory response was no longer eosinophilic, but rather dominated by mononuclear cells and neutrophils, along with abundant IFNγ in the BAL [8]. We used these models of *bona fide* Th1- and Th2-polarized airways inflammation to examine the expression of a variety of molecules allegedly affiliated with Th1 or Th2 responses *in vivo*, at the primary site of T cell activation, using real-time quantitative PCR (TaqMan™). Our data demonstrate that distinct patterns of gene expression are readily observed in the thoracic lymph

nodes during Th1- and Th2-polarized inflammatory responses. Moreover, we show that cytokine transgene expression compartmentalized in the airway is able to fundamentally alter molecular events in the thoracic lymph nodes, in spite of the absence of transgene expression in these lymph nodes. Flow cytometric analysis indicated that the divergence of the immune responses may be, in part, attributable to differing patterns of costimulatory molecule expression by lung dendritic cells.

MATERIALS & METHODS

Subjects. 6-8 week-old female Balb/c mice were obtained from Charles River (Ottawa, ON, Canada), and maintained under specific pathogen-free conditions, with a 12h light/dark cycle and food and water *ad libitum*. All experiments described herein were approved by the McMaster Animal Research Ethics Board.

Administration of adenoviral constructs. As previously described [7,8], airway expression of cytokines was achieved by the intranasal delivery of a replication-deficient human type 5 adenovirus with the cytokine gene inserted into the E1 region of the viral genome. The viral dose was administered intranasally to isoflurane-anaesthetized animals in 30 μl PBS on day -1. For Ad/GM-CSF, the dose was $3x10^7$ pfu; for Ad/IL-12, the dose was $1x10^7$ pfu. As a control, an empty replication-deficient adenovirus (RDA) was administered. In order to assess transgene expression, an Ad vector expressing a mouse-exogenous gene (OVA) was delivered at a dose of $3x10^7$ pfu.

Sensitization to OVA. 1 day after administration of Ad/GM-CSF alone or Ad/GM-CSF and Ad/IL-12, mice were sensitized to ovalbumin (OVA) by exposing them to a 1% OVA aerosol (1% wt/vol in 0.9% saline, Sigma-Aldrich, Oakville ON) for 20 minutes daily for 10 days. The aerosol was generated using compressed medical air at 7 l/min through a Bennet/Twin nebulizer, into a plexiglas chamber.

Preparation of cDNA samples. At various time points during OVA aerosolization mice were anaesthetized by isoflurane and killed by exsanguination. Thoracic lymph nodes and lung tissue were collected, pooled, and stored in RNAlater (Ambion Inc., Austin TX). Total RNA was extracted using TriPure (Roche, Indianapolis IN) using a Polytron Aggregate homogenizer (Kinematica, Luzern Switzerland). Genomic DNA was removed from these samples using the Qiagen RNeasy kit (Qiagen Inc., Mississauga ON). RNA was reverse transcribed to cDNA using the Qiagen OMNIscript kit (Qiagen) using random hexamers (Gibco, Rockville MD) and oligo-dT (Gibco) as primers.

Real-time quantitative PCR analysis. PCR primers and FAM-labelled probes for GATA-3, IP-10, I-309, t-bet, and OVA (table 1) were designed using the PrimerExpress v1.5 software package (Applied Biosystems, Foster City CA). Primer and FAM-labelled probe sets for IFNγ, IL-4, CCR3, CCR4, CCR5, and TARC were obtained as pre-developed assay reagents (PDARs) from Applied Biosystems. GAPDH primers and VIC-labelled probes were obtained from Applied Biosystems. PCR was carried out in the ABI Prism 6700 Sequence Detection System, operated by Sequence Detector v1.7 software (Applied Biosystems), using TaqMan Universal PCR Master Mix (Applied Biosystems) for all PCR reagents. 1 μg of cDNA was added to each well, and all measurements were done in triplicate wells. Gene expression was quantitated relative to the expression of the housekeeping gene GAPDH, and normalized to that measured in naive control mice (where applicable).

Flow Cytometric Analysis. At various timepoints during OVA exposure, mice were anaesthetized with isoflurane, killed by exsanguination, and lungs removed. Lung tissue was perfused by injecting 10 ml HBSS into the right atrium of the heart. After cutting into ~3 mm pieces, pooled lung tissue from several mice was incubated in collagenase III (Worthington Biochemical, Freehold NJ) in HBSS (Gibco BRL, Grand Island NY)(150 U/ml) for 1 hour, and then ground through a tissue screen into HBSS. This

cell suspension was layered atop a 30%/60% Percoll density gradient, and centrifuged at 2500 rpm for 25 minutes at room temperature. Cells at the 30%/60% interface were collected, washed twice and resuspended in PBS. 10^6 cells were stained for flow cytometry in polystyrene tubes. After blocking with Fc block (1 ug/10^6 cells)(PharMingen), cells were stained with FITC-conjugated anti-CD11c (1 ug/10^6 cells)(PharMingen), PE-conjugated anti-CD11b, and either biotin-conjugated anti-I-A^d, anti-B7.1 or anti-B7.2 (1 ug/10^6 cells)(PharMingen); streptavidin-PerCP (20 ul/10^6 cells)(Becton Dickinson, San Jose CA) was added to fluorescently label the biotin-conjugated antibodies. Cells were stored in 1% paraformaldehyde overnight, run on a FacSCAN flow cytometer (Becton Dickinson) running CellQuest acquisition software, and data analyzed using WinMDI (Scripps Institute, La Jolla CA).

Data analysis. Real-time quantitative PCR data are expressed as mean ± SD of triplicate wells; 1 representative experiment is shown. Statistical analysis was performed using SigmaStat v2.03. Differences were considered statistically significant when $p \leq 0.05$ by ANOVA.

RESULTS

Detection of Transgene Expression in the Lungs and Thoracic Lymph Nodes of Intranasally Infected Mice. In order to determine the tissue distribution of transgene expression after intranasal instillation of the Ad vectors, we administered an Ad vector expressing a completely exogenous sequence (OVA) to mice in a fashion identical to that by which the cytokine vectors are administered. Mice were sacrificed at various time points after intranasal Ad/OVA administration, and expression of the transgene detected in lung or lymph node tissue by TaqMan™ (Figure 1). As expected, transgene expression was completely undetectable in the lungs or lymph nodes of uninfected mice. Expression of the transgene was readily detected in the lung at the earliest time point examined (day 3) through to day 9, peaking on day 6 after infection. However, even after 40 cycles of

PCR amplification, absolutely no transgene expression was detectable in the lymph nodes at any time point after intranasal infection.

Transcription Factor Expression. The transcription factor t-bet is an important activator of the IFNγ gene [9-11], whereas GATA-3 is known to initiate IL-5 and IL-13 transcription [12-14]; thus, differential expression of these factors is likely to be important in the acquisition of the Th1 or Th2 phenotypes, respectively. We examined t-bet and GATA-3 expression in the lymph nodes during the Th1 and Th2 models. Figure 2A demonstrates that t-bet expression is significantly upregulated only in the Th1 model. In contrast, GATA-3 expression is upregulated only in the Th2 model on day 4 (figure 2B); although this upregulation is transient and modest in extent (~2-fold over naive), this observation has been made consistently between repeated TaqMan assays and between multiple experiments in this study, as well as in other studies [15].

Cytokine and Chemokine Expression in the Thoracic Lymph Nodes During Th1 and Th2 Polarization. Our previous work [7,8] demonstrates that mucosal exposure to GM-CSF/IL-12/OVA elicits a *bona fide* Th1 response, while GM-CSF/OVA elicits Th2 immunity. These conclusions were based on findings in the lung and spleen. Here, we have investigated cytokine expression in the thoracic lymph nodes during sensitization to OVA in these Th1 and Th2 models to determine whether alteration of the lung microenvironment fundamentally alters Th polarization events in the lymph nodes. Lymph node expression of IFNγ is dramatically increased in the Th1 model on days 4 and 7, returning to near-naive levels by day 11 (figure 3A). However, in the Th2 model IFNγ mRNA is never detected above that seen in naive animals. In contrast, IL-4 gene expression is significantly upregulated at all time points examined in the Th2 model, and modestly but significantly upregulated only on day 11 of the Th1 model (figure 3B).

We also examined the expression of supposedly Th1- and Th2-affiliated chemokines in the lymph nodes during sensitization to OVA *in vivo*. IP-10 was significantly upregulated in the Th1 model, peaking on day 4, but was not upregulated at all in the Th2 model (figure 4A). Expression of MIP-1α was enhanced in both models on day 4 to a similar degree, but remained heightened in the Th1 model on day

7 whereas it had returned to naive levels in the Th2 model (figure 4B). mRNA for I-309 was moderately but significantly increased in the Th2 model on day 4, but returned to naive levels by day 7, and was never upregulated in the Th1 model (figure 4C). TARC expression was substantially upregulated in the Th2 model on day 4 and day 7, returning to naive levels by day 11, whereas TARC was only detected above naive levels on day 4 of the Th1 model at a considerably lower level than that seen in the Th2 model (figure 4D).

Chemokine Receptor Expression. Different patterns of chemokine receptor expression have been associated with Th1- and Th2-polarized cells. Here we examined the expression of the Th1-affiliated CCR5 and the Th2-affiliated CCR3 and CCR4 in the lymph nodes during *in vivo* immune responses. As seen in figure 5A, CCR5 expression was upregulated only during the Th1 protocol, and not expressed above naive levels in the Th2 model. In contrast, mRNA for CCR3 and CCR4 were significantly upregulated only during the Th2 protocol and not during the Th1 model (figure 5B and 5C).

Flow Cytometric Analysis of Lung Dendritic Cells. Assessment of costimulatory molecule expression on lung dendritic cells was performed at various timepoints during the Th1 and Th2 models. B7.1 and B7.2 expression was analyzed on $CD11b^+$ $CD11c^+$ lung-derived mononuclear cells; 89.9% of these cells were positive for MHC class II expression, indicating that they were dendritic cells of the myeloid phenotype. In naive mice, 18.9% and 5.4% of $CD11b^+$ $CD11c^+$ lung mononuclear cells expressed B7.1 and B7.2, respectively (table 2). By day 4 of the Th1 model, 44.4% of $CD11b^+$ $CD11c^+$ lung mononuclear cells expressed B7.1, and this remained at a similar level on day 7 (39.8%), returning to baseline levels by day 11 (20.8%). Fewer $CD11b^+$ $CD11c^+$ lung mononuclear cells expressed B7.1 in the Th2 model, peaking at 29.1% on day 4, and returning to below naive levels on day 7 and 11. B7.2 expression was also upregulated in the Th1 model, peaking by day 4 (31.5%), and remaining relatively stable through day 7 (28.0%) and day 11 (29.4%). In the Th2 model, 21.9% of lung $CD11b^+$ $CD11c^+$ lung mononuclear cells expressed B7.2 by day 4, increasing to 32.7% on day 7, and remaining

high at day 11 (33.8%).

DISCUSSION

Although Th1 and Th2 cells were originally defined solely by their cytokine production profile [1], recent work has identified a variety of other genes that are differentially expressed between the two Th subtypes [2-5]. In the present series of experiments we have used models of Th1- and Th2-polarized airway inflammation to examine whether the expression of allegedly Th1- and Th2-associated genes can be observed *in vivo* in the thoracic lymph nodes during the time of T cell activation and differentiation. Use of these models in parallel gave us the opportunity to make *direct* comparisons between *equivalent* time points during *in vivo* Th1- and Th2-polarized sensitization to the *same antigen*, something that has not been possible using other approaches.

For this study, we elected to analyze mRNA derived from whole lymph nodes, rather than to use purified populations of cells; this approach has both apparent advantages and limitations. The first major advantage of this approach is that we do not introduce any potential for experimental artefact due to *ex vivo* stimulation of cells during any sorting procedure, nor during an *in vitro* culture step. Since the lymph nodes were removed and immediately placed in RNALater, with no other manipulation, we can be confident that TaqMan™ measurements precisely represented the status of gene expression *in vivo* at each time point. This cannot be said of any previously published literature that we are aware of comparing gene expression during Th1 and Th2 polarization, which have all used *ex vivo* sorting or *in vitro* culture steps. However, the concomitant limitation is that we are unable to ascribe observed changes in gene expression to a particular cell type; thus we have avoided describing our data in terms of Th1 or Th2 *cells* but rather speak about Th1- or Th2-*polarized responses*. The second advantage is, given the absence of reliable commercially-available antibodies against a number of these proteins, that real-time quantitative PCR is a very powerful, sensitive

and specific technique for measuring gene expression. The corollary is that by examining mRNA expression, we cannot be sure that the changes we observed are reflected by a change in protein expression or biological activity. However, we believe that it is reasonable to presume that changes in gene expression are at least to some degree reflected in protein expression; for example, the changes we observed in IFNγ and IL-4 mRNA expression (figure 3) correspond very well with our previously published ELISA data in BAL, as well as with divergent cellular responses in the lung [7,8].

Based on our previous work, we contend that airway expression of GM-CSF during exposure to aerosolized OVA results in a Th2-polarized immune response [7], whereas concurrent overexpression of IL-12 and GM-CSF during OVA exposure directs the response to the Th1 end of the spectrum [8]. TaqMan analysis of IL-4 and IFNγ gene expression in the thoracic lymph nodes (figure 3) further corroborates that these are *bona fide* models of Th1- and Th2-polarized immune responses. Whereas our previous work documented that these cytokines were differentially present in the BAL fluid, the data presented here indicate that the effector programme is acquired in the thoracic lymph nodes during the time of T cell activation and differentiation, confirming that the differences seen in the BAL were not simply attributable to differential recruitment of T cells, but rather reflect the emergence of distinct populations in the lymph nodes.

Transcription factors, cytokines, chemokines, and chemokine receptors were all differentially expressed *in vivo* in the lymph nodes during Th1- or Th2-polarized immunological sensitization to OVA, in a manner consistent with that previously described in *in vitro* studies of CD4+ cells [3]. Such a divergence in gene expression in the lymph nodes likely has important functional consequences for the subsequent development of immune-inflammatory responses in the target organ. To summarize, in the present study, Th1-polarized responses were associated with increased expression of t-bet, IFNγ, IP-10, MIP-1α, and CCR5, whereas GATA-3, IL-4, I-309, TARC, CCR3, and CCR4 were more prevalently expressed during Th2-polarized responses.

The transcription factors GATA-3 and t-bet have been described as "master switches" in the development of Th1 and Th2 cells, since they transactivate expression of key Th1 and Th2 genes, including the prototypic cytokines IFNγ (t-bet) and IL-5 and IL-13 (GATA-3). As reviewed in [11], expression of t-bet or GATA-3 not only determines Th differentiation, but can actually override the influence of exogenous polarizing stimuli or previous polarization. Hence, the differential expression of t-bet or GATA-3 we observed in the lymph nodes in Th1- and Th2-polarized responses likely represents a critical step in the development of these responses. If indeed differences in the levels of t-bet and GATA-3 are responsible for Th1 or Th2 lineage committment [10,13,16-20], these may be attractive therapeutic targets in Th1- or Th2-mediated immunopathological processes such as autoimmunity and allergy [9,21].

Disparate production of cytokines is the defining feature of Th subsets. The present analysis demonstrates differential mRNA expression of the prototypical Th1 and Th2 cytokines, IFNγ and IL-4, respectively, as expected. We have previously shown that protein expression is also different in these models, examining BAL fluid and splenocyte culture supernatants, and observing predominantly IFNγ in the Th1 model, and IL-4, IL-5 and IL-13 in the Th2 model [7,8]. These observations are not incidental, but rather reflect the primary role of Th cells in determining the nature of immune responses through their production of cytokines, which orchestrate adaptive immune responses through numerous pathways: regulating the development of leukocytes in the bone marrow; influencing the differentiation and effector function of other T cells and NK cells; signalling for isotype switching in B cells; and regulating the expression of other genes[22-26].

It is well established that cells of the myeloid lineage have different patterns of chemokine responsiveness due to disparate chemokine receptor expression; for example, neutrophils tend to express chemokine receptors which bind to the CXC family of ligands, whereas eosinophils express a pattern of receptors which bind CC chemokines. Likewise, chemokine receptors are differentially expressed on Th1 and Th2 cells [4,5]; here we show that this pattern holds true *in vivo* during Th1-and Th2-

polarized inflammation. Such biased expression of chemokines and chemokine receptors allows for the preferential attraction and retention particular Th subsets at sites of inflammation, thereby modifying the nature of the inflammatory process. This has been exemplified in studies showing that overexpression of Th1-associated chemokines can alter the inflammatory response in an otherwise Th2-driving milieu [27], or where chemokine or chemokine receptor knockout animals generate aberrant inflammatory processes in the target organ [28-34].

These experiments also demonstrate that molecular events taking place in the lymph nodes can be substantially affected by cytokine expression occuring at distant sites. As shown in figure 1, intranasal instillation of an adenoviral vector encoding a completely exogenous protein resulted in robust expression of the transgene in lung tissue, but there was no detectable transgene present in the draining thoracic lymph nodes, even after 40 cycles of PCR amplification. This suggests that the impact of GM-CSF and IL-12 overexpression in the airways on the molecular events taking place in the thoracic lymph nodes is not due to the expression of these cytokines locally in the lymph nodes themselves. Table 2 shows that the expression of these cytokines in the airway mucosa conditions the antigen presenting cells (APC) residing there, associated with different patterns of expression of the costimulatory molecules B7.1 and B7.2; we do not presume that the observed differences in B7 expression are solely responsible for the divergent immune responses that emerge, but are rather a 'proof-of-principle' that the local cytokine milieu can substantially alter the phenotype of antigen presenting cells. Upon migration to the draining lymph nodes, we speculate that these differently conditioned APCs can then present antigen to naive T cells along with an appropriate complement of signals (including B7 molecules, other costimulatory molecules, and cytokines) which elicit Th1 or Th2 differentiation [35-37]. Our data indicate that in the absence of other modulating factors, GM-CSF alone conditions airway APCs to present antigen in the lymph nodes in a Th2-privileging fashion; if IL-12 is additionally present in the lung, these APCs promote Th1 differentiation instead. Our analysis of molecular events in the lymph nodes clearly demonstrates that the impact of GM-CSF and/or IL-

12 expression in the airway microenvironment is not merely the result of local effects on leukocyte recruitment to the airways, but rather that it fundamentally alters the very character of the adaptive immune response as it develops in the thoracic lymph nodes.

This finding has important implications for our understanding of the initiation of airways disease and approaches to its treatment. Even if cytokine expression is wholly localized within the lung, this can still have a profound impact on immunodifferentiation events taking place at a distant site, such as the lymph nodes. Therefore, exposure to agents which stimulate airway epithelial cells or alveolar macrophages to produce cytokines could facilitate immunological sensitization, through the effects on lung APCs, as we have proposed previously [38]. For example, agents which can induce GM-CSF in the airway, such as various allergens [39,40] and pollutants [41,42], could thereby facilitate Th2-polarized allergic sensitization. Additionally, these data suggest that we may be able to target immunotherapeutic strategies locally to the target organ, and thereby affect not only local events in that organ, but fundamentally affect the systemic response to an antigen without administering the therapy systemically.

To conclude, our study demonstrates that gene expression in the thoracic lymph nodes during immunologic sensitization is fundamentally different in *in vivo* Th1- and Th2-polarized models of airway inflammation. Using these models, we were able to *directly* compare gene expression between Th1 and Th2 models *in vivo* during the primary immune response. Our data support previous findings showing that IP-10, MIP-1α, CCR5, and t-bet, in addition to IFNγ, are preferentially produced during Th1-skewed immune responses, whereas Th2-polarized immune responses are characterized by the expression of I-309, TARC, CCR3, CCR4, and GATA-3, in addition to IL-4 and other Th2 cytokines. Importantly, such divergent profiles of gene expression in the lymph nodes were driven by cytokine expression at a distant site, without evidence of local transgene expression within the lymph nodes themselves. These data enhance our understanding of molecular events during Th1- and Th2-polarization *in vivo*, and suggest that cytokine expression distant from the sites of T cell activation can have a major

impact on the nature of the immune responses that subsequently develop.

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GLOSSARY

Ad	**ad**enoviral vector
BAL	**b**ronchoa**l**veolar **l**avage
MIP-1α	**m**acrophage **i**nflammatory **p**rotein 1 alpha
TARC	**t**hymus and **a**ctivation **r**egulated **c**hemokine
IP-10	**I**FNγ-inducible **p**rotein 10

Figure 1: Expression of a mouse-exogenous transgene in lung and lymph node was measured by real-time quantitative PCR (TaqMan™) at various time points after intranasal administration of $3x10^7$ pfu of an adenoviral vector carrying the transgene. Transgene expression was quantitated relative to the presence of a housekeeping gene, GAPDH. Black circles (●) show transgene expression in the lung, while white squares (□) indicate transgene expression in the lymph nodes. Points are mean ± SEM of individual mice, n=4-5. * indicates a significant difference ($p < 0.05$) from naive mice by ANOVA.

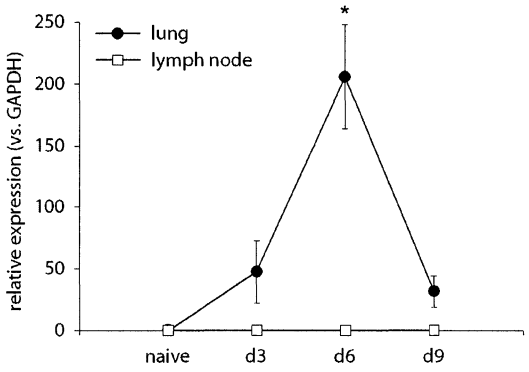

Figure 2: Expression of the transcription factors t-bet and GATA-3 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time points during respiratory sensitization to OVA. Cytokine expression was quantitated relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from 8-10 mice for analysis. Bars are mean ± SD of triplicate measurements. * indicates a significant difference ($p < 0.05$) from naive mice; † indicates a significant difference ($p < 0.05$) from the Th1 model at the same time point.

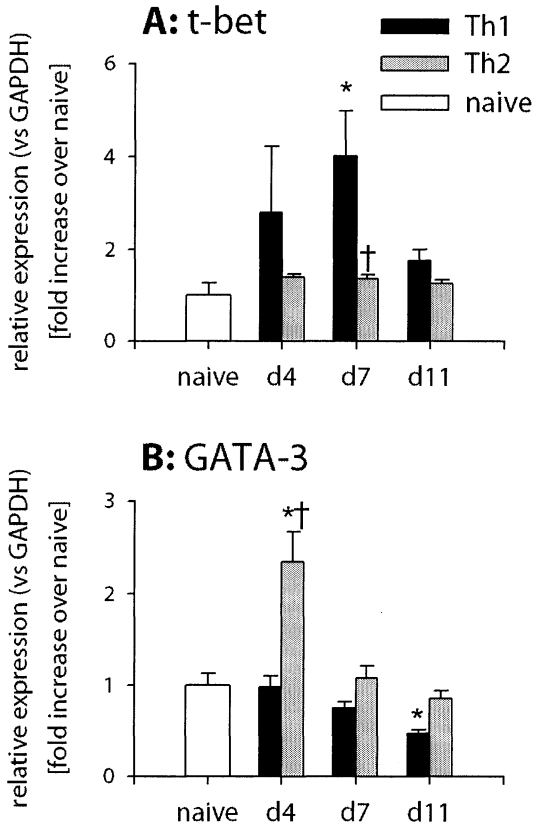

Figure 3: Expression of the prototypical Th1 and Th2 cytokines IFNγ and IL-4 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time points during respiratory sensitization to OVA. Cytokine expression was quantitated relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from 8-10 mice for analysis. Bars are mean ± SD of triplicate measurements. * indicates a significant difference ($p < 0.05$) from naive mice; † indicates a significant difference ($p < 0.05$) from the Th1 model at the same time point.

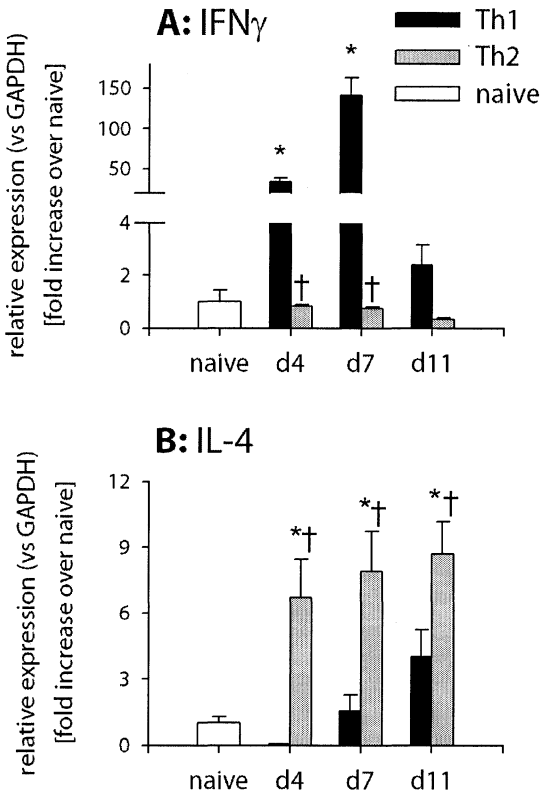

Figure 4: Expression of the chemokines IP-10, MIP-1α, I-309, and TARC were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time points during respiratory sensitization to OVA. Chemokine expression was quantitated relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from 8-10 mice for analysis. Bars are mean ± SD of triplicate measurements. * indicates a significant difference ($p < 0.05$) from naive mice; † indicates a significant difference ($p < 0.05$) from the Th1 model at the same time point.

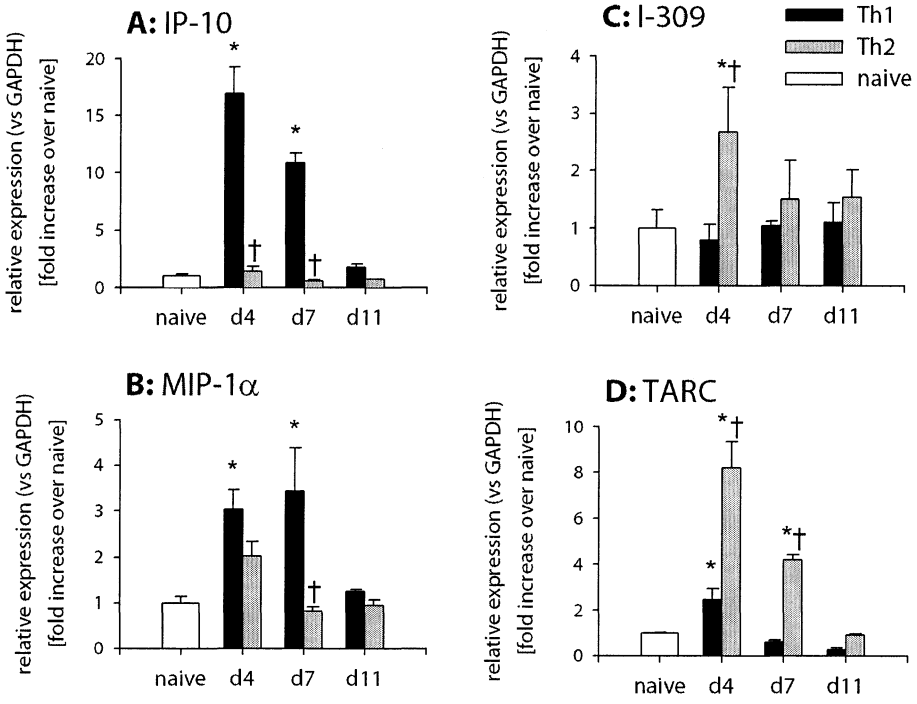

Figure 5: Expression of the chemokine receptors CCR5, CCR3, and CCR4 were measured by real-time quantitative PCR (TaqMan™) in the lymph nodes at various time points during respiratory sensitization to OVA. Chemokine receptor expression was quantitated relative to the presence of GAPDH, and normalized in relation to levels seen in naive mice. White bars indicate naive mice; black bars show expression in mice exposed to OVA in the presence of GM-CSF and IL-12 (Th1 model); grey bars show expression in mice exposed to OVA in the presence of GM-CSF alone (Th2 model). Lymph nodes were pooled from 8-10 mice for analysis. Bars are mean ± SD of triplicate measurements. * indicates a significant difference ($p < 0.05$) from naive mice; † indicates a significant difference ($p < 0.05$) from the Th1 model at the same time point.

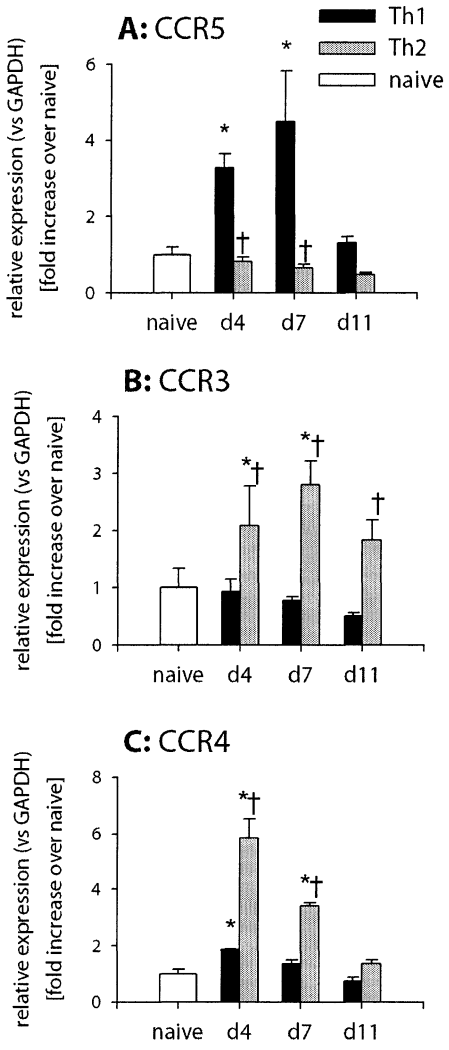

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Table 1: Sequences of Custom Primers & Probes for Real-Time Quantitative PCR

gene	forward primer	reverse primer	probe
GATA-3	CTA CCG GGT TCG GAT GTA AGT C	GTT CAC ACA CTC CCT GCC TTC T	AGG CCC AAG GCA CGA TCC AGC
I-309	ACA AAA CGT GGG TTC AAA ATC A	GGG AAG GTG GCT CAT CTT CA	CTG AAG AAG GTG AAC CCC TGC TAA CCG
IP-10	GGA TGG CTG TCC TAG CTC TGT AC	TGG GCA TGG CAC ATG GT	AGG GCGT TCG CAC CTC CAC ATA GCT
t-bet	ACC AGA ACG CAG AGA TCA CTC A	CAA AGT TCT CCC GGA ATC CTT	CTG AAA ATC GAC AAC AAC CCC TTT GCC
OVA	CCA TGC AGC ACA TGC AGA A	GGA ATG GAT GGT CG CCC TAA	AGA GAC GCT TGC AGC ATC CAC TCC A

Table 2: Flow Cytometric Analysis of Costimulatory Molecule Expression

	costimulatory molecule expression on lung-derived $CD11b^+$ $CD11c^+$ cells (%)					
	$B7.1^+$			$B7.2^+$		
naive	18.9			5.4		
	d4	d7	d11	d4	d7	d11
Th1 (GM-CSF/IL-12/OVA)	44.4	39.8	20.8	31.5	28.0	29.4
Th2 (GM-CSF/OVA)	29.1	11.4	10.9	21.9	32.7	33.8

Mice were sensitized to OVA by daily aerosol exposure in the context of airway expression of GM-CSF and IL-12 (Th1) or GM-CSF alone (Th2). Lungs were pooled from 5-10 mice for each time point, and mononuclear cells were isolated, stained and analyzed by flow cytometry at the indicated time points. Data shown is from one representative experiment.

CHAPTER 3:
GRANULOCYTE/MACROPHAGE COLONY-STIMULATING FACTOR-DRIVEN RESPIRATORY MUCOSAL SENSITIZATION INDUCES TH2 DIFFERENTIATION AND FUNCTION INDEPENDENTLY OF INTERLEUKIN-4.

The following manuscript describes our investigation of the role of IL-4 in the development of Th2 responses during respiratory mucosal sensitization to OVA. During primary aerosol exposure to OVA in the context of respiratory GM-CSF expression, immune-inflammatory responses of wild-type or IL-4 knock-out Balb/c mice were examined for evidence of Th2 polarization.

From these studies, we concluded that GM-CSF-driven respiratory mucosal sensitization: [*A*] does not require IL-4 for the expression of Th2-associated genes in the thoracic lymph nodes; [*B*] does not depend on IL-4 for the generation of eosinophilic inflammatory responses and goblet cell hyperplasia in the airways; and [*C*] does not rely on IL-13 for IL-4-independent Th2 responses.

This paper appeared in the *American Journal of Respiratory Cell & Molecular Biology*, 27:428 October 2002. I am responsible for the design and interpretation of all of the experiments presented, as well as the execution of the majority of them, and for writing the manuscript. My co-authors contributed by assisting with the establishment of TaqMan™ analysis, carrying out a key experiment during my comprehensive examinations, supplying technical advice, reagents, and mice, and providing supervisory and funding support (see the *Preface* for more details).

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GM-CSF-DRIVEN RESPIRATORY MUCOSAL SENSITIZATION INDUCES TH2 DIFFERENTIATION AND FUNCTION INDEPENDENTLY OF IL-4

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Running Title: GM-CSF-Driven IL-4-Independent Th2 Differentiation

ABSTRACT

The development of Th2 responses is a key step in the pathogenesis of asthma. IL-4 is thought to be important, although not strictly necessary, for Th2 differentiation, although triggers of IL-4-independent Th2 polarization have not been identified. We examined whether IL-4 is necessary for Th2-polarized responses during GM-CSF-driven respiratory mucosal sensitization. Balb/c wild type (WT) or IL-4 knockout (4KO) mice were exposed to aerosolized ovalbumin (OVA) in the context of airway GM-CSF expression. We examined the extent of Th2 polarization using real-time quantitative PCR on lymph node mRNA, flow cytometric analysis of lung T helper cells, and measurement of cells, cytokines and immunoglobulins in bronchoalveolar lavage (BAL) and serum. GATA-3, CCR3, -4, and -8 were expressed in the lymph nodes of WT and 4KO mice at similar levels, as were IL-5 and IL-13 levels in the BAL, T1/ST2 on lung T helper cells, and BAL eosinophils after recall challenge. With the exception of immunoglobulin production, expression of GATA-3, CCR-3, -4, -8, IL-5, T1/ST2 and the generation of blood eosinophilia were intact in mice doubly deficient in both IL-4 and IL-13. We conclude that IL-4 is not required for the generation of Th2-polarized responses in the presence of GM-CSF.

Abstract word count: 198

KEYWORDS: asthma, GM-CSF, Th2, IL-4 knockout mice, GATA-3, CCRs, eosinophils, cytokines

INTRODUCTION

In spite of their innocuous character, aeroallergens induce asthma in a growing proportion of the population[1]. Asthma is a syndrome distinguished by a distinct cytokine and immunoglobulin profile, eosinophilic airway inflammation, and bronchial hyperreactivity. Studies in murine models of asthma have established a critical role for T helper (Th) cells[2,3], and it is well established that IL-4, IL-5 and IL-13 are the preeminent effector molecules [4-10]. Insofar as Th2 cells are the primary producers of these cytokines, the differentiation of precursor Th cells into Th2 cells is of paramount importance to the generation of asthmatic inflammation.

It is frequently asserted that IL-4 is necessary for the development of Th2-polarized responses, in spite of evidence that these responses can sometimes be generated in the absence of IL-4. In murine models of allergic inflammation and infectious disease models, some studies show that Th2 responses are apparently hampered in the absence of IL-4 signaling [11-22], while others demonstrate that they are not impaired [17,23-27]. However, none of these latter studies have established a mechanism by which Th2 responses are elicited in the absence of IL-4.

Until recently Th2 responses were defined entirely by the cytokine profile observed, and previous documentation of Th2 responses in the absence of IL-4 have relied almost exclusively on these cytokine readouts. However, more recently a number of non-cytokine markers of Th2 responses have been described, including the transcription factor GATA-3, the chemokine receptors CCR3, -4, and -8, and the cell surface protein T1\ST2. We have used these markers to more comprehensively assess the presence of Th2 responses in mice in which the prototypical Th2 cytokine, IL-4, is absent.

Murine models have been very fruitful for investigating mechanisms of human allergic inflammation, but not sensitization, because sensitization in these

models is typically accomplished by the introduction of antigen into the intraperitoneal cavity in conjunction with alum as an adjuvant, which is very dissimilar to the conditions under which allergic sensitization occurs in humans. In contrast, this study is unique in that we examined the differentiation and activity of Th2 cells *in vivo* in a model of allergic airways inflammation in which mice are exposed to an innocuous antigen, exclusively via the respiratory tract, and in the absence of adjuvants. Such a system recapitulates the route of allergen sensitization in humans. GM-CSF is employed because exposure to antigen only has been shown to induce inhalation tolerance[28,29], and we have previously shown that expression of GM-CSF in the airways during antigen exposure overrides the tendency to elicit tolerance, and promotes the generation of an immune-inflammatory response reminiscent of asthma[30].

In order to examine the role of IL-4 in the generation of Th2-polarized responses via the respiratory tract, IL-4 knockout (4KO) mice were compared to their wild type (WT) controls. In addition, IL-4 and IL-13 double-knock-out (DKO) mice were used to investigate the possibility that IL-13 could drive Th2 responses in the absence of IL-4[31]. We found that indices of Th2 differentiation and function were intact in 4KO and DKO mice. These studies suggest a novel role for GM-CSF in the generation of Th2 responses in the mucosae, independently of IL-4 and IL-13.

MATERIALS & METHODS

Subjects. Female Balb/c mice (6-8 wk old) were obtained from Charles River Laboratories (Ottawa, ON). Female 4KO mice on a Balb/c background were obtained from Jackson Laboratories (Bar Harbor, ME). IL-4 and IL-13 DKO mice on the Balb/c background[31] were bred in the McMaster facility. Mice were kept under specific pathogen-free conditions, with a 12 h light/dark cycle, and food and water *ad libitum*. These experiments were approved by the Animal Research Ethics Board of McMaster University.

Respiratory mucosal sensitization and challenge. Airway expression of GM-CSF was achieved by delivery of a replication-deficient human type 5 adenovirus (Ad) with the gene for GM-CSF in the E1 region of the genome[30]. $3x10^7$ pfu of Ad/GM-CSF in 30 μl PBS were delivered intranasally to isoflurane-anaesthetized animals, 1 d prior to OVA exposure (d -1). We have previously demonstrated that this dose of the Ad\GM-CSF vector results in airway expression of GM-CSF for 10 days, with BAL expression peaking at ~80-100 pg\ml on day 7[30]; this level of expression is well within physiologically achievable concentrations.

For sensitization, mice were exposed to OVA for 20 min daily from d 0 to d 9. For rechallenge, OVA was delivered for 1 h daily for 3 d. Mice were placed in a Plexiglas chamber, and OVA aerosol (1% wt/vol in 0.9% saline, Grade V, Sigma-Aldrich, Oakville, ON) delivered using compressed medical air at 10 l/min through a Bennet/Twin nebulizer. We have previously reported an extensive characterization of this model[30] in which we observed that neither exposure to OVA only, nor exposure to OVA in the presence of an empty Ad vector, results in immunologic sensitization to OVA. Similarly, 4KO mice exposed to OVA in the context of a control adenoviral vector did not exhibit any indication of antigen-specific immune-inflammatory responses to OVA; total BAL inflammation in these mice was

comparable to that seen in naïve 4KO controls (total cells 6.8±0.7 vs 4.3±0.8 x10^5 cells/ml, respectively), and no eosinophilia was observed. *Isolation of mRNA and analysis of mRNA expression by real-time quantitative PCR.* Thoracic lymph nodes were pooled from 4-6 mice and placed in RNAlater (Ambion Inc., Austin, TX), and RNA extracted with TriPure (Roche, Indianapolis, IN). Genomic DNA was removed using the Qiagen RNeasy kit (Qiagen Inc., Mississauga, ON). RNA was reverse transcribed using the Qiagen OMNIscript kit (Qiagen) using random hexamers (Gibco, Rockville, MD) and oligo-dT (Gibco) as primers. Primers and FAM-labeled probes were designed with PrimerExpress v1.5 software (Applied Biosystems, Foster City, CA). For GATA-3, the forward primer was 5'-CTACCGGGTTCGGATGTAAGTC; the reverse was 5'-GTTCA CACACTCCCTGCCTTCT; and the probe was 5'-AGGCCCAAGGCACGATC CAGC. For t-bet the forward primer was 5'-ACCAGAACGGACAGATCACTCA; the reverse was 5'-CAAAGTTCTCCCGGAATCCTT; and the probe was 5'-CTGAAAATCGACAACAACCCTTTGCC. CCR3, -4, and -8, primers and FAM-labeled probes, and GAPDH primers and VIC-labeled probe were obtained from Applied Biosystems. PCR was done in the ABI Prism 6700 Sequence Detection operated by Sequence Detector v1.7 software (Applied Biosystems), using TaqMan Universal PCR Master Mix (Applied Biosystems). Gene expression was quantitated relative to the expression of GAPDH. The value of the relative expression for the WT naïve samples are defined as 100%, and all other values are plotted compared to them.

Collection and measurement of specimens. Mice were anaesthetized by isoflurane and killed for sample collection. Peripheral blood was collected, total cell counts determined using a haemocytometer, and leukocyte smears prepared. Blood samples were incubated at 37°C for 30 min, and the serum supernatant stored at -20°C.

Lungs were dissected and BAL performed. 2 aliquots of PBS (250 μl and 200 μl) were delivered and recovered through a polyethylene tube (Becton Dickinson, Sparks MD) inserted in the trachea; 300 ul are consistently recovered. Total cell counts were determined with a haemocytometer. The BAL was centrifuged, and the supernatant stored at -20°C for cytokine analysis. The cell pellet was resuspended in PBS and smears were prepared by cytocentrifugation (Shandon Inc, Pittsburgh, PA) at 300 rpm for 2 min.

Peripheral blood and BAL smears were stained with the Protocol Hema 3 Stain Set (Fisher Scientific, Toronto, ON). Differential cell counts were made from 300 leukocytes.

Lung tissue was fixed in 10% formalin and embedded in paraffin. 3 μm-thick sections were stained with haematoxylin and eosin (H&E), or with periodic acid-Schiff (PAS).

Flow cytometric analysis of lung cells. Lungs were perfused with 10 ml warmed HBSS, cut into 2mm pieces, and agitated for 1 h at 37°C in 150 U/ml collagenase III (Life Technologies, Rockville, MD). Pieces were ground through a metal screen into HBSS, filtered through fine-gauge nylon mesh, washed and resuspended in HBSS, and layered over 30% and 60% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) for density gradient centrifugation at 2000 rpm (25 min at room temperature (rt)). Cells at the 30/60% interface were collected, and washed with PBS. 10^6 cells in 50 μl PBS were incubated with 1 μg Fc block (anti-CD16/CD32, PharMingen, Mississauga, ON) on ice for 15 min, and then stained with fluorescently-conjugated Ab: anti-CD3ε-PE (clone 2C11, PharMingen)(2 μg/10^6 cells); anti-CD4-cychrome C (clone L3T4, PharMingen)(0.05 μg/10^6 cells); anti-T1/ST2-FITC (clone 3E10)(1.25 μg/10^6 cells) or its isotype control. Flow cytometry was performed on a FACScan (Becton Dickinson, Sunnyvale, CA) and analyzed

using WinMDI version 2.8 (Scripps Research Institute, La Jolla, CA).

ELISA measurement of cytokines and immunoglobulins. Cytokine levels in BAL were measured by ELISA using commercially available kits for IL-13, eotaxin, MCP-1, and IFNγ (R&D, Minneapolis, MN), and IL-5 (Amersham, Buckinghamshire, UK). The level of sensitivity for these assays was ~5 pg/ml.

Sandwich ELISA was used to measure OVA-specific IgG1 and IgG2a in serum. 100 μl/well of 5 mg/ml OVA in a borate buffer was applied to 96-well plates and incubated for 1 h at 37°C, 3 h at rt, and overnight at 4°C. Plates were blocked with 1% BSA (Sigma) in PBS (150 μl/well) for 2 h at rt. 50 ul of each sample was added and left overnight at 4°C. After washing, 50 μl/well of 0.25 mg/ml biotinylated anti-mouse IgG1 or IgG2a Ab (Southern Biotechnology Associates, Birmingham, AL) was added. After 2 h at rt, plates were washed, and alkaline phosphate/streptavidin was added for 1 h at rt (diluted to 1:1000, 50 μl/well). *p*-nitrophenyl phosphate in diethanolamine buffer was used for colour development. OVA-specific IgE levels were determined using an antigen-capture ELISA method, which has been previously described[30]. Briefly, anti-mouse IgE Ab was in the solid phase, serum samples were added, and OVA-specific IgE detected using biotinylated OVA. Units of OVA-specific Ig were determined relative to standard sera. Units of the standard serum are defined as the largest dilution factor giving an OD reading greater than background plus 2 SD; therefore, by definition, the sensitivity of the assay is 1 U/ml.

Data analysis. Data are expressed as mean ± SEM. Statistical analysis was performed using SigmaStat version 2.03. Differences were considered statistically significant when $p \leq 0.05$ by ANOVA, with Fisher's PLSD where applicable.

RESULTS

Analysis of transcription factor and chemokine receptor mRNA expression. We used real-time quantitative PCR to measure expression of mRNA for Th2-associated transcription factors and chemokine receptors in the thoracic lymph nodes at various time points during sensitization.

Compared to naïve, WT mice had increased expression of the Th2-associated transcription factor GATA-3 [32,33] by d4 of the sensitization phase, returning to near naïve levels by d7 (figure 1A). The kinetic and magnitude of GATA-3 expression in 4KO mice was similar to that observed in the WT. Although the fold-increases in GATA-3 expression are not dramatic, they were consistently reproducible in repeated experiments. Neither WT nor 4KO mice significantly upregulated the Th1-associated transcription factor t-bet [34] at any time point (figure 1B).

The Th2-associated chemokine receptors CCR3, -4, and -8 [32,35] were also examined. In WT mice, CCR3 expression was increased on d4, and peaked on d7 (figure 2A); although basal expression of CCR3 was lower in 4KO mice, expression was equivalent to that seen in WT mice on d4 and d7. CCR4 expression peaked on d4 in WT mice (figure 2B), and was identical to the expression seen in 4KO mice. Expression of CCR8 also peaked on d4 in WT mice (figure 2C); the pattern of expression was the same in 4KO mice, although CCR8 expression was higher in 4KO mice at all time points examined.

Measurement of cytokines and chemokines in the bronchoalveolar lavage. BAL cytokine and chemokine levels were measured on d7 of the sensitization protocol, as we previously determined that this is the peak of cytokine expression[30].

BAL from naïve WT and 4KO mice had no detectable levels of IL-5 or IL-13 as measured by ELISA (data not shown). On d7, both WT and 4KO mice had upregulated IL-5 protein expression to the same extent (figure 3A). On d7, IL-13

expression was lower in 4KO mice than in WT mice, although this difference did not reach statistical significance; furthermore, this amount, while reduced compared to WT, was still significantly greater than that seen in naïve mice. IFNγ was not detected in the BAL of WT or 4KO mice at any time point examined (data not shown); similarly, real-time quantitative PCR analysis of lymph node RNA did not demonstrate any upregulation of mRNA for IFNγ above naive levels in WT or 4KO mice at any time point examined (data not shown).

No eotaxin was detectable in the BAL of naïve mice of either strain and MCP-1 was not detectable in naïve WT mice (data not shown), but was detectable at low levels in 4KO mice (12.9 ± 2.6 pg/ml). Eotaxin (figure 3C) and MCP-1 (figure 3D) levels in the BAL on d7 were significantly increased above naïve levels, and were not significantly different between WT and 4KO mice.

T1/ST2 expression on lung T helper cells. On d9, T1/ST2 expression was analyzed on lung Th cells by flow cytometry to identify effector Th2 cells[36-38]. Th cells were defined as $CD3^+$ $CD4^+$ cells in the lymphocyte gate (as defined by forward and side scatter characteristics). ~2% of Th cells from the lungs of naïve WT and 4KO mice were $T1/ST2^+$ (data not shown). On d9, 10.7% of lung Th cells were $T1/ST2^+$ in WT mice, compared to 10.4% in 4KO mice (table 1). These data were reproducible in 3 separate experiments.

Inflammation and eosinophilia. As a hallmark of allergic asthma, we assessed eosinophils in blood and BAL during sensitization, in BAL after rechallenge, and in histological sections.

WT and 4KO mice had similar numbers of total blood leukocytes on d11 (figure 4A). 4KO mice had a slight reduction in peripheral blood eosinophilia compared to WT mice (4B), but this was not statistically significant.

Both WT and 4KO naïve mice had similar total cell numbers in the BAL (6.9 ± 0.6 vs 4.3 ± 0.8 x10^5 cells/ml), and no eosinophils. On d11, WT and 4KO mice had statistically significant increases in total BAL leukocytes (4C) and eosinophils (4D) compared to naïve, although 4KO mice had 40% fewer total cells and 65% fewer eosinophils than WT. Histological sections of lung tissue at this time point revealed peribronchial and perivascular inflammatory leukocytes in both strains, and eosinophils were apparent among the infiltrating cells in H&E-stained sections (5A & 5B). PAS stain revealed mucous-containing goblet cells in the bronchial lining of both WT and 4KO mice (5C & 5D).

In some experiments, the inflammation caused by sensitization was allowed to resolve for 1 month, and the mice were rechallenged with OVA, in order to examine antigen-specific memory responses. Inflammation in the BAL was assessed at the peak of the inflammatory response, 72h after the third OVA rechallenge. In WT mice the eosinophilic inflammation was recapitulated (4E & 4F). In contrast with the observations at d11, after long term rechallenge 4KO mice had eosinophilic inflammatory responses indistinguishable from that seen in WT mice.

Immunoglobulin profile. After sensitization, OVA-specific Ig were measured in the serum of WT and 4KO mice by ELISA. As expected, no OVA-specific Ig were detected in naïve WT or 4KO mice (data not shown). OVA-specific IgE, IgG1, and IgG2a were all present in WT mice after sensitization to OVA (table 1). In 4KO mice, no OVA-specific IgE was detected and IgG1 was significantly decreased, whereas IgG2a levels were significantly increased.

Evidence of Th2-polarized responses in DKO mice. We used DKO mice to determine whether IL-13 can substitute for IL-4 in the generation of Th2-polarized responses. None of GATA-3 (6A), CCR-3 -4 or -8 (6B), IL-5 (6C), or peripheral blood eosinophilia (6D) were significantly different between WT, 4KO, and DKO

mice. Furthermore, T1/ST2 expression on lung Th cells from DKO mice was, at 9.2%, upregulated to an extent comparable to that seen in WT and 4KO mice (table 1). In contrast, BAL eosinophilia (6E) was completely absent in DKO mice at d11. Whereas eotaxin expression was expressed to a comparable degree in the BAL of WT and 4KO mice, it was reduced by ~85% in DKO mice (6F).

DISCUSSION

Molecular signals delivered to a Th cell during primary activation critically influence its differentiation pathway, and ultimately determine the nature of the immune-inflammatory response that will ensue. The primary objective of this study was to investigate the role of IL-4 in the development of Th2 polarized responses during respiratory exposure to aerosolized OVA in the context of a GM-CSF-enriched airway microenvironment. This model mimics the natural route of exposure to antigen while providing a signal (GM-CSF) which overrides the tendency of the lung to induce inhalation tolerance when exposed to harmless antigens[28,29].

The Th2-associated transcription factor GATA-3 and Th2-affiliated CCRs were analyzed to evaluate whether Th2 differentiation had taken place. Our data show that GATA-3 is upregulated in the lymph nodes of WT mice; importantly, our data also show that GATA-3 is upregulated in 4KO mice. Furthermore, analysis of chemokine receptors associated with Th2 cells demonstrated the upregulation of CCR3, -4, and -8 in both WT and 4KO mice. These findings have several implications. First, insofar as these molecules are markers of Th2 cells, these data confirm that Th2 cells are present. Second, the upregulation of GATA-3, CCR3, -4,

and -8 in 4KO mice indicates that their expression can occur independently of IL-4 *in vivo*.

Expression of the prototypical Th2 cytokines in the BAL was analyzed to examine whether these Th2 cells were functional. Previous studies using 4KO mice have generally shown a marked decrease in IL-5 and/or IL-13 production in *in vitro* recall assays [11,13,16,19,20-22,24-25]. However, the findings reported here are consistent with reports[17,23,27] in which these cytokines are present at equivalent levels in WT and 4KO mice. We did not directly measure the T cell contribution to cytokine levels, and hence cannot rule out the possibility that cells other than T cells may have supplemented the overall cytokine levels; however, given that GATA-3 was equivalently expressed in WT and 4KO mice, and since GATA-3 expression is sufficient for the transcription of these cytokines[39], we infer that Th2 cells are most likely responsible for the cytokine levels measured in the BAL.

With respect to eosinophilia, a number of groups have reported a drastic reduction in BAL eosinophils when IL-4 was blocked or absent during allergen sensitization[11,13-16], while others have not demonstrated such a change[16]. Our data show that the ability of 4KO mice to generate peripheral blood eosinophilia, and BAL eosinophilia after recall challenge, is intact, consistent with the fact that IL-5 production is intact in 4KO mice. The extent of the BAL eosinophilic response in 4KO mice observed on d11 was considerably greater than that observed by some others[11,14-16], but still significantly less than in WT controls; this transient inhibition of eosinophilia remains unexplained. However, since this reduction occurred while the adenoviral vector was present, we suspect that this was due to an unknown process induced in response to the virus. This suspicion was confirmed when we used recombinant GM-CSF to induce sensitization instead of the Ad/GM-CSF; in this case, the number of eosinophils was statistically indistinguishable between WT and

4KO mice (8.0±2.0 vs 6.0±1.9 x10^5 eosinophils/ml; p=0.496, n=4). One possibility is that the vector induced a concurrent Th1 response, antagonizing the Th2 effector response in the lung[40]. However, our data suggest that is unlikely, since neither the Th1-associated transcription factor t-bet nor IFNγ were upregulated. Thus the mechanism by which the viral vector reduced BAL eosinophilia at day 11 is not obvious at present.

Immunoglobulin profiles are often used as indices of the nature of Th responses, since Th1 and Th2 cytokines signal for distinct patterns of isotype switching in B cells. As shown in table 1, we observed no production of antigen-specific IgE in 4KO mice, as expected, given the requirement for IL-4 for ε isotype switching[4]. In addition, OVA-specific IgG1 production was lower in 4KO mice than in WT mice, whereas IgG2a production was increased. Although IgG2a was increased in 4KO mice, it is not likely that this is indicative of a Th1-polarized response because we were unable to detect t-bet or IFNγ using very sensitive methods (real-time quantitative PCR) in the lymph nodes of WT or 4KO mice; more probably, in the absence of IL-4, isotype switching to IgG2a was not inhibited in 4KO mice. This skewed pattern of Ig production, taken by itself, is suggestive of an absence of Th2 differentiation in 4KO mice. However, in the context of our more direct markers indicating that Th2 polarization indeed occurred, we conclude that Ig profiles do not fully reflect the nature of Th responses.

IL-4 and IL-13 have a number of redundant biological functions, and it is possible that IL-13 may have been responsible for our observation of Th2 differentiation in the absence of IL-4[31]. We used DKO mice to establish whether indices of Th2 polarization in the absence of IL-4 were attributable to IL-13. It is clear that they were not; the equivalent expression of GATA-3, CCR-3, -4, -8, T1/ST2, IL-5, and blood eosinophils in DKO mice are compelling evidence that

neither IL-4 nor IL-13 are required for Th2 polarization *per se*, although a downstream effect of Th2 responses, BAL eosinophilia, was not fully realized in their absence. IL-13 is known to upregulate eotaxin production [41-43]; therefore, in this case, it would seem that eosinophils were not recruited from the blood into the lung tissues because of the absence of eotaxin, which in turn was likely due to the congenital absence of IL-13.

GM-CSF has been documented to be present in a variety of allergic airway diseases[44-47]; polymorphisms in the GM-CSF gene have been identified in allergic populations[48]; and airway expression of GM-CSF appears to be differentially regulated in atopic and non-atopic individuals[49-51]. Furthermore, the known functions of GM-CSF (such as the expansion of antigen-presenting cells and upregulation of antigen-presenting functions and costimulatory molecules[52-54], and downregulation of the immunosuppressive functions of alveolar macrophages[55,56]) are events conducive to the initiation of adaptive immune responses through the ability of such antigen-presenting cells to activate naïve T cells. Others have previously shown that GM-CSF expands a population of myeloid dendritic cells which are able to preferentially induce Th2 responses [57], independently of IL-4 [58], and we speculate that such a mechanism could be at work in this model.

Collectively, our data provide compelling evidence that Th2 differentiation occurred *in vivo* in the absence of IL-4. We demonstrate that respiratory exposure to antigen in the context of GM-CSF expression overrides the requirement for IL-4 to induce Th2-polarized immune-inflammatory responses. We further propose that the ability of GM-CSF to elicit Th2 responses suggests an important and novel role for this cytokine in the initiation of allergic responses.

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Figure 1: Expression of GATA-3 and t-bet in the thoracic lymph nodes of WT and 4KO mice. WT (black bars) and 4KO mice (grey bars) were exposed to OVA in the context of GM-CSF. At days 4, 7 and 11 RNA was isolated from thoracic lymph nodes and GATA-3 and t-bet were analyzed by real-time quantitative PCR. Panel A shows the relative expression of GATA-3, and panel B shows the relative expression of t-bet. Data shown is mean ± SD of triplicate measurements; * indicates a statistically significant ($p \leq 0.05$) difference from the expression levels of the naïve control, as determined by ANOVA.

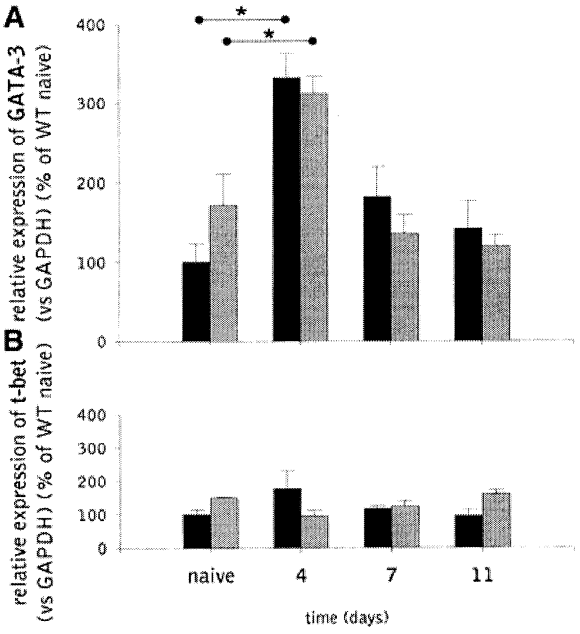

Figure 2: Expression of CCR3, CCR4, and CCR8 in the thoracic lymph nodes of WT and 4KO mice. RNA was isolated from pooled thoracic lymph nodes of WT (black bars) or 4KO mice (grey bars) at day 4 and day 7 of exposure to OVA in the context of GM-CSF expression. Real-time quantitative PCR was carried out to assess CCR3, CCR4 and CCR8 expression. Relative expression of CCR3 is shown in panel A, of CCR4 in panel B, and of CCR8 in panel C. Data shown is mean ± SD of triplicate measurements; * indicates a statistically significant ($p \leq 0.05$) difference from the expression levels of the naïve control, as determined by ANOVA.

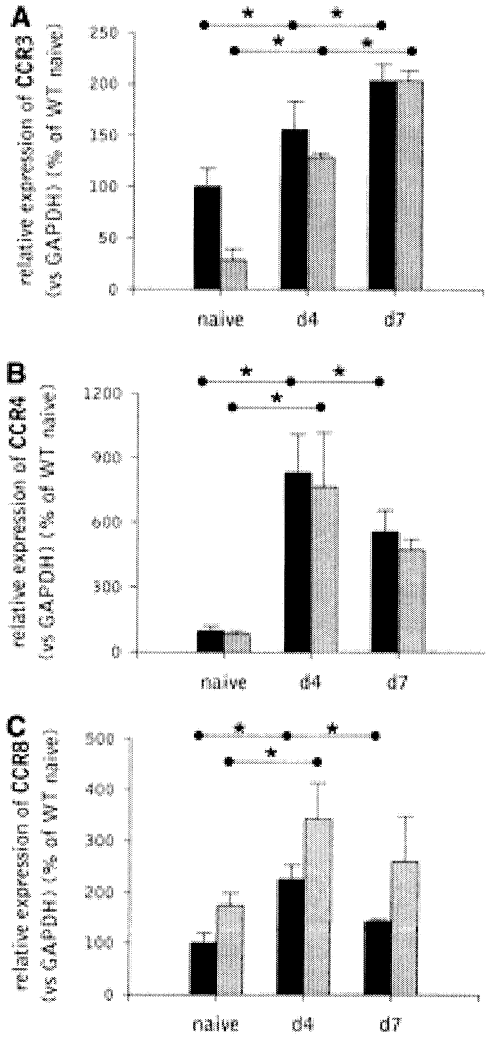

Figure 3: Levels of IL-5, IL-13, eotaxin and MCP-1 in BAL fluid of WT and 4KO mice. BAL samples were obtained on day 7 of exposure to OVA in the context of GM-CSF expression, and cytokine levels measured by ELISA. IL-5 levels are depicted in panel A, IL-13 in panel B, eotaxin in panel C, and MCP-1 in panel D. Data shown is mean ± SEM, n=4-9.

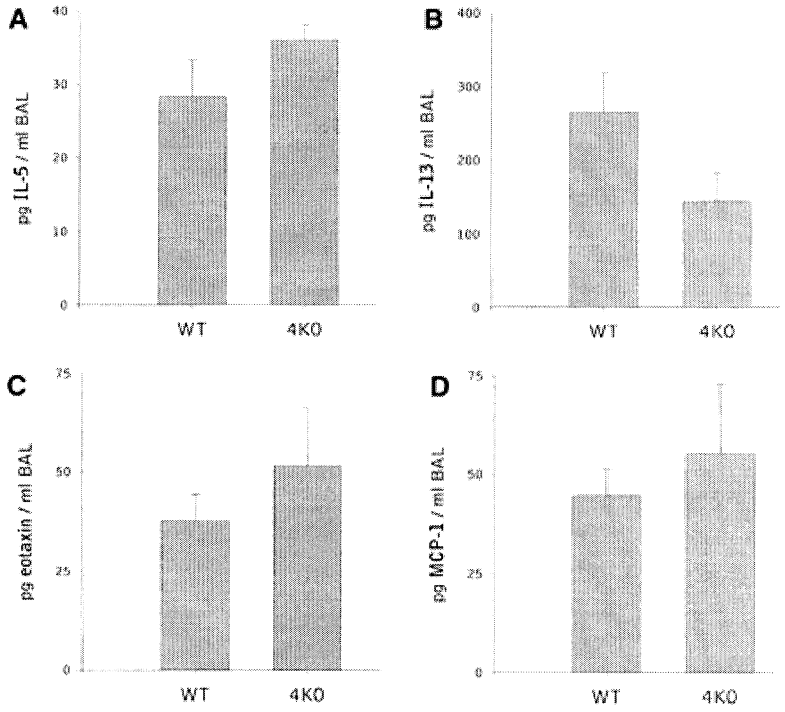

Figure 4: Eosinophilia in the peripheral blood and BAL of WT and 4KO mice. WT and 4KO mice were exposed to OVA aerosol in the context of GM-CSF expression. On day 11, peripheral blood (panels A & B) and BAL (panels C & D) were collected from both mouse strains; another group of WT and 4KO mice were rechallenged on days 40-42, and BAL collected on day 47 (panels E & F). Data show total leukocytes (A, C, & E) and eosinophils (B, D, & F) in the blood or BAL. Data shown is mean ± SEM, n=4-14; * indicates a statistically significant ($p \leq 0.05$) difference from WT mice as determined by ANOVA.

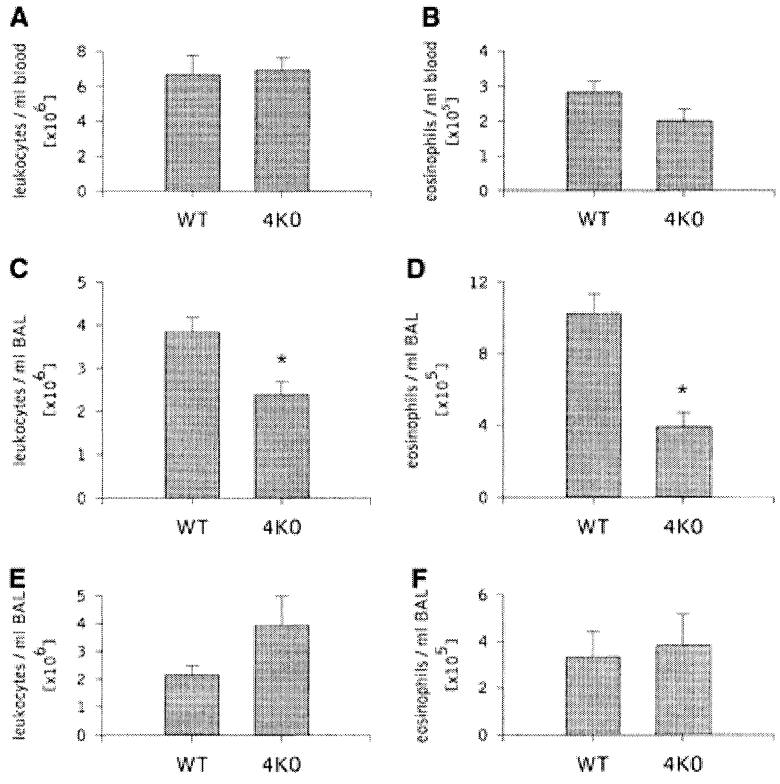

Figure 5: Light photomicrograph of lung tissues from WT and 4KO mice. Lung tissues from WT (panels A & B) and 4KO mice (panels C & D) exposed to OVA aerosol in the context of GM-CSF were obtained on day 11, fixed in formalin, and embedded in paraffin. Sections were stained with haematoxylin and eosin (A & C) to reveal general morphology, or periodic acid-Schiff (B & D) to stain for mucous. Note that peribronchial and perivascular inflammation are present both in WT (A) and 4KO mice (C), and that eosinophils are present in both strains (arrows). Also, magenta-stained mucous is apparent in both WT (B) and 4KO mice (D). "A" indicates airways, while "V" indicates blood vessels. Magnification: (A) and (C) 250x; (B) and (D) 100x.

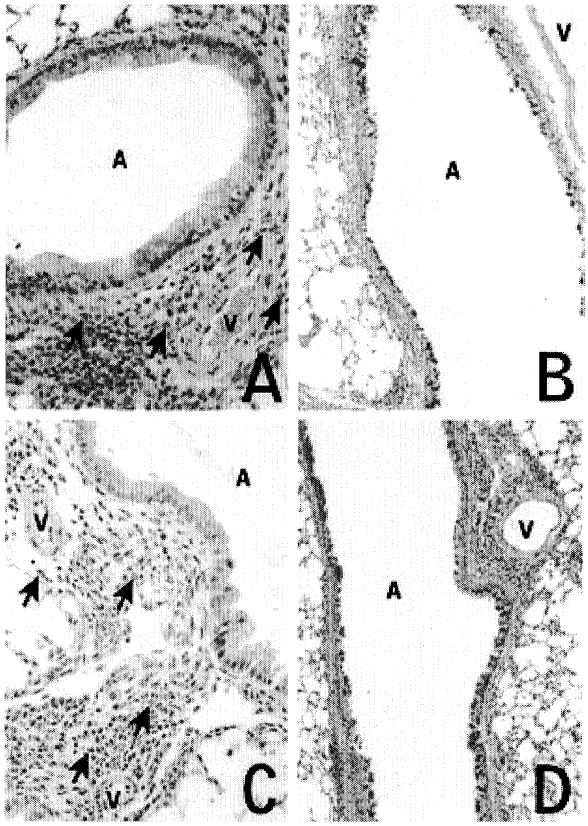

Figure 6: Indices of Th2 polarization and eosinophilic inflammation in IL-4/IL-13 double knockout mice. WT, 4KO and DKO mice were exposed to OVA aerosol in the context of GM-CSF expression. mRNA expression of GATA-3 (panel A), CCR3, -4, and -8 (panel B) was analyzed in the thoracic lymph nodes by real-time quantitative PCR on day 7. IL-5 (panel C) and eotaxin (panel F) were measured by ELISA on day 7. Peripheral blood (panel D) and BAL (panel E) eosinophilia was assessed on day 11. In panel C, black bars indicate WT mice, light grey bars are 4KO mice, and dark grey bars are DKO mice. Data in (A) and (B) are mean ± SD of triplicate measurements; data in (C-F) are mean ± SEM, n=4-13. * indicates a statistically significant ($p \leq 0.05$) difference as determined by ANOVA.

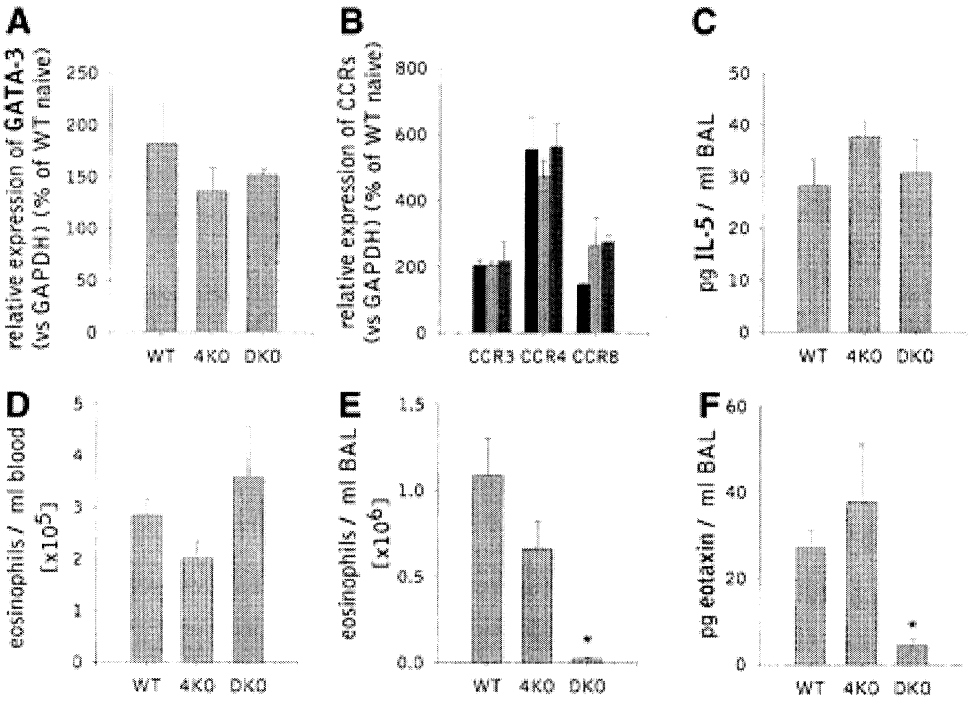

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Table 1: Flow cytometric and immunoglobulin analysis in WT and 4KO mice

	WT	4KO
OVA-specific IgE	96.6±42.2	n.d.
OVA-specific IgG1 [x10^5]	3.16±0.59	1.01±0.29*
OVA-specific IgG2a [x10^3]	1.07±0.12	2.59±0.46*
T1/ST2$^+$ T helper cells (%)	10.7	10.4

OVA-specific immunoglobulins were measured by ELISA in the serum of WT and 4KO mice, 2 days after the final exposure to OVA in the context of GM-CSF expression. Data shown are mean ± SEM, n=3-8. * indicates a statistically significant ($p \leq 0.05$) difference from WT mice, as determined by ANOVA; n.d. = not detectable. Expression of T1/ST2 on pooled lung T helper cells was assessed by flow cytometry. Data shown are percent of CD3$^+$ CD4$^+$ lymphocytes positive for T1/ST2, as defined relative to an isotype control antibody; these results were reproduced in 2 additional experiments.

CHAPTER 4:
IL-10 CONTRIBUTES TO TH2 POLARIZATION DURING GM-CSF-DRIVEN RESPIRATORY MUCOSAL SENSITIZATION IN MICE

The following manuscript examines the role of IL-10 in the development of Th2-polarized immune-inflammatory responses during respiratory mucosal sensitization to OVA. We used 2 approaches to ascertain the function of IL-10 in these responses. In the first, we injected blocking antibodies against IL-10 during sensitization in WT and 4KO Balb/c mice. In the second, we utilised WT, 4KO, 10KO and 4/10KO mice on the C57BL/6 background. At various time points during the protocol we examined the development of Th2-polarized responses in the lungs and spleen to determine whether the presence of IL-10 influenced these outcomes.

From these studies, we concluded that: [*A*] IL-10 contributes to the development of Th2 responses in Balb/c mice, particularly in the absence of IL-4; [*B*] in C57BL/6 mice, the absence of IL-10 does not allow for the development of Th2 responses; [*C*] the requirement for IL-4 in Th2-polarization may depend on the genetic background of the host.

The manuscript has been prepared for submission to the *American Journal of Respiratory Cell & Molecular Biology*. I am responsible for the design, execution, and interpretation of the experiments presented, as well as the writing of the manuscript. My co-authors provided technical advice, reagents, and mice, as well as supervision and funding for the work (see the *Preface* for more details).

IL-10 Contributes to Th2 Polarization in the Absence of IL-4 During GM-CSF-Driven Respiratory Mucosal Sensitization in Mice

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ABSTRACT

IL-4 is widely recognized as a key mediator of Th2 differentiation. However, IL-4-independent Th2 responses have been observed in several *in vivo* systems, suggesting that other factors are also involved.. Here we examined the role of IL-10 in mediating IL-4-independent Th2-polarized responses during GM-CSF-driven respiratory mucosal sensitization. Balb/c wild type (WT) or IL-4 knockout (4KO) mice were exposed to aerosolized ovalbumin (OVA) in the context of a GM-CSF-enriched airway microenvironment, and treated with either anti-IL-10 antibodies or control serum. Although it had no effect on total airway inflammation, anti-IL-10 treatment was associated with a significant reduction in eosinophilia in 4KO, but not WT, mice. Anti-IL-10 treatment reduced levels of the Th2-affiliated cytokines IL-5 and IL-13 in the bronchoalveolar lavage (BAL) both in WT mice and in 4KO mice compared to control serum-treated animals. This same pattern was also observed with respect to splenocyte production of IL-5 after *in vitro* restimulation with OVA. In addition, anti-IL-10 treatment had no effect on the production of OVA-specific IgE or IgG1 in WT or 4KO mice. Finally, we also used IL-4 IL-10 double knock-out (4/10KO) mice on the C57BL/6 background as an alternative approach, and showed that compared to C57BL/6 WT controls, none of 4KO, 10KO nor 4/10KO mice generated a substantial inflammatory response characterized by eosinophilia. We conclude that in this model of allergic airways inflammation, both IL-4 and IL-10 are involved in mediating Th2 polarization of the response.

NTRODUCTION

Allergic asthma is comprised of a constellation of pathophysiological abnormalities, including bronchial hyperreactivity, eosinophilic airway inflammation, and a distinct cytokine and immunoglobulin profile. T helper (Th) cells are consistently present in human asthma patients, and studies in murine models of asthma have demonstrated that Th cells are crucial to the development of the asthma phenotype (Garlisi 1995)(Gonzalo 1996), primarily through their production of the Th2-associated cytokines IL-4, IL-5, and IL-13 (Foster 2002). Given the centrality of Th2 cells in the pathogenesis of allergic disorders such as asthma, enhancing our understanding of the mechanisms by which precursor Th cells are directed to differentiate into Th2 cells could be of great importance for the development of preventative and therapeutic strategies in allergic disease.

IL-4 is usually identified as the single most important factor for the development of Th2-polarized responses. Indeed, there have been many reports in murine models of allergic inflammation and infectious disease in which Th2 responses are impaired in the absence of signalling through the IL-4 pathway (Brusselle 1994)(Coyle 1995)(Herrick 2000)(Kips 1995)(Kopf 1993)(Kuperman 1998), and IL-4 (in the presence of antibodies against Th1-polarizing cytokines) is sufficient to drive Th2 differentiation of activated T cells *in vitro* (Swain 1990)(LeGros 1990). However, there are also a significant number of studies, by ourselves and others, which indicate that *in vivo* Th2-polarized responses are not always substantially inhibited in the absence of IL-4 (Ritz 2002)(Brewer 1999)(Chensue 1997)(Herrick 2000)(Hogan 1997)(Hogarth 1998)(Kopf 1996)(Kropf 1999)(Mohrs 2000)(Noben-Trauth 1997). Hence it is clear that the development of Th2 responses is not entirely dependent on the presence of IL-4.

Indeed, rather than being exclusively dependent on a single pathway, it is likely that many different factors interact to determine the outcome of Th differentiation. Aside from IL-4, low antigen doses, low avidity TCR-antigen-MHC interactions, preferential costimulation by B7.2 and OX40, and decreased levels of the negative regulator SHP-1 have been shown to privilege polarization toward the Th2 pathway (Akiba 2000)(Constant & Bottomly 1997)(Glimcher & Murphy 2000)(Jones 2001)(Guery 1996)(Kamata 2003). Soluble signals other than IL-4 may also contribute to Th2 polarization, and there are indications in the literature that IL-10 may be one such cytokine (Maldonado-Lopez 2001)(Iwassaki 1999).

IL-10 is known to have powerful immunogreulatory effects. IL-10 downregulates the production of the Th1-polarizing cytokine IL-12 by antigen-presenting cells (Moore 2001), thereby shifting the cytokine microenvironment away from a Th1-inducing milieu. IL-10 also affects antigen presentation itself, by downregulating MHC class II and costimulatory molecule expression on APCs (Willems 1994)(Koppelman 1997)(Moore 2001); such a change in the density of antigen presentation may also facilitate Th2 responses. In addition, the ability of APCs themselves to produce IL-10 may also be important in the induction of Th2 polarization, as dendritic cells from IL-10 KO mice were not able to efficiently induce Th2 responses *in vitro* (Maldonado-Lopez 2001). Recently, IL-10 has been identified as an important factor mediating susceptibility to *Leishmania major* infection in mice (Noben-Trauth 2003), which is associated with Th2 responses.

We previously demonstrated that IL-4 was not necessary to generate Th2-polarized responses in a model of GM-CSF-driven respiratory mucosal sensitization, as assessed by the expression of a variety of Th2-associated markers including cytokines, chemokine receptors, and transcription factors (Ritz 2002b). Here, we have examined the extent to which IL-10 contributes to this IL-4-independent Th2

polarization through the administration of anti-IL-10 antibodies to WT and IL-4 KO mice during GM-CSF-mediated sensitization to OVA. In a second approach, we have used IL-4/IL-10 double knock-out mice in this model. These studies indicate that IL-10 plays a role in the development of Th2 polarization *in vivo* during respiratory mucosal sensitization.

MATERIALS & METHODS

Subjects. Female Balb/c or C57BL/6 wild type mice were purchased from Charles River Laboratories (Ottawa, ON, Canada) at 6-8 weeks of age. Female IL-4 knock-out mice on the Balb/c background were purchased from Jackson Laboratories (Bar Harbor, ME). Female IL-4, IL-10, and IL-4/IL-10 knock-out mice on the C57BL/6 background were obtained from Taconic (Rockville, MD). Mice were housed under specific pathogen-free conditions, with food and water *ad libitum*, and kept on a 12 h light/dark cycle. The experiments described here were approved by the Animal Research Ethics Board of McMaster University.

Respiratory Mucosal Sensitization. $3x10^7$ pfu of replication-deficient human type 5 adenovirus carrying the gene for murine GM-CSF in the E1 region of the genome was delivered intranasally to isoflurane-anaesthetized mice, in order to achieve airway expression of GM-CSF. As we have described previously (Stampfli 1998), this dose of Ad/GM-CSF results in the sustained expression of GM-CSF in the airways for ~10 days, with peak expression occurring on day 7 at 80-100 pg/ml GM-CSF detected in the BAL by ELISA. These levels of GM-CSF are comparable to physiologic expression of GM-CSF during inflammation in the lung (Ohkawara 1996).

Beginning 1 day after Ad/GM-CSF administration, mice were exposed to aerosolized OVA solution daily, for 20 minutes per day, for up to 10 days. OVA aerosol (1% wt/vol in 0.9% saline; Grade V; Sigma-Aldrich, Oakville, ON, Canada) was generated using a Bennet/Twin nebulizer (Kansas City, MO) and compressed medical air at a flow rate of 7 l/min. This model of respiratory mucosal sensitization has been extensively characterized, and it was observed that neither exposure to OVA only, nor OVA along with a control Ad vector, results in immunologic sensitization to OVA in either WT (Stampfli 1998) or 4KO (Ritz 2002b) mice.

Anti-IL-10 Treatment. In order to block IL-10, polyclonal rabbit anti-mouse IL-10 sera were administered to mice; normal rabbit sera (Sigma) were administered as a control. Mice were lightly anaesthetized with isoflurane, and 250 μl of serum injected intraperitoneally with a 27 gauge needle. Injections were initiated concurrently with Ad/GM-CSF instillation, and given every second day immediately prior to OVA aerosol exposure.

Collection & Measurement of Specimens. Following anaesthesia with isoflurane, blood was obtained by retroorbital bleeding, and mice were then killed by exsanguination from the descending aorta. To obtain serum, blood was incubated at 37°C for 30 min, centrifuged, and supernatants collected and stored at -20°C until analysis.

The lungs were removed, and bronchoalveolar lavage was performed. PBS was delivered and recovered in 2 aliquots (250 μl and 200 μl) via a polyethylene tube (# 427410, Becton Dickinson, Sparks, MD) inserted intratracheally and secured with surgical thread. Total cell counts were determined using a hemocytometer, cells pelleted by centrifugation (~5 s at 1200 rpm), and supernatants recovered and stored at -20°C until analysis. BAL cells were resuspended in PBS and slides prepared by cytocentrifugation (2 min at 300 rpm)(Shandon Inc., Pittsburgh, PA).

Blood and BAL smears were stained using the Protocol Hema 3 Stain Set (Fisher Scientific, Toronto, ON, Canada), and differential counts made from 300 leukocytes using standard morphological criteria to identify mononuclear cells, neutrophils, and eosinophils.

Splenocyte Cultures. Spleens were removed from mice postmortem, and placed in Hanks' balanced salt solution (HBSS) on ice. Under sterile conditions, the spleens were cut into several pieces, and ground between the frosted ends of glass slides into HBSS. After centrifugation (12 min at 1200 rpm), supernatants were discarded. Red blood cell lysis was accomplished by incubating each pellet with ACK lysis buffer (0.15 M NH_4Cl (Sigma), 10 mM $KHCO_3$ (Sigma), 0.1 nM Na_2EDTA (Sigma); pH adjusted to 7.2-7.4)(1 ml/spleen) for 1 minute, followed by the addition of HBSS (9 ml/spleen), and centrifugation. After an additional wash with HBSS, splenocytes were resuspended in RPMI 1640 (Gibco) with 10% FCS (Gibco), 1% penicillin/streptomycin, and 1% L-glutamine, and cultured in flat-bottomed 96-well plates at $8x10^5$ cells/well, in the presence or absence of 40 μg/well OVA, at 37°C for 5 days.

ELISA Measurement of Cytokines & Immunoglobulins. Commercially-available ELISA kits were used to measure IL-5, IL-13, and IFNγ (R&D, Minneapolis, MN) in BAL and splenocyte culture supernatants. The level of sensitivity for these assays was ~5 pg/ml.

OVA-specific IgG1 was measured by sandwich ELISA as previously described (Ritz 2002b). OVA-specific IgE was measured using an antigen-capture ELISA, as previously described (Okhawara 1996); briefly, serum IgE was captured by anti-mouse IgE Ab in the solid phase, followed by detection of OVA-specific IgE using biotinylated OVA. Quantitation of OVA-specific Ig was determined by comparison to a standard sera, in which 1 unit is defined as the largest dilution factor

giving an optical density reading greater than background + 2 SD; therefore, by definition, the sensitivity of these assays is 1 U/ml.

Data Analysis. Data are expressed as mean ± SEM. SigmaStat version 2.03 was used for statistical analysis. Differences were considered to be statistically significant when $p \leq 0.05$ by analysis of variance (ANOVA), with Fisher's PLSD where applicable.

RESULTS

IL-10 Expression During Respiratory Mucosal Sensitization. We measured IL-10 in the BAL of WT and 4KO mice by ELISA over sensitization to ascertain whether IL-10 was being expressed during this process. As shown in figure 1, IL-10 was not detectable in the BAL of naive mice. IL-10 expression increased by day 4 of the sensitization protocol, and reached peak levels at day 7 in WT mice. 4KO mice had levels of IL-10 expression not significantly different from WT mice, peaking at 21.1 ± 3.0 pg/ml on day 7.

Airway Inflammatory Responses. Total inflammation and eosinophils were assessed in the BAL on day 11 (2 days after the 10th exposure to aerosolized OVA). We have previously shown that naive WT and 4KO mice have similar numbers of total BAL cells and no eosinophils (Ritz 2002b).

Substantial inflammatory responses were evident at day 11 in both WT and 4KO control mice (figure 2A). Anti-IL-10 treatment had no effect on total cell numbers in the BAL in either strain of mice. WT control mice had notable eosinophilia in the BAL at day 11, comprising approximately 25% of BAL cells (figure 2B), and anti-IL-10 treatment did not depress eosinophilia in WT mice. Control-treated 4KO mice had similar levels of airway eosinophilia as their WT

counterparts, at ~20%. In contrast with WT mice, anti-IL-10 treatment of 4KO mice led to a significant reduction in BAL eosinophils, by 60%.

BAL Cytokine Profile. BAL cytokine levels were measured on day 7, as we have previously shown that this time point is the peak of cytokine expression in this model (Stampfli 1998). Naive WT and 4KO mice did not have any detectable IL-5 or IL-13 in the BAL (data not shown). At day 7, WT control mice had ~200 pg/ml IL-5 in the BAL, whereas 4KO control mice had ~70 pg/ml (figure 3A). Anti-IL-10 treatment was associated with a decrease in BAL IL-5 levels in both strains of mice. The same pattern was observed with respect to BAL IL-13 (figure 3B), with lower expression in 4KO controls than in WT controls, and decreased IL-13 expression in both strains with anti-IL-10 treatment.

IL-5 Production by Cultured Splenocytes. After 5 days of *in vitro* culture in the presence or absence of OVA, splenocyte supernatants were recovered and analyzed for IL-5 content by ELISA.

Splenocytes from WT control mice produced approximately 150 pg/ml IL-5 when cultured with medium alone, whereas 4KO control splenocytes had slightly lower spontaneous production (figure 4A). Anti-IL-10 treatment increased spontaneous IL-5 production in both strains of mice, significantly so in WT mice; however, the difference did not quite reach statistical significance in 4KO mice ($p=0.076$).

When cultured with OVA, splenocytes from all groups significanly upregulated their production of IL-5 when compared to the levels of spontaneous production from the same group (figure 4B). OVA-stimulated WT control splenocytes expressed the highest levels of IL-5, reaching ~7500 pg/ml. Anti-IL-10 treatment was associated with a significant reduction in IL-5 production from WT splenocytes. Levels of IL-5 produced by 4KO control splenocytes were significantly

lower than from WT control splenocytes. Splenocytes from 4KO mice treated with anti-IL-10 produced marginally lower levels of IL-5 than 4KO control splenocytes or WT anti-IL-10 treated splenocytes, but these differences were not statistically significant.

Given the significant differences in spontaneous production of IL-5 in different groups, we elected to analzye these data as fold increases in IL-5 production (OVA-stimulated/spontaneous) as well. Splenocytes from WT control mice produced ~50x more IL-5 when stimulated with OVA compared to their spontaneous production (figure 4C). In WT mice treated with anti-IL-10, OVA-stimulated production of IL-5 *in vitro* was significantly less than in control mice, approximately 5x greater than their spontaneous production. A ~30x increase in IL-5 production was observed in splenocytes from 4KO control mice when stimulated with OVA *in vitro*, whereas anti-IL-10 treatment was associated with only a 3x increase in IL-5 compared to their spontaneous production.

OVA-Specific Immunoglobulin Profile. OVA-specific immunoglobulins were measured in the serum by ELISA. We have previously shown that no OVA-specific immunoglobulins were detected in naive WT or 4KO mice (Ritz 2002b). OVA-specific IgE and IgG1 were both detected in WT control mice after sensitization (figure 5). 4KO control mice had significantly lower levels of both of these immunoglobulin isotypes. Anti-IL-10 treatment had no effect on antibody production in either strain of mice.

Immune-Inflammatory Responses in IL-4/IL-10 Double Knock-Out Mice. In order to examine the effect of an *absolute* (as opposed to relative) deficiency in IL-10, we also exposed 4KO, 10KO, and 4/10KO mice on the C57BL/6 background to aerosolized OVA in the context of GM-CSF overexpression in the airways. As seen in figure 6, WT C57BL/6 mice generated a significant inflammatory response in the

BAL characterized by eosinophilia. In contrast, neither 4KO, 10KO, nor 4/10KO mice generated such a response.

We also performed *in vitro* splenocyte recall responses to OVA in these mice to examine their production of IL-5. As seen in figure 7, in contrast with mice on the Balb/c background, virtually no IL-5 was produced spontaneously when splenocytes were cultured in medium alone in any of the groups. 5 days of culture with OVA resulted in antigen-specific IL-5 production in WT and 4KO mice, but not in 10KO or 4/10KO mice.

DISCUSSION

Polarization of immune-inflammatory responses toward the Th2 pathway is undoubtedly a critical step in the development of allergic disease. We have previously shown that IL-4 is not required for Th2 polarization in a model of GM-CSF-driven respiratory mucosal sensitization (Ritz 2002b). In the present study, we have examined the influence of IL-10 on the development of Th2 responses in this model, in the presence or absence of IL-4. The model of respiratory mucosal sensitization we utilized employs GM-CSF as a 'natural adjuvant' to provoke allergic sensitization against an otherwise innocuous antigen, OVA, delivered exclusively via the respiratory tract. This model has several distinct advantages over other models of allergic airway disease, in that it does not utilize chemical adjuvants such as alum, and the route of antigen delivery is comparable to the mode of allergen exposure in humans.

It has been previously demonstrated that respiratory exposure to aerosolized OVA alone results in inhalation tolerance, and not pathological immune-inflammatory responses (Holt 1982)(Swirski 2002). However, we have previously shown that the addition of exogenous GM-CSF to the airway cytokine milieu

overrides the development of tolerance (Stampfli 1998), likely through the GM-CSF-mediated expansion of the local population of myeloid dendritic cells and enhancement of their antigen-presenting capabilities (Wang 2000)(Christensen 1995)(Daro 2000)(McKenna 2001)). In the context of the work presented here, it is worth noting that myeloid dendritic cells have been shown to preferentially elicit Th2-polarized responses *in vitro* and *in vivo* (Maldonado-Lopez 2001)(Lambrecht 2000)(Pulendran 1999), and that they are able to do so in a manner that is independent of IL-4 but dependent on IL-10 (Maldonado-Lopez 2001). It was this evidence that led us to speculate that IL-10 might be responsible for IL-4-independent Th2 polarization in this model of GM-CSF-driven sensitization.

We have previously observed that GM-CSF-driven respiratory mucosal sensitization does not require IL-4 for the development of Th2-polarized responses using 4KO mice on the Balb/c background (Ritz 2002b). In that study, we examined the expression of the Th2-associated markers GATA-3, IL-5, IL-13, eotaxin, CCR3, CCR4, CCR8 and T1/ST2, and found that there was no difference between WT and 4KO mice, and that BAL eosinophilia was equivalent after OVA recall. In this study we obtained similar, but not identical, results in the 4KO Balb/c mice; unlike our original study, here we observed that 4KO Balb/c mice had reduced levels of IL-5 in the BAL, although BAL eosinophilia was unaffected. However, unlike the previous study, mice in this study received repeated injections of rabbit serum, which likely induced a degree of serum sickness in the mice after 12 days; immune responses to the rabbit serum may have had some cross-regulatory effect on the responses to OVA, thereby reducing the production of IL-5 in the BAL and spleen. Such interference may be more pronounced in the 4KO mice because, although IL-4 is not necessary for Th2 responses, it likely plays a role in consolidating those responses once initiated (Jankovic 2001).

Notably, unlike 4KO mice on the Balb/c background, 4KO mice on the C57BL/6 background showed no evidence of the development of Th2-polarized responses in this model, indicating a differential dependence on IL-4 for these responses in different mouse strains. Given that C57BL/6 mice are conventionally thought of as "Th1 mice," and Balb/c as "Th2 mice," it may be that the threshold of signalling required to initiate Th2 responses is higher in C57BL/6 mice than in Balb/c, and the necessary threshold cannot be met in the absence of IL-4 in C57BL/6 mice (Hsieh 1995). However, investigating such strain differences is beyond the scope of the current investigation.

The data shown here support the hypothesis that IL-10 can, at least in part, mediate the development of Th2 responses, and particularly so in the absence of IL-4 in Balb/c mice. Treatment of WT Balb/c mice with anti-IL-10 antibodies had no impact on the ultimate development of eosinophilic airway inflammation, but did have significant effects on the production of Th2 cytokines in BAL and from cultured splenocytes. Moreover, anti-IL-10 treatment of 4KO mice was associated with a significant reduction in airway eosinophilia, and Th2 cytokine production was even further suppressed than in control 4KO mice or in anti-IL-10-treated WT mice. Since anti-IL-10 treatment was associated with a diminishment in such Th2-affiliated phenomena, we conclude that IL-10 does indeed contribute to the development of Th2 responses *in vivo* in this model. Moreover, our observations in 10KO and 4/10KO mice, where the deficiency in IL-10 is absolute, suggest that, at least in this mouse strain, IL-10 is absolutely required for the generation of Th2-polarized immune inflammatory responses.

IL-10 is a cytokine with pleiotropic effects. On one hand, IL-10 is known to be a potent immunosuppressive factor, able to inhibit inflammatory responses in a variety of immunological contexts. We and others have previously shown that IL-10

can have immunosuppressive effects in contexts including eosinophilic airways inflammation (Bellinghausen 2001)(Stampfli 1999)(Tournoy 2000), transplantation (Baker 1999)(Asderakis 2001), and autoimmunity. However, IL-10 has also been shown to be an immunostimulatory factor, mediating $CD8^+$ T cell responses against tumours (Moore 2001), and particpating in the induction of Th2 responses as observed in models of *Leishmania major* infection (Mentink Kane 2001)(Noben-Trauth 2003).

It may be argued that the expansion and activation of myeloid dendritic cells is a critical step in the initiation of allergic sensitization and disease. Although there is little doubt that Th2 cells and their mediators are necessary for allergic inflammation, the fact that myeloid dendritic cells can preferentially induce Th2 polarization indicates a role for these cells that is upstream of Th2 differentiation, and therefore a potential control point in Th2 development. The idea that Th2 polarization may depend more on myeloid dendritic cells and IL-10 than on IL-4 is appealing because it may resolve the unsettled question about what the 'original source' of IL-4 is for Th2 differentiation; that is to say that there may in fact be no need for an 'original source' of IL-4. Of course, we are not trying to argue that there is no important role for IL-4 in Th2-mediated diseases. On the contrary, it is likely that the presence of IL-4 can impel Th2 polarization and consolidate the Th2 phenotype at a population level, as proposed by Jankovic *et al* (2000), rather than at the single cell level. Moreover, IL-4 is very important for the production of antigen-specific IgE (Finkelman 1988), a hallmark of allergic disease states. However, IL-4 alone does not appear to be sufficient to drive Th2 responses to antigen, as Ad-mediated gene transfer of IL-4 to the airways of mice did not facilitate allergic sensitization to aerosolized OVA (unpublished data)(Ritz 2000); nor does IL-4 appear to be necessary for Th2 responses, as we and others have previously shown.

More likely, IL-4 is one of a myriad of factors which can collaborate to produce Th2 responses.

In contrast with our speculation that GM-CSF-induced myeloid DCs are the key source of IL-10 for Th2 development, Noben-Trauth *et al* suggest that $CD4^+$ T cells are the most important source of the IL-10 which mediates susceptibility to *Leishmania major*, based on their demonstration that $CD4^+$ T cell depletion had similar effects on resistance to *Leishmania* as anti-IL-10R treatment (2003). However, this interpretation is somewhat problematic, since depletion of all T helper cells certainly eliminates Th2 cells, which are important determinants of susceptibility, and essentially neglects the distinction between the role of IL-10 in the *induction* of Th2-polarized responses, and as a *product* of Th2 cells. Data elsewhere in their paper indicate that it is reasonable to conclude that IL-10 plays an important role in the induction of Th2 responses, since anti-IL-10R treatment inhibited the production of IL-4 from Ag-stimulated LN cells (2003).

The data presented here support the growing notion in the literature that factors other than IL-4 can mediate Th2 polarization *in vivo*, and that IL-10 is one of those factors. We hypothesize that myeloid dendritic cells, expanded by GM-CSF, produce this IL-10 during their activating interactions with naive T cells, contributing to a milieu of soluble factors and surface protein interactions which preferentially give rise to a Th2 response.

Figure 1: BAL levels of IL-10 during respiratory mucosal sensitization in WT mice. BAL was collected from naive mice, and on days 4 and 7 of exposure to OVA in the context of airway GM-CSF expression, and measured by ELISA. Data shown is mean ± SEM, n=3-5. IL-10 levels were not statistically significantly different in 4KO mice, peaking at 21.1 ± 3.0 pg/ml on day 7.

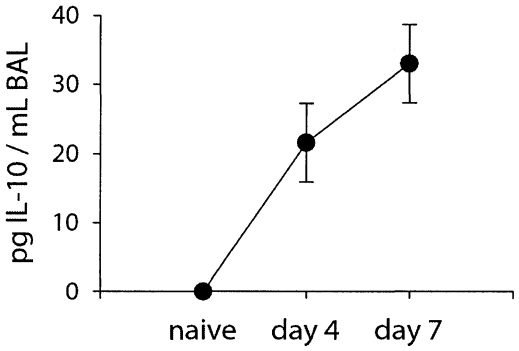


Figure 2: Total inflammation and eosinophilia in the BAL of WT and 4KO mice during respiratory mucosal sensitization. WT and 4KO mice were exposed to OVA aerosol in the context of airway GM-CSF expression, and treated every second day with either polyclonal anti-IL-10 antibodies, or control rabbit serum. BAL was collected on day 11 for assessment of inflammation. Data show total leukocytes (*A*) and eosinophils (*B*) in the BAL. Data shown is mean ± SEM, n=9-12; * indicates a statistically significant ($p \leq 0.05$) difference between the indicated groups, as determined by ANOVA.

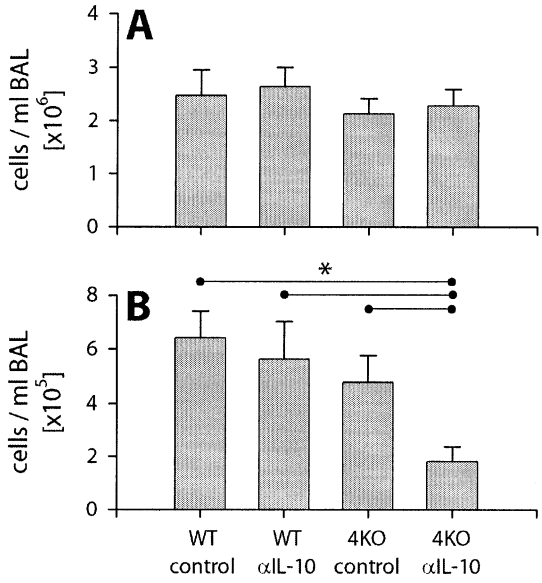


Figure 3: IL-5 and IL-13 levels in the BAL of WT and 4KO mice during respiratory mucosal sensitization. WT and 4KO mice were exposed to OVA aerosol in the context of airway GM-CSF expression, and treated every second day with either polyclonal anti-IL-10 antibodies, or control rabbit serum. BAL was collected on day 7 for cytokine measurements by ELISA. Data show IL-5 (*A*) and IL-13 (*B*) in the BAL. Data shown is mean ± SEM, n=8; * indicates a statistically significant ($p \leq 0.05$) difference between the indicated groups, as determined by ANOVA.

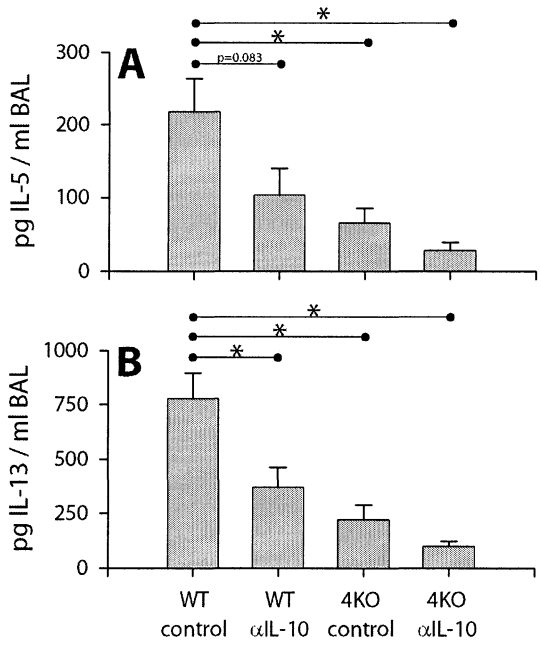


Figure 4: IL-5 production by cultured splenocytes from WT and 4KO mice during respiratory mucosal sensitization. WT and 4KO mice were exposed to OVA aerosol in the context of airway GM-CSF expression, and treated every second day with either polyclonal anti-IL-10 antibodies, or control rabbit serum. Spleen cells were isolated on day 11, placed into culture with medium alone or with OVA (40 μg/well) for 5 days, and supernatants collected for IL-5 measurement by ELISA. Data show spontaneous production of IL-5 in the presence of medium alone (*A*), IL-5 production by splenocytes cultured in the presence of OVA (*B*), and the fold-increase in IL-5 production over spontaneous (*C*). Data shown is mean ± SEM of triplicate measurements; * indicates a statistically significant ($p \leq 0.05$) difference between the indicated groups, as determined by ANOVA.

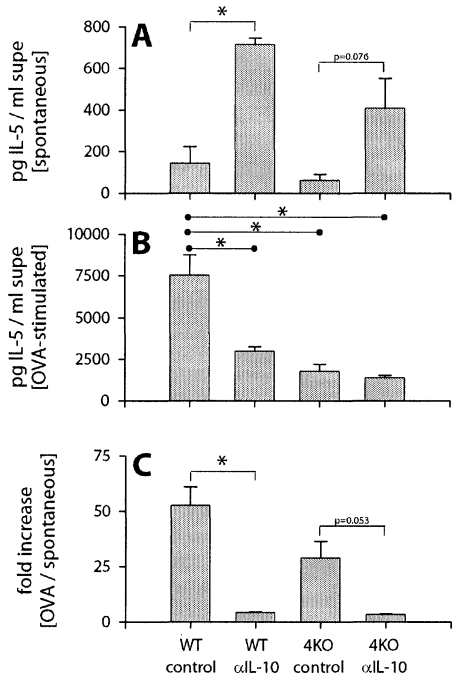


Figure 5: Immunoglobulin production by WT and 4KO mice during respiratory mucosal sensitization. WT and 4KO mice were exposed to OVA aerosol in the context of airway GM-CSF expression, and treated every second day with either polyclonal anti-IL-10 antibodies, or control rabbit serum. Serum was collected on day 11 for measurement of OVA-specific IgE and IgG1 by ELISA. Data show OVA-specific IgE (*A*) and IgG1 (*B*) in the serum. Data shown is mean ± SEM, n=8; * indicates a statistically significant ($p \leq 0.05$) difference between the indicated groups, as determined by ANOVA.

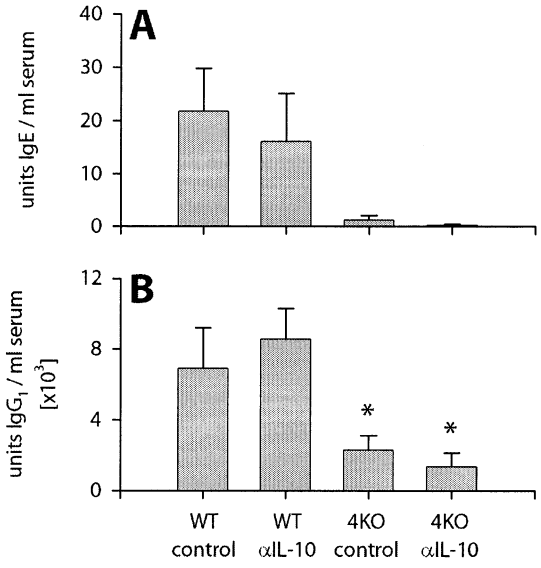


Figure 6: Total inflammation and eosinophilia in the BAL of WT, 4KO, 10KO, and 4/10KO mice during respiratory mucosal sensitization. Mice were exposed to OVA aerosol in the context of airway GM-CSF expression, and BAL was collected on day 11 for assessment of inflammation. Data show total leukocytes (*A*) and eosinophils (*B*) in the BAL. Data shown is mean ± SEM, n=8; * indicates a statistically significant ($p \leq 0.05$) difference from WT, as determined by ANOVA.

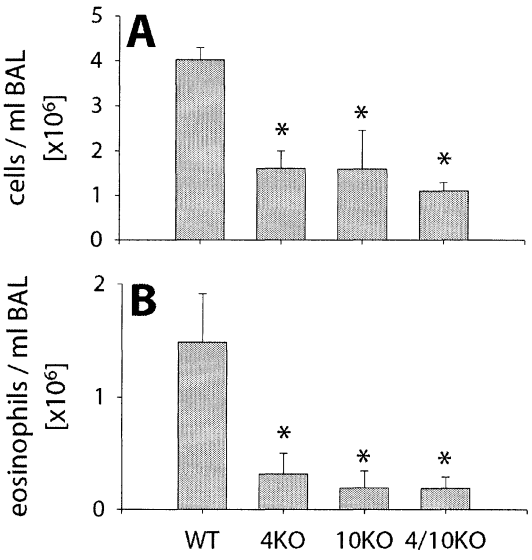
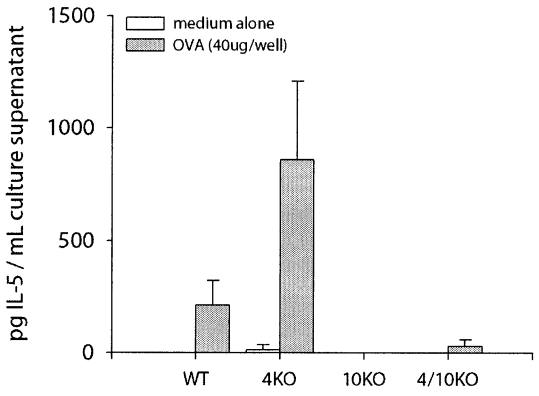


Figure 7: IL-5 production by cultured splenocytes from WT, 4KO, 10KO, and 4/10KO mice during respiratory mucosal sensitization. Mice were exposed to OVA aerosol in the context of airway GM-CSF expression; spleen cells were isolated on day 11, placed into culture with medium alone or with OVA (40 μg/well) for 5 days, and supernatants collected for IL-5 measurement by ELISA. Data show spontaneous production of IL-5 in the presence of medium alone (*white bars*) and IL-5 production by splenocytes cultured in the presence of OVA (*grey bars*). Data shown is mean ± SEM of triplicate measurements.



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CHAPTER 5: DISCUSSION

One of the most common questions laypeople have about current research in allergy and asthma is "why do so many people have asthma now?" In spite of intensive research efforts investigating this question, only theoretical answers are yet available. The major hypotheses currently under popular discussion in the field include: decreased rates of childhood infection; pasteurization and irradiation of food products; concern about 'germs' and resultant over-cleanliness; increased exposure to allergens due to sealed homes and recycled indoor air; and air pollution. However, currently there is insufficient evidence to decisively implicate any of these in what is often called our 'epidemic' of asthma.

The work of this thesis has not attempted to directly prove or disprove any of these hypotheses regarding the causes of asthma. Instead, this research has investigated immunological conditions of the airways that are conducive to allergic sensitization. This work has provided insight into the mechanisms through which allergic sensitization takes place in the respiratory tract, particularly with respect to the roles of a number of cytokines in these processes, and the molecular and cellular events in the lymph nodes and respiratory tract associated with respiratory mucosal sensitization. Through our understanding of these mechanisms, we are better positioned to appreciate the origins of allergic respiratory diseases, appraise the likely impact of various kinds of environmental and genetic factors that may contribute to their pathogenesis, and to critically evaluate the potential merit of proposed intervention strategies.

Since each of the manuscripts that form the body of this thesis includes a discussion of the results observed in each of them, I will avoid reiterating those

discussions again, and focus instead on aspects which have not been previously discussed, and on the implications of this body of work as a whole.

LOCAL CYTOKINE EXPRESSION AFFECTS DISTANT EVENTS IN THE LYMPH NODES

In the original investigations of the GM-CSF- and GM-CSF/IL-12-driven models of respiratory mucosal sensitization to OVA (Stampfli 1998)(Stampfli 1999b), our efforts were focussed primarily on the characterization of responses in the airways. However, at the time we did not understand the mechanisms through which polarized antigen-specific adaptive immune responses were generated via the respiratory tract, though there were a number of possibilities. The research of this thesis has contributed to our understanding of the mechanisms through which respiratory mucosal sensitization takes place, allowing us to reconsider the possibilities we had originally envisioned.

First was that cytokine expression driven from the adenoviral vector given intransally was sufficiently great to allow the cytokines to enter the systemic circulation and reach the secondary lymphoid organs, thereby influencing T cell differentiation events directly or indirectly through the biological activity of the cytokine itself in the lymph nodes. However, ELISA measurements of GM-CSF or IL-12 in the peripheral blood showed little or no systemic spillover of the cytokines (unpublished data), leading us to believe that this was not the correct explanation.

A second possibility was that the adenoviral vector itself was disseminating from the airways, either as independent viral particles, or after infecting migratory cells which then moved to the lymph nodes, and expressed the transgenic cytokines in the lymph nodes themselves. However, using a highly sensitive technique (real-time quantitative PCR), we demonstrated that although there was robust expression of the transgene in the lung tissue, no transgene expression was detectable in the

lymph nodes of mice at any time point after intranasal infection with the adenoviral vector at the doses used in these studies (figure 1, *Chapter 2*). Hence it is unlikely that cytokine protein is reaching the lymph nodes by this means either.

Thirdly, it could be that T cell activation was occurring locally within the respiratory tract itself, rather than in the lymph nodes, for example within bronchial-associated lymphoid tissue (BALT). It is difficult to completely rule out this possibility. However, we do not normally observe BALT in the lungs of naive mice, although it develops after chronic stimulation with antigen. Moreover, our observations in *Chapter 2* and *Chapter 3* indicate that expression of Th1- or Th2-associated genes is indeed altered in the lymph nodes themselves, and hence there would seem to be no need to invoke BALT as an explanation for our observations.

A fourth hypothesis was that expression of cytokines in the respiratory tract did not affect lymph node events at all, but that the local expression of GM-CSF or GM-CSF and IL-12 in the lung led to differential recruitment of polarized T cell subsets to the target organ, due to locally-mediated effects on the expression of chemokines and adhesion molecules. This theory was informed, in part, by experiments directed by Ryan Wiley in our lab investigating the effects of chemokine expression on inflammatory responses in the airways during GM-CSF-driven sensitization to OVA. That work demonstrated that when mice were exposed to OVA in the context of both GM-CSF and the Th1-chemoattractant IP-10, that the character of the ensuing inflammatory response in the airways was remarkably altered, such that eosinophilia was almost completely abrogated in favour of a more mononuclear and neutrophilic response (Wiley 2001). Moreover, BAL cytokine measurements and *in vitro* recall assays of splenocytes demonstrated that the predominant cytokine response shifted toward a Th1 profile upon treatment with IP-10 (Wiley 2001). Since IP-10 is not known to be able to influence Th1

differentiation, it was speculated that the mechanism of these effects was that IP-10 mediated biased recruitment of Th1-polarized OVA-specific T cells to the airways, and that these T cells were preferentially maintained as memory/effector cells in the secondary immune responses measured *in vitro* and after *in vivo* recall with OVA. This potential explanation for these results was not explored further in that study. It is at least theoretically possible that this could be the mechanism for the effect of IL-12 on GM-CSF-driven responses to OVA as well. However, the results reported in *Chapter 2* of this thesis would tend to argue against this explanation. By comparing gene expression during the course of respiratory mucosal sensitization to OVA in the context of airway expression of either GM-CSF alone or GM-CSF and IL-12, we observed that differentiation events in the lymph nodes were affected by the cytokines present in the airways, in a fashion consistent with polarization toward the Th2 and Th1 phenotypes, respectively. These data clearly demonstrated that the impact of cytokine expression in the airways does not simply alter leukocyte recruitment into the respiratory tract, but rather it fundamentally alters the nature of adaptive immune responses during their development in the secondary lymphoid tissues. Therefore, even when cytokine expression is completely localized at tissue sites, it can still have profound effects on immune events taking place in distant tissues. Of course, this still does not answer the question of *how* such effects are remotely mediated.

Our favoured hypothesis to explain these observations is that local cytokine expression at non-lymphoid sites conditions the tissue-resident antigen-presenting cells to become mature and activated in such a way that upon migration to the draining lymph nodes they elicit particular kinds of T cell responses. Although we have not exhaustively pursued this hypothesis as part of this thesis (examination of antigen presentation in these models has been the domain of my fellow PhD student

Beata Gajewska), Table 2 in *Chapter 2* indicates that indeed antigen-presenting cells in the lung do acquire a different profile of costimulatory molecule expression in the presence of GM-CSF versus that observed in the presence of GM-CSF and IL-12 during OVA exposure: with GM-CSF alone, B7.1 was expressed on a lower proportion of dendritic cells, and more transiently, than with GM-CSF and IL-12. Conditioning of APCs at the local tissue site could have a number of other effects on the APCs themselves which could influence T helper cell polarization, including the level of MHC class II expression, expression of other costimulatory molecules of the B7 family, expression of other costimulatory molecules such as OX40, expression of adhesion molecules such as ICAM-1, or the cytokines produced by the APC (Akiba 2000)(Constant & Bottomly 1997)(Glimcher & Murphy 2000)(Jones 2001)(Guery 1996).

Regardless of the precise details of the mechanism through which it occurs, the ability of cytokines present in the lung to affect lymph node differentiation events is not inconsequential, and could have important implications for understanding mucosal immunity, and for treatment or immunization strategies for many diseases. First of all, it highlights the significance of events taking place at the site of initial encounter with antigen as being determinative in the development of the type of immune response that ensues. From the data contained in this thesis, as well as other work from our lab, we know that the presence of GM-CSF in the airways is sufficient for the induction of allergic airway inflammation reminiscent of asthma. This suggests that agents which are able to induce endogenous GM-CSF expression in the airways may be capable of triggering pathogenic responses to airborne antigens, thereby leading to asthma; this will be discussed at length in the next section. In addition, these observations indicate that it is possible to direct immunotherapeutic strategies locally to the target organ itself, and thereby affect not

only local events in that organ, but also to modify the systemic responses to the antigen, without the risks associated in administering immunomodulatory agents systemically.

GM-CSF AS A CAUSATIVE FACTOR IN ALLERGIC AIRWAY DISEASE

A number of different environmental factors have been proposed to explain the increase in the incidence of allergic respiratory diseases. These include increased exposure to pollution or allergens, changes in diet and other lifestyle changes associated with Westernization, and changes in the prevalence of Th1- and Th2-polarizing infectious agents (Matricardi 2001)(Bardana 2001)(Martinez 2001). As for the latter of these, Peter Black has argued that it is unlikely that a shift away from Th1 responses and toward Th2 responses due to changes in infectious disease is responsible for the increase in asthma and allergy, since this explanation would also imply that the incidence of autoimmune disease would have decreased as allergic disease increased (2001), when in fact the incidence of autoimmune disease has increased in parallel with allergic disease (Stene 2001). We have proposed that increased exposure to GM-CSF-eliciting stimuli may have contributed to the increased incidence of both of these types of diseases (Ritz 2002a).

There is no question that GM-CSF plays an important role in the pathogenesis of allergic airway diseases, and evidence for this assertion abounds in the literature: for example, allergen challenge of asthmatic subjects elicited GM-CSF expression by BAL T cells (Bodey 1999); peripheral blood mononuclear cells from asthmatic patients make more GM-CSF when stimulated with IL-2, LPS, or dust mite antigens than normal control subjects (Nakamura 1993)(Hallsworth 1994)(Okano 1998); GM-CSF expression in BAL fluid was significantly associated with BAL eosinophilia and the severity of the late asthmatic response (Woolley 1995)

and with the severity of the clinical severity of asthma (Vignola 1999). There is also some suggestion that steroid treatment of asthma may operate in part through downregulation of GM-CSF expression by airway epithelial cells (Adkins 1998). Moreover, a genetic association has been identified, in that a variant in the GM-CSF gene has been correlated with atopic asthma in a population of Swiss children (Rohrbach 1999). We have more exhaustively described this evidence in a recent review article (Ritz 2002a), which can be found in *APPENDIX 1* of this thesis. Given the variety of immunological functions relevant to asthmatic processes that GM-CSF is known to play (as described in the *Introduction*), it is perhaps not surprising that it has been so frequently associated with asthmatic inflammation.

What is novel about the work presented in this thesis, however, is that it contributes to the evidence that GM-CSF may be a *causative* factor in the initiation of allergic airway diseases, and not just another cytokine that happens to be present in asthmatic inflammation. In conjunction with other work from our laboratory (Stampfli 1998)(Cates 2003a)(Cates 2003b), the research comprising this thesis elaborates the observation that the presence of GM-CSF alone can apparently create conditions in the airways which are conducive to the generation of Th2-polarized inflammatory responses against inhaled antigens. We have repeatedly shown that adenovirally-mediated transgenic expression of GM-CSF in the airways can generate Th2-polarized inflammation against the innocuous protein antigen OVA (Stampfli 1998)(Stampfli 1999a)(Stampfli 1999b)(Wiley 2001)(Ritz 2002b). We are confident that this is due to the GM-CSF itself, and not the adenoviral vector, because we have also delivered recombinant GM-CSF into the respiratory tract and found the same effect (Ritz 2002b), and because exposure to OVA after intranasal infection with the control adenovirus vector or with other cytokine-expressing vectors does not lead to the development of antigen-specific immune responses (Ritz 2000). Moreover, the

endogenous production of GM-CSF in response to certain agents can also stimulate adaptive immune responses, since allergic sensitization to both ragweed pollen and house dust mite antigens were largely prevented by treatment with antibodies against GM-CSF (Cates 2003a)(Cates 2003b). Taken collectively, these observations definitively support the hypothesis that the presence of GM-CSF in the airway microenvironment is sufficient to cause the development of antigen-specific immune responses to airborne allergens, at least in mice. Hence we are compelled to consider that aberrant GM-CSF expression or exposure to GM-CSF-eliciting stimuli in the environment during initial exposure to airborne antigens may be responsible for the initiation of allergic airway diseases in humans as well.

The airway epithelium is the primary interface between the external environment and the respiratory tract, and forms an enormous surface area. The epithelial area available for gas exchange alone is estimated to be 70 m^2 in an average adult, and this does not include the epithelium of the conducting airways (Levitzky 1999). During respiratory ventilation, this large area is in contact with all of the contents of inhaled air, including a variety of gaseous and particulate entities in addition to the air itself. Some of these environmental agents are able to effectively induce the expression of GM-CSF from airway-resident cells, particularly epithelial cells and alveolar macrophages. Allergens themselves, some kinds of pollution, and many viruses and bacteria are potentially relevant environmental stimuli for the production of GM-CSF, and may thereby have the ability to trigger allergic responses to airborne allergens.

• *Allergens*

Although allergens are not pathogens, they are not always completely innocuous substances either; many aeroallergens are known to have enzymatic and

proteolytic activity (Shen 1999)(Stewart 1993), including house dust mite (Hewitt 1998). These proteolytic allergens are known to be able to cleave proteinase-activated receptors (PARs) on airway epithelial cells (King 1998)(Vliagoftis 2001)(Sun 2001). Knight *et al* have observed that PAR-2 is more highly expressed on airway epithelium from asthmatic subjects than normals (2001), raising the possibility that increased constitutive expression of PAR-2 may enhance susceptibility to the development of asthma. Cleavage of PAR-2 activates the receptor, and causes the expression of GM-CSF (King 1998)(Vliagoftis 2001)(Sun 2001); based on our findings that GM-CSF facilitates allergic sensitization, the PAR-2-mediated expression of GM-CSF provides a mechanism through which proteolytic allergens could mediate sensitization to themselves. However, even protease-deficient allergens, such as rye grass pollen, are able to induce the expression of GM-CSF from airway epithelial cells (Knight 2000), indicating that there are also protease-independent pathways through which allergens can elicit cytokine expression. Blocking antibodies against GM-CSF prevented sensitization to ragweed and house dust mite in a murine model of respiratory sensitization, demonstrating conclusively that allergen-induced GM-CSF can provoke allergic sensitization *in vivo* (Cates 2003a)(Cates 2003b).

• *Pollution*

Pollution has been often dismissed by experts as a cause of allergic disease (Teotonio 2003), although they usually acknowledge that it can exacerbate existing disease. In support of their case, such experts frequently cite the study of von Mutius *et al*, who studied the incidence of allergic respiratory disease in the former East and West Germany, and showed that the 'heavily' polluted East actually had a lower incidence of these diseases than in the 'clean' West (1994). However, such an

interpretation of this data does not take into account the disparate nature of the pollution present in each country, nor other factors which may account for the observed differences. A more comprehensive examination of the literature indicates that such dismissal is, in fact, not well-founded. Although still controversial, there is substantial evidence that exposure to pollution can elicit allergic sensitization, at least under experimental conditions. Moreover, there is epidemiologic and correlative evidence which, although not conclusive, certainly warrants further investigation into this possibility.

Motor vehicle-generated air pollution has increased progressively since the 1950s, causing the accumulation of fine particulate matter and ozone in heavily populated areas (Parnia 2001)(D'Amato 2001), in concert with the observed increase in the incidence of allergic diseases. Diesel exhaust particles may be a particularly important pollutant with respect to the initiation of allergic airway disease, and in fact diesel exhaust has been shown to cause allergic sensitization in experimental systems in mice and in humans (Steerenberg 2003)(Fernvik 2002)(Diaz-Sanchez 1999). Studies with other types of pollution such as ozone and cigarette smoke have shown that these entities can also induce allergic inflammation in animals (Tsai 1998)(Rumold 2001), and a recent study demonstrated a link between ozone exposure and asthma incidence in children (McConnell 2002).

The ability to elicit GM-CSF production may be a key mechanism through which airborne pollutants may contribute to asthma susceptibility. A number of different pollutants have been shown to be able to induce GM-CSF expression from airway epithelial cell lines, primary bronchial epithelial cells *in vitro* (Bayram 2001)(Bonvallot 2001)(Devalia 1993)(Fujii 2001), alveolar macrophages (van Eeden 2001), and *in vivo* (Aris 1993), including ozone, nitrogen dioxide, ambient particulate matter, and diesel exhaust. Moreover, the airway hyperreactivity induced by

exposure to diesel exhaust particles was reversed by treatment with anti-GM-CSF in a murine model (Ohta 1999). Unfortunately, to date there have been no studies which have examined whether blocking GM-CSF can prevent the development of allergic inflammation in these models.

• *Viruses and Bacteria*

During defensive immune responses against a variety of microbes, GM-CSF production is elicited in the airways. GM-CSF expression is particularly well documented in the case of infections with rhinovirus and various kinds of Gram-negative bacteria (LPS is a particularly potent stimulus for GM-CSF production in the airways), but is undoubtedly present in many other kinds of infection as well. However, the ability of microbes generally to facilitate allergic sensitization is a very complex issue, owing, in part, to the diversity of immune responses that they can elicit (Renz 2002). Although they are often able to elicit GM-CSF production, the ability of microbial infections to instigate Th2-polarized allergic sensitization is also likely to depend on whether or not they concurrently evoke other signals (such as IL-12), which may check the development of Th2 responses.

Of course, environmental factors alone do not determine the outcome of antigen exposure; exposure to aeroallergens and pollutants is virtually ubiquitous, while only a subset of the population becomes sensitized, and a still smaller group actually develops clinically-evident allergic disease. Thus it is obvious that it is the interaction between host factors and environmental stimuli which critically determine the outcome of antigen exposure; in other words, the ability of environmental agents to promote or inhibit allergic tendencies will depend largely on the specific characteristics of the interaction between any given agent and any given

host at a given time. However, minimizing exposure to potentially harmful environmental agents would likely be an effective strategy in reducing the incidence of asthma and allergic respiratory diseases at the population level.

MODIFIED MODEL OF TH2 POLARIZATION: GM-CSF CREATES CONDITIONS CONDUCIVE TO IL-4-INDEPENDENT TH2 POLARIZATION

I would argue that the Th1/Th2 paradigm has been the most important concept shaping our immunological view of asthma over the last 15 years. The model has been widely accepted and reified in the community of researchers studying asthma and allergy, and frequently utilized as a conceptual framework for the interpretation of data and the development of novel strategies for intervention. For example, it has been frequently suggested that affecting the Th1/Th2 balance in favour of Th1-polarized responses could be a treatment (or even a cure) for asthma.[1] Also, given the importance of Th2-polarization in allergic airway diseases, finding ways to prevent these responses from developing in the first place should be able to prevent disease; in order for this to become a reality, however, we must define those conditions which are necessary or sufficient for the development of Th2-polarized responses.

As discussed extensively in the *Introduction*, the traditional Th1/Th2 paradigm holds that IL-4 is particularly critical for the induction of Th2-polarized responses. Early studies in animal models of asthma indicated that blocking IL-4 during the sensitization phase could prevent the subsequent development of pathological airway inflammatory responses (Kips 1995). This possibility was attractive enough to motivate clinical trials into the use of IL-4 antagonists for the treatment of asthma;

[1] This idea persists, in spite of the likelihood that superfluous Th1-polarized inflammatory responses could be equally damaging.

results of these studies have shown only modest effects (Borish 2001). From our present perspective, however, these findings are not especially surprising, since most of the pathophysiological hallmarks of asthmatic inflammation and airway dysfunction can be caused by cytokines other than IL-4, such as IL-5 and IL-13; therefore, blocking IL-4 in established disease is unlikely to be very effective due to the presence of these other molecules. Moreover, there is a growing amount of data indicating that IL-4 is not necessary for the generation of Th2-polarized immune-inflammatory responses in the first place. *Chapter 3* of this thesis documents our observations that, in our model of GM-CSF-driven respiratory mucosal sensitization, indices of Th2 polarization are evident despite the congential absence of IL-4 in 4KO mice. The indicators of Th2 polarization that we elected to analyze cover a wide range of molecular and cellular readouts: mRNA expression of the transcription factor GATA-3; protein analysis of protypic Th2 cytokines in the BAL; mRNA expression of Th2-affiliated chemokine receptors; surface expression of the Th2 marker T1/ST2 on the surface of T helper cells; and eosinophilic inflammation. We find these data themselves to be convincing evidence that Th2 polarization occurred in IL-4 knock-out mice. In the context of other reports of IL-4-independent Th2 phenomena that have been published in the literature (Ritz 2002)(Brewer 1999)(Chensue 1997)(Herrick 2000)(Hogan 1997)(Hogarth 1998)(Kopf 1996)(Kropf 1999)(Mohrs 2000)(Noben-Trauth 1997), any assertion that IL-4 is required for Th2 differentiation is clearly not correct. As expressed by Noben-Trauth *et al*, "[i]t is now clear...that IL-4Rα/STAT6 signaling is not essential for priming of $CD4^+$ T cells to produce Th2 cytokines in vivo" (2003). Hence, it would appear that the traditional paradigm of Th2 differentiation requires refinement to account for these observations.

Over the past several years, other factors have been identified which promote Th2 polarization. One such factor is the cytokine IL-10. Usually regarded as an immunoregulatory factor, IL-10 likely plays pleiotropic roles in the regulation of allergic airway inflammation, depending on the context of its expression. We and others have previously demonstrated that IL-10 can downregulate eosinophilic inflammatory responses in the airways (Stampfli 1999a)(Tournoy 2000). Clinical evidence also indicates that severe asthma is associated with attenuated production of IL-10 from peripheral blood monocytes (Tomita 2002), and that successful treatment with specific immunotherapy is associated with increased expression of IL-10 (Bellinghausen 2001). These observations tend to insinuate that IL-10 plays a protective role in allergic disease; some versions of the hygiene hypothesis put forward this theory explicitly (Wills-Karp 2001). However, there is other evidence suggesting that IL-10 can in fact promote Th2 responses under certain circumstances.

In *Chapter 4* of this thesis, we examined the role of IL-10 during GM-CSF-driven respiratory mucosal sensitization, particularly in the absence of IL-4. Administration of anti-IL-10 antibodies was associated with a significant decrease in the number and percentage of BAL eosinophils in 4KO but not WT Balb/c mice. Moreover, levels of the Th2 cytokines IL-5 and IL-13 were reduced in WT and 4KO mice treated with anti-IL-10 antibodies, both in the BAL and in *in vitro* recall assays with splenocytes. Most striking was the effect of anti-IL-10 treatment on the fold-increase in IL-5 production from splenocyte cultured with OVA. When these experiments were repeated in IL-4, IL-10, or IL-4/IL-10 gene knock-out mice on the C57BL/6 background, we observed no eosinophilic inflammation in the BAL of 4KO, 10KO, or 4/10KO mice. IL-5 production by splenocytes from WT and 4KO mice was upregulated, but 10KO and 4/10KO splenocytes did not produce IL-5

upon antigen restimulation. Collectively, these data indicated to us that IL-10 was contributing to the polarization of Th2 responses in this model

These observations are consistent with other recent reports defining a role for IL-10 in the development of Th2 responses. Studies in murine models of *Leishmania major* infection (where susceptibility to *Leishmania* is associated with Th2 responses) indicate that IL-10 knock-out mice are better able to control infection than WT mice (Mentink Kane 2001), and a recent paper suggests that there is a additive effect of IL-4 and IL-10 in the development of Th2 responses, since "neither IL-10$^{-/-}$ mice nor wild-type mice treated with anti-IL-10R mAb approached the striking level of resistance displayed by IL-4Rα KO mice that were also deficient in IL-10 or IL-10R signalling" (Noben-Trauth 2003). Moreover, the production of Th2 cytokines in this model was significantly decreased in the absence of IL-10 signalling, and this was further reduced in the absence of both IL-4 and IL-10 signalling (Noben-Trauth 2003). These observations and our own, in 2 independent experimental models, strongly implicate IL-10 in the development of Th2 responses.

How IL-10 may motivate Th2 responses is not clear, but there is suggestive data that myeloid dendritic cells secrete IL-10 during their interactions with naive T cells. Myeloid dendritic cells have been shown to be able to preferentially elicit Th2-polarized immune-inflammatory responses when adoptively transferred *in vivo* (Pulendran 1999)(Lambrecht 2000)(Lambrecht 2001). Dendritic cells derived from mouse Peyer's patches produce IL-10 and preferentially induce Th2 responses (Iwasaki 1999). Moreover, in 2001, Maldonado-Lopez *et al* observed that myeloid (CD8α$^-$) dendritic cells from IL-10 knock-out mice were unable to efficiently induce the differentiation of Th2 cells *in vitro*, whereas IL-4 knock-out dendritic cells had a much smaller (non-significant) defect in the ability to elicit Th2 differentiation. These data are consistent with our observations that Th2 polarization during GM-

CSF-driven respiratory mucosal sensitization is largely IL-4 independent, but relies on IL-10 for full expression of the Th2 immune-inflammatory response. However, it is unlikely that dendritic cells with a myeloid phenotype are *only* able to elicit Th2 responses; others have shown that they can induce either Th1 or Th2 responses depending on immunological context (Lopez 2001). This is also compatible with our observation that the addition of IL-12 to the airway milieu can change the costimulatory phenotype of $CD11b^+$ $CD11c^+$ myeloid dendritic cells (table 2, *Chapter 2)*, and also lead to a different inflammatory response (Stampfli 1998b).

Based on these observations, we would propose a modification of the traditional Th1/Th2 paradigm, particularly with respect to the generation of Th2 responses. We would argue that the outcome of T helper cell activation and differentiation is largely determined by the conditions under which antigen presenting cells are activated at the original site of encounter between antigens and the host (usually the mucosae). Upon interaction with environmental entities, tissue-resident cells (such as epithelial cells, macrophages, fibroblasts, keratinocytes, mast cells, smooth muscle) can secrete a variety of soluble mediators into the surrounding area; the variety of mediators secreted will vary with the nature of the stimulus which elicited them. This causes local antigen-presenting cells to become activated and differentiate into mature APCs, with potentially diverse phenotypes determined by the types of mediators which are present. For example, the presence of certain allergens and airborne pollutants (such as ozone, nitrogen dioxide, diesel exhaust and other particulate matter) can elicit the production of GM-CSF from airway epithelial cells, as discussed earlier. As elaborated in the *Introduction*, GM-CSF is the most potent known stimulus for the expansion, activation, and maturation of myeloid dendritic cells. Having taken up antigen at the mucosal site, these mature dendritic

cells then migrate to the local draining lymph nodes, where they interact with and activate antigen-specific naive T cells.

The pathway of T helper cell differentiation after this point likely depends on the interaction of a number of different factors, rather than on a single determinant. The observations described in *Chapter 4* of this thesis indicate that IL-10 is one such factor that can influence the differentiation of T cells along the Th2 pathway, but there are likely many others as well (see *Introduction*). Given the observations of Maldonado-Lopez *et al* (2001) and Iwasaki *et al* (1999), we hypothesize that in our model of respiratory mucosal sensitization, the presence of GM-CSF in the airways during OVA antigen exposure caused expansion of a population of myeloid dendritic cells which were able to produce IL-10 in the lymph nodes, thereby promoting Th2 polarization. Blocking IL-10 inhibited this process, by altering the balance of Th2-promoting signals below the threshold necessary to efficiently elicit Th2 differentiation, particularly in the absence of IL-4, where consolidation of the Th2 phenotype may not effectively occur. In contrast, the additional presence of IL-12 during antigen exposure alters the phenotype of maturing dendritic cells in such a way that they preferentially induce Th1 responses instead of Th2; the different pattern of B7.1 and B7.2 expression on dendritic cells from GM-CSF/OVA-exposed and GM-CSF/IL-12/OVA-exposed mice support this contention.

This model differs from the conventional model of Th1/Th2 differentiation in that it places greater emphasis on the role of the antigen presenting cell itself, and the conditions under which *it* was activated, in determining the outcome, rather than exclusively focussing on the T cell. Such a shift in perspective is not trivial, particularly in envisioning ways and means through which we could intervene in these processes. For example, under the old paradigm, efforts to abort Th2 differentiation as a potential preventative or treatment for allergic disease tended to

focus on interfering with IL-4; as mentioned earlier, anti-IL-4-directed therapy was the subject of intense investigation for a number of years, and even made it to the stage of clinical trials, although this approach proved to be largely disappointing (Borish 2001). Enlarging our perspective, both spatially and temporally, to consider the importance of circumstances earlier in the chain of events and at sites distant from the lymph nodes, provides additional opportunities to intervene and prevent the development of pathological immune-inflammatory responses.

CONCLUSIONS

The rise in the incidence of allergic respiratory diseases in the last 30 years (Omran 1996)(Sly 1999)(Ulrik 1996)(Ciprandi 1996)(Ulrik 2000) raises the question as to which factors contribute to the pathogenesis of such diseases. The genomic revolution has launched a massive research effort into understanding the genetic basis of disease, and indeed a great deal has been invested in the search for 'asthma' and 'allergy' genes. However, the identification of genes associated with allergy and asthma does not help us to understand the rising prevalence of these diseases, since the increase has taken place over too short a period of time to be explained by genetic causes. More likely, the increase in allergy and asthma is related to changes in the environment, which differentially affect those individuals who are genetically susceptible to such changes. Although there is little we can do to affect the heritable vulnerability of populations to allergic disease, universally reducing exposure to environmental factors which can trigger disease is likely to be a fruitful approach. Hence it is of importance to elucidate the mechanisms of allergic sensitization so that we can identify factors able to set these cascades in motion, and limit our exposure to them.

The work contained in this thesis accentuates the importance of the cytokine microenvironment in the lung at the time of antigen exposure in determining the outcome of the ensuing immune-inflammatory response, describes some of the key molecular and cellular events that take place during the evolution of these immune responses, and examines the necessity for particular cytokine signals in the generation of the Th2 responses strongly implicated in allergic disease. These observations provide novel insight into the mechanisms mediating allergic sensitization in the respiratory tract, and contribute to our understanding of respiratory immunobiology more generally. Among the challenges before us is to translate this knowledge into clinically useful approaches for the prevention and treatment of allergic disease, whether that be through direct interference with the mediators identified as necessary or sufficient for their pathogenesis, or via the further elucidation of agents which can elicit the expression of these mediators and the minimization of exposure to such agents.

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APPENDIX 1:
ON THE GENERATION OF ALLERGIC AIRWAY DISEASES: FROM GM-CSF TO KYOTO

The following manuscript is a review article, reprinted from *Trends in Immunology* 23:396 August 2002, with permission from Elsevier (see Appendix 2). In it, we: [*A*] review evidence that GM-CSF is an important factor in the pathogenesis of allergic airway diseases; [*B*] elaborate our hypothesis that GM-CSF may be able to induce allergic sensitization and inflammation in the airways in responses to otherwise non-pathogenic agents; and [*C*] summarize evidence that environmental agents such as allergens and pollutants can elicit GM-CSF expression, and may therefore be responsible for the increased incidence of allergic airway diseases seen in the last several decades.

My role in the preparation of this review article was to extensively review the published literature for relevant research, and to take primary responsibility for the writing of the manuscript, as well as the preparation of figures. My co-authors contributed to the writing as well, and we functioned as a team in terms of the conceptualization of the paper.

On the Generation of Allergic Airway Diseases: from GM-CSF to Kyoto

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TEASER

Why is asthma prevalence increasing in the West? Exposure to certain environmental agents can trigger the expression of GM-CSF in the airways. Is an elevation of GM-CSF in part responsible for Th2-mediated allergic asthma?

SUMMARY

The sharp increase in the prevalence of asthma over the last three decades suggests an important contribution of environmental factors in the generation of this disease, and compels a search for molecular pathways by which such factors may facilitate Th2 immune-inflammatory airway responses; GM-CSF may be one such signal. Here, we appraise the evidence with respect to the presence of GM-CSF in asthma, the roles played by GM-CSF in these immune responses, and environmental triggers that can induce GM-CSF expression. Further, we propose a paradigm that unites these divergent observations, and postulate that GM-CSF produced in response to environmental agents may establish an airway microenvironment that foments the initiation, influences the evolution and supports the maintenance of an aeroallergen-specific adaptive Th2 immune response.

KEYWORDS: GM-CSF, asthma, Th2, epithelial cells, dendritic cells, allergen, pollution

INTRODUCTION

Over millions of years, the immune system has evolved sophisticated mechanisms to eliminate harmful replicating pathogens such as viruses, bacteria and nematodes. From this perspective, the survival advantage of generating immune-inflammatory responses against allergens we breathe in all the time is not apparent. It is therefore a

challenge to understand why aeroallergens play such an important role in airway diseases such as asthma. This issue is imperative given that the worldwide prevalence of asthma has risen and continues to rise. That such a dramatic change has occurred over the last 20-30 years indicates that this is unlikely due to a population-level genetic shift, and strongly incriminates environmental factors (reviewed in [1]).

It is widely accepted that the histopathological and functional abnormalities characterizing asthma are largely mediated by the cytokines IL-3, IL-4, IL-5, IL-9 and IL-13 produced primarily, but not exclusively, by Th2 cells. That aeroallergen exposure is ubiquitous yet only 10% of the population develops asthma has stimulated research to investigate circumstances that prevent the development of asthma and allied allergic airway diseases in the majority of people.

The current pre-eminent hypothesis, referred to as the Hygiene Hypothesis, (reviewed in [2] and [3]), proposes that Th1-polarizing infections in early childhood abort the development of Th2-mediated airway diseases, and implies that the current asthma epidemic is related to the decreased incidence of such infections in the developed Western world. While the strength of the epidemiological evidence for the decrease in early childhood infections and the increase in asthma prevalence is undisputed, the connection remains at this point correlative.

The counterregulatory hypothesis suggests that the presence of immunosuppressive cytokines such as IL-10 and TGF$ will blunt the development of allergic diseases. An implication of this hypothesis is that a relative defect in either constitutive or induced IL-10 production in some individuals may be related to the emergence of asthma. Murine studies of the phenomenon of inhalation tolerance have demonstrated that respiratory exposure to an innocuous protein antigen (ovalbumin, OVA) induces immune activation, resulting in the generation of regulatory T cells which are able to produce cytokines such as IL-10 and TGF$, and thereby prevent

future inflammatory responses against that antigen [4]; recently, a subset of CD4+ CD25+ T cells producing such regulatory cytokines has been identified in humans [5-7]. It has also been proposed that IL-10 generated during chronic infections can suppress the development of allergic tendencies (reviewed in [8]).

Both theories, immune-deviation and counter-regulation, assume that in the absence of a regulatory mechanism, the allergen-specific response is biased towards a type 2 cytokine pattern. While there is a significant amount of data in the human literature that supports such a notion, this contention effectively bypasses the question of why and how aeroallergens might elicit deleterious Th2 immunity. Moreover, the extent to which this Th2 bias per se influences the generation of the 'asthmatic phenotype' (as opposed to the 'allergic phenotype') is not resolved (reviewed in [9]). It is of interest to note that while the prevalence of asthma has risen to approximately 10%, the prevalence of allergic sensitization is currently estimated at 40%; these data suggest that allergic sensitization per se is not sufficient for the development of asthma, and that additional factors may be required to provoke airway disease in an allergic individual.

We contend that the ability of aeroallergens to cause allergic airway inflammation is contingent upon the presence of an immunological milieu in the airway microenvironment that privileges Th2 responses. In this document, we survey the evidence that GM-CSF may be one factor with such an instructive signature that is well-documented to be present in asthma. The potential immunological impact of a GM-CSF-rich microenvironment is considerable, given that GM-CSF is one of the most powerful natural activators of dendritic cells, the most important antigen-presenting cells for the activation and differentiation of naive T cells. That epithelial cells and alveolar macrophages can produce substantial amounts of GM-CSF in response to a variety of stimuli presents a paradigm for how the innate

mucosal system (that is, macrophages, dendritic cells, epithelial cells, and the underlying mesenchymal cells) may determine the initiation, influence the evolution and support the maintenance of adaptive immune responses.

ESTABLISHING A PLAUSIBLE LINK BETWEEN GM-CSF AND TH2-MEDIATED ALLERGIC AIRWAY DISEASES

In the 19th century, the German microbiologist Robert Koch devised a set of postulates which, when fulfilled, furnished a firm basis upon which to appraise the connection between a given microorganism and a particular disease. As a result of the explosion in genetics over the past 25 years, and especially since the completion of the Human Genome Project, biologists have been confronted with a plethora of genes for which the function is not known or only superficially understood. Thus, we have taken the liberty of modifying Koch's postulates for the genomic era, such that they can be applied to investigate the functional contribution of endogenous molecules in disease pathogenesis (Box 1). This adaptation of Koch's postulates is not intended to provide unequivocal proof for causality, but rather, to provide a framework for appraising the likelihood of a causal relationship between a molecule and a pathological process. Using these postulates as a guide, we have appraised the evidence for a link between GM-CSF and Th2-mediated allergic airway diseases, particularly asthma.

Postulate 1: Presence in Disease Cases

Detection of GM-CSF in asthma and other allergic airway diseases is the first requisite to ascertain whether this growth factor might be important in the pathogenesis of these disorders. In asthma, increased production of GM-CSF at both the RNA and protein levels has been consistently observed in bronchial epithelial

cells [10], both the cellular and fluid components of BAL [11-12], sputum [13], sputum cells [14], and in antigen-stimulated peripheral blood mononuclear cells [15]. Moreover, airway expression of GM-CSF has been found to correlate with eosinophilia and the severity of the late asthmatic response [16] as well as with asthma severity scores [17]. GM-CSF expression has also been observed in allied allergic airway diseases such as allergic rhinitis [18], and nasal polyposis [19]. That GM-CSF expression has been constantly detected in many studies, in a variety of pathological specimens, and in different allergic airway pathologies, compels us to evaluate further the potential role of GM-CSF in the pathogenesis of all allergic airway disorders.

Postulate 2: Differential Expression

The consistent detection of GM-CSF in patients with asthma and other allergic airway diseases is unlikely to be of pathogenetic relevance unless it is distinct from that observed in non-asthmatic subjects. In this regard, a number of studies have demonstrated that both cells in the circulation and airway resident cells isolated from asthmatic subjects produce more GM-CSF than cells from non-asthmatic subjects in response to a variety of stimuli. For example, peripheral blood mononuclear cells from asthmatic patients produced more GM-CSF than did cells from non-asthmatic subjects, constitutively [20] as well as in response to LPS [20,21], and antigen [22]. In addition, bronchial epithelial cells from asthmatic patients produced significantly greater amounts of GM-CSF than did cells from normal controls, not only constitutively [23], but also when cultured with various stimulants [23-25]. Recently, a polymorphism in the gene for GM-CSF has been described in an asthmatic population, suggesting that there may be at least one genetic basis for this differential expression [26].

Importantly, among airway inflammatory diseases, the high expression of GM-CSF is unique to asthma. Indeed, GM-CSF is not detectable in sputum from patients with COPD [27], and direct comparisons of biopsy tissue demonstrates that GM-CSF is present at much higher levels in asthma than in chronic bronchitis [17].

Postulate 3: Experimental Induction and Exacerbation of Disease

The evidence presented to this point for a role for GM-CSF in asthma and other allergic airway diseases is credible yet circumstantial. Inherent limitations of human research preclude experimentation to obtain direct evidence in vivo for a causative role of GM-CSF in the generation of the asthmatic phenotype. However, it is precisely in these instances that the use of experimental animal models can provide unique information.

It has been repeatedly demonstrated in mice that aerosolization of a prototypic innocuous antigen such as ovalbumin (OVA) alone evokes inhalation tolerance. However, if exposure to OVA takes place in the context of an airway microenvironment containing physiological levels of GM-CSF (80-100 pg/mL BAL fluid) the result is not tolerance, but rather an active immune-inflammatory response that is Th2-polarized and characterized by the production of Th2 cytokines, IgE, blood and lung eosinophilia, goblet cell hyperplasia, antigen-specific Th2 memory, and bronchial hyperreactivity [28]. These data are unequivocal evidence that GM-CSF can condition an airway environment conducive to Th2 responses and allergic airway inflammation. That the role of GM-CSF in allergic airway diseases may extend beyond facilitating allergic sensitization is illustrated by the observation that administration of exogenous GM-CSF during aeroallergen challenge in previously sensitized mice exacerbates airway eosinophilic inflammation, and delays the resolution of this response [29].

Postulate 4: Experimental Inhibition of Disease

The contention that GM-CSF is important in the generation and perpetuation of allergic inflammation in the airways would predict that its inhibition should lead to an amelioration of disease. There are, at this point, no studies directly examining this question. However, Ohta et al. have demonstrated that the use of an antibody to block GM-CSF prevented airway hyperresponsiveness elicited upon exposure of mice to diesel exhaust particles [30]. Unfortunately, this study did not examine immune-inflammatory outcomes in response to antigen; therefore, whether the blockade of GM-CSF would prevent allergic sensitization, airway inflammation or both remains unresolved. In addition, Borchers et al. recently observed that eosinophilic allergic airway responses were abrogated in Gq knock-out mice (which hampers G-protein-mediated signal transduction), in association with low levels of lung GM-CSF; importantly, airway eosinophilia was completely reconstituted in these mice by administration of GM-CSF [31].

Thus far, no studies have been published evaluating the potential value of a GM-CSF neutralization strategy for allergic diseases in humans. However, although no such direct evidence is available, data from intervention studies with corticosteroids show a correlation between amelioration of symptoms and decreased expression of GM-CSF [14,32]. Similarly, treatment with cyclosporin A attenuated the allergen-induced late asthmatic response, and also reduced GM-CSF production [33]. The therapeutic benefits of these strategies are certainly not realized exclusively through their effects on GM-CSF production, but the concomitant reduction both in asthma symptoms and GM-CSF expression are at least consistent with the possibility that GM-CSF is important in asthma pathogenesis. Moreover, in vitro studies have shown that blocking GM-CSF in BAL supernatants has a major effect in reducing the survival and persistence of human eosinophils [34]. In summary, while the

relationship between GM-CSF downregulation and improvement of asthmatic symptoms is consistent, it remains correlative; conclusive evidence proving a causal connection is not presently available in either human or experimental systems.

IMPACT OF GM-CSF ON ANTIGEN PRESENTATION, T CELL DIFFERENTIATION, AND EFFECTOR EVENTS IN THE AIRWAYS

As elegantly reviewed by Lanzavecchia and Sallusto [35], our current understanding of the generation of T cell responses is that such responses are initiated in the draining lymph nodes, where naïve T cells interact with dendritic cells (DCs) that have migrated from peripheral sites carrying antigen and are equipped with a particular package of immunological instructions. While specific recognition of antigens via the T cell receptor/MHC interaction defines the specificity of the immune response, the additional instructions carried by the DCs determine the nature of the immune response generated. That the acquisition of the mature DC phenotype is influenced by signals at the mucosal site highlights the importance of events that occur in the airway microenvironment in the ultimate outcome of antigen exposure.

Dendritic cells, the most potent professional antigen-presenting cells, are ubiquitously found in the airway submucosa, projecting their dendrites through the interepithelial spaces into the airway lumen in order to survey antigens in the environment. In the absence of "danger" signals, they reside in a resting, immature state. However, contact with pathogens, necrotic cells or pro-inflammatory cytokines launches a series of events that equip DCs with the necessary apparatus to migrate to the regional lymph nodes and activate T cells. Since aeroallergens are neither pathogens nor necrotic cells, the cytokine milieu in the airway environment at the time of exposure is presumably critical in the processes of DC activation and

maturation (Figure 1). In this context, GM-CSF is known to enhance antigen presentation by inducing proliferation, activation, and maturation of dendritic cells ([36], and reviewed in [37]). The functional impact of such events is illustrated by the demonstration that GM-CSF enhances the ability of DCs to generate antigen-specific B and T cell responses [38,39]. Thus, the presence of a GM-CSF-rich airway environment would seem to promote the development of an immune response even if the antigen per se does not warrant such response. Importantly, respiratory exposure to prototypic innocuous antigens such as OVA in the context of other cytokines (such as TNF", IL-2, IL-4, and IL-6) does not lead to Th2 sensitization or antigen-specific allergic airway inflammation (unpublished data reviewed in [40]), suggesting that this effect is specific to GM-CSF and not a general feature of all pro-inflammatory cytokines.

GM-CSF is not known to influence the differentiation of naïve T cells directly, but is rather more likely to do so through its effects on antigen presenting cells (APCs), especially DCs, which can in fact regulate the development of Th1 and Th2 subsets (reviewed in [41]). For example, GM-CSF preferentially promotes the differentiation and proliferation of dendritic cells of the myeloid lineage [42], and adoptive transfer of such GM-CSF-stimulated myeloid DCs into mice elicited in vivo immune responses characterized by the presence of Th2-associated cytokines and immunoglobulins [42,43]. Ultimately, the outcome of antigen presentation to T cells will be determined by the convergence of signals delivered to them by APCs; it is, then, plausible that the default immune pathway facilitated by GM-CSF is a Th2 response, and that Th1 differentiation requires additional signals, for example IL-12 [44], or the presence of a replicating pathogen [45].

As asthma becomes consolidated, there occurs a dynamic interaction between the inflammatory and remodelling components that serves to enhance and

prolong the inflammatory response (Figure 2). Th2 cells and eosinophils can themselves make GM-CSF, and also produce mediators which can stimulate epithelial cells to produce GM-CSF [46,47]; that GM-CSF is a significant factor for the survival and function of T cells and eosinophils further contributes to the establishment of a self-sustaining immune-inflammatory environment. In addition to inflammation, airway dysfunction in asthma is the result of structural changes to the airway wall with increases in smooth muscle, matrix, and mucous-producing cells being most relevant (reviewed in [48]). Injury to the epithelium caused by a wide range of environmental insults leads to epithelial damage and aberrant repair. Growth factors produced by the epithelium cause the proliferation and differentiation of mucosal mesenchymal cells, leading to airway wall thickening consequent upon increased matrix production and smooth muscle proliferation [49,50]. In this chronic cycle of epithelial injury/repair the bronchial epithelium becomes a highly active source of growth factors including GM-CSF. Underlying mesenchymal cells that differentiate into myofibroblasts and smooth muscle also become important sources of GM-CSF [51,52].

ENVIRONMENTAL TRIGGERS OF GM-CSF PRODUCTION IN THE AIRWAYS

A fundamental step in the proposed sequence of events leading to asthmatic responses remains unaddressed: what environmental entities can interact with cells in the respiratory mucosa and trigger the production of GM-CSF?

Allergens themselves are able to elicit GM-CSF production by the bronchial epithelium. Airway epithelial cells from asthmatic donors but not from control subjects produced GM-CSF when cultured in the presence of house dust mite antigens, suggesting that the ability to produce GM-CSF upon exposure to allergen

may be unique to asthmatics [24]. Mechanistic insight for this observation comes from considering that a substantial number of the most relevant aeroallergens are proteases, and can cleave protease activated receptors (PAR) on airway epithelial cells, thereby causing the production of cytokines, including GM-CSF [53]. Moreover, PAR-2 has been demonstrated to be expressed at higher levels on the respiratory epithelium from asthmatic subjects [54], raising the possibility that high constitutive expression of PAR-2 may be a risk factor for asthma. However, even allergens that are deficient in protease activity, such as rye grass pollen, are able to elicit GM-CSF expression from lung epithelial cells [55], suggesting that there may be protease-independent pathways through which allergens can elicit GM-CSF production by epithelial or other airway cells.

There is extensive evidence that a number of pollutants induce GM-CSF expression in airway resident cells in vitro. For example, ambient urban air particles, diesel exhaust particles, NO2, and ozone are all known to elicit GM-CSF production from human bronchial epithelial cells in vitro [56-59]. Of particular interest are data showing that GM-CSF production from epithelial cells of asthmatic patients in response to pollutants is substantially greater than that from normals [23,25]. Moreover, the ability of such pollutants to induce allergic airway disease has been established in experimental systems both in animals and in humans [60-63], and a recent prospective cohort study in The Lancet provides epidemiological evidence that exposure to high-ozone environments significantly increases the risk of developing asthma in children [64]. The mechanisms leading to such pollution-induced allergen sensitization have not been explored, but given that the pollutants in question are known to induce airway expression of GM-CSF, and that allergen exposure in the context of GM-CSF expression has been shown to lead to

allergic sensitization [28], it is reasonable to hypothesize that sensitization may be mediated through the effects of GM-CSF in these systems.

As compared to aeroallergens and environmental pollutants, there is much less information with respect to the ability of microbes to facilitate allergic sensitization and asthmatic responses. This is a complex issue due, in part, to the diversity of immune responses that microbes can elicit (reviewed in [65]). Various pathogens (and their constituents, like LPS) can indeed stimulate GM-CSF production by airway epithelial cells, but whether microbial infections are able to instigate allergic responses is likely related to their ability to evoke airway microenvironments also rich in IL-12 or IFN(, which would be expected to inhibit Th2 processes.

CONCLUSIONS

GM-CSF is, in all likelihood, not the only factor that could predispose an individual to airway disease, and moreover it is not the only cytokine that is dysregulated in asthma or that is upregulated by exposure to environmental entities. In this paper, we have focussed on the role of GM-CSF in asthma because we believe that the potentially important role of GM-CSF in the pathogenesis of asthma has been largely overshadowed by its Th2-affiliated cousins.

Research in the past 10 years has repeatedly implicated GM-CSF as a factor in the pathogenesis of asthma and other allergic airway diseases. As a paradigm shift in the field of allergy, we propose here that GM-CSF is a major instructive signal, produced by cells of the mucosal innate system in response to environmental stimuli, which is conducive to the development and sustenance of Th2 responses against aeroallergens. However, the ultimate immunological outcome of antigen exposure is

determined in conjunction with the information acquired by dendritic cells, a process that is dependent on the nature of the antigen (aeroallergens, harmful pathogens, self antigens) and influenced by the presence of other immunoregulatory molecules in the mucosal environment. GM-CSF may indeed be important in the pathogenesis of some Th1-mediated autoimmune disorders as well [66], which is consistent with evidence that the prevalence of both allergic and autoimmune diseases have been increasing in developed countries [67,68].

Immunologically, the evidence presented here provides a compelling argument for the role of the innate mucosal system in the evolution of an adaptive Th2 immune response. This should impel research into a number of relatively understudied areas. For example, it would be informative to elucidate the pathways through which cells of the innate mucosal system, notably airway epithelial cells, interact with environmental agents to facilitate adaptive immune responses. Similarly, to fully understand the respiratory mucosa as a functional defense unit will require dissecting the molecular interplay between epithelial cells, the underlying mesenchyme, dendritic cells, and macrophages. Of particular interest would be to carefully define the cellular sources, and investigate the molecular targets, of GM-CSF in various innate cells. These are likely to be extremely productive areas as they focus our inquiry into the generation of allergic airway diseases at the very site of initial encounter between aeroallergens and the respiratory mucosal immune system. Insofar as this research contributes to the identification and investigation of novel cellular and molecular targets for pharmacological intervention, a therapeutic benefit will likely ensue. But is this where our responsibility as researchers ends?

Failing to acknowledge environmental contributions to disease implies that we restrict conceptualization of treatment to symptomatic relief of established disease, without addressing the factors underlying the increasing incidence of asthma.

It is true that living conditions in the industrialized West have decreased the incidence of early childhood infections and parasite infestations, but they have also heightened exposure to a variety of environmental pollutants and aeroallergens which can stimulate cytokine and growth factor production (including GM-CSF), if not universally, then at least in substantial segments of the population. The data that we have appraised here suggest that GM-CSF could be a molecular pathway through which environmental agents may convey asthma susceptibility. Our contention is that research efforts to elucidate molecular and cellular pathways must be complemented by social and political efforts to reduce exposure to the relevant breathable triggers. For example, the Kyoto protocol attempts to secure international cooperation for a modest reduction of greenhouse gas emissions (including NO2), but also symbolizes, to us, a growing recognition that human activities have detrimentally altered this planet's environment and, as the case of asthma may illustrate, the health of its inhabitants. We suggest that researchers ought to be inspired by the spirit of Kyoto and consider broadening the old bench-to-bedside model to a more comprehensive bench-to-bedside-to-policy paradigm.

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BOX 1: KOCH'S POSTULATES, ADAPTED

In their original formulation, Koch's postulates established criteria for linking a specific microorganism to a specific disease. We have adapted Koch's postulates in such a way that they can be utilised to evaluate the likelihood that a given endogenous molecule plays a role in the pathogenesis of disease. We contend that the stronger the available evidence in support of each postulate, the more likely it is that the molecule is of central importance to the aetiology of the disease.

(1) The molecule is consistently expressed in disease cases.
(2) The molecule is differentially expressed in susceptible and resistant populations.
(3) Experimental administration of the molecule can initiate or exacerbate the disease.
(4) Experimental or therapeutic blockade of the molecule can prevent or treat the disease.

Figure 1: GM-CSF in the generation of Th2 responses. A number of aeroallergens, environmental pollutants and some pathogens interact with epithelial cells and macrophages thereby stimulating the production of GM-CSF. In the absence of concurrent Th1-polarizing signals such as IL-12, the presence of a GM-CSF-rich airway milieu will activate/mature dendritic cells (DCs) along a pathway which privileges Th2 responses. Activated DCs carrying allergens then migrate to the thoracic lymph nodes, interact with naive T cells, and evoke allergen-specific Th2 responses.

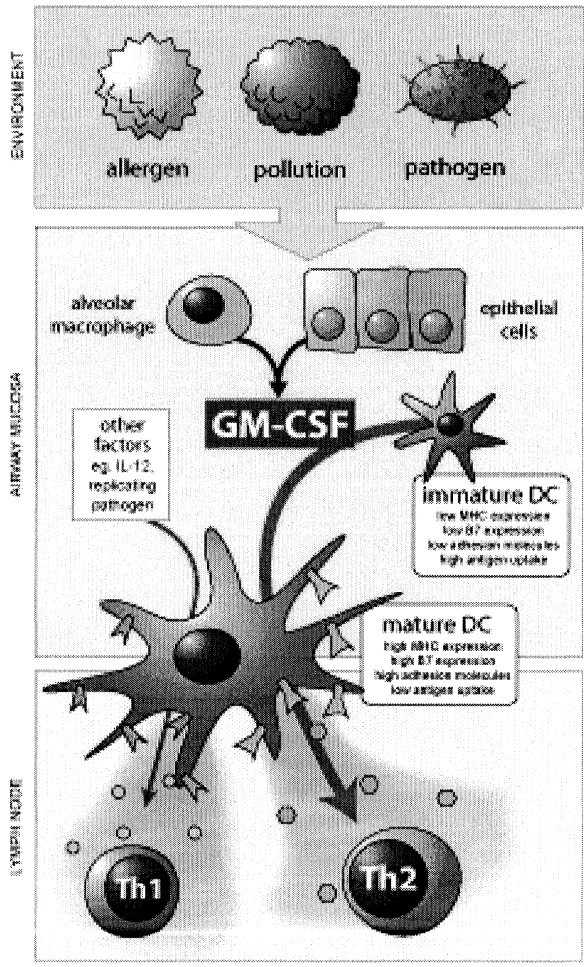
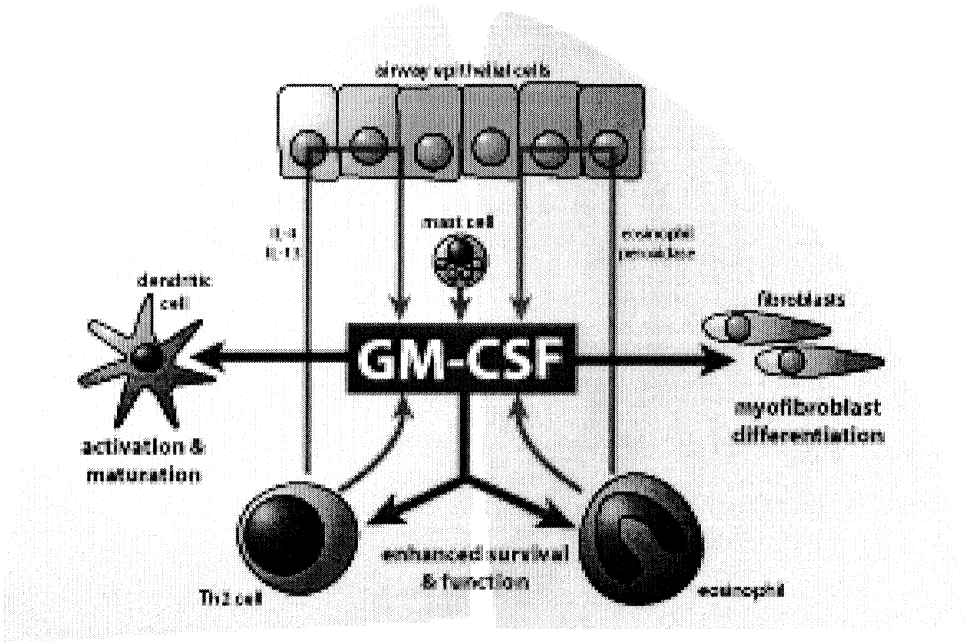


Figure 2: GM-CSF in the maintenance of the immune-inflammatory response. Eosinophils and T cells, main effector cells in allergic airway responses, can produce GM-CSF, as well as molecules that interact with epithelial cells and stimulate further the production of GM-CSF. GM-CSF sustains immune-inflammatory responses through its effects on dendritic cells (DCs), but also enhances the survival and activation of eosinophils and T cells. Along with other growth factors, GM-CSF additionally facilitates the differentiation of fibroblasts to myofibroblasts and smooth muscle cells which, in turn, become producers of GM-CSF.



APPENDIX 2:

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