GENERATION OF IMMUNE RESPONSES IN EXPERIMENTAL ALLERGIC AIRWAY INFLAMMATION

BY

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GENERATION OF ALLERGIC AIRWAY INFLAMMATION

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ABSTRACT

Allergic diseases, including asthma, result from airway inflammatory responses directed against ubiquitous antigens-allergens. The detailed immunological mechanisms underlying the development of allergy (allergic sensitization) have not been fully elucidated. Our understanding of the complexity of cellular interactions underlying allergic inflammation originates mainly, but not exclusively, from studies in experimental animal models. The studies presented in this thesis utilize experimental mouse models of antigen-induced allergic airways inflammation in order to investigate 1) the events occurring during sensitization (primary immune responses) and following re-challenge (secondary immune responses) in two immunologically important sites: lungs and lymph nodes following experimental allergen (ovalbumin; OVA) exposure; 2) the role of the secondary lymphoid organs *vs.* lungs in elicitation of immune responses to allergen; 3) the importance of two major costimulatory pathways - CD28/B7 and ICOS/B7RP-1 - in the generation of allergic airways inflammation.

Findings presented here indicate that introduction of antigen leads to vigorous T and B cell activation in the draining lymph nodes. Such activation translates into the acquisition of a Th2 phenotype, an important step in the generation of allergic sensitization. Considering the multitude of changes occurring in the draining lymph nodes, the importance of lymph nodes during sensitization was investigated in mice devoid of lymph nodes - lymphotoxin α deficient mice. The study demonstrated the absolute requirement of lymphoid organs, either lymph nodes or spleen in generating of Th2-type inflammatory responses. Finally, studies on CD28 and B7RP-1 deficient mice indicated

that, whereas the CD28/B7 pathway is necessary for the establishment of allergic airway inflammation, the ICOS/B7RP-1 pathway is redundant.

The data presented in this thesis identifies several important aspects by which the immune system generates efficient allergic airway inflammation. As we suspect that new-sensitization occurs after each exposure to allergens, information in this thesis may provide insights into novel therapeutic strategies.

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CHAPTER 1 : INTRODUCTION

IMMUNOLOGICAL MECHANISM OF HOMEOSTASIS MAINTENANCE IN THE LUNG

For a long time the lung has been perceived almost exclusively as an organ involved in gas exchange. However, its extensive relationship with the environment implies constant exposure to a multitude of pathogenic and non-pathogenic entities (Lambrecht *et al.*, 2001). Consequently, it became imperative to develop a very efficient system capable of differentiating between harmful and harmless antigens, and of mounting immune responses accordingly. The effectiveness of this tight immunological regulation in the lung relies on the presence of a complex network of antigen-presenting cells (APC), primarily dendritic cells (DC) that can induce such contrasting states as immunity or tolerance depending on the signals that they receive. The initial interaction between DC and naïve T cells in the process of antigen recognition represents the first step in the generation of adaptive immune responses (Banchereau *et al.*, 2000).

Under homeostatic conditions, the immune cells residing in the lung are strictly controlled. An inherent controlling step is the phenotypic immaturity of DC specialized, by definition, in antigen uptake rather than antigen presentation to naïve T cells (Cochand *et al.*, 1999; Stumbles *et al.*, 1998). It is thought that, in the absence of inflammation, immature DC sample inhaled innocuous antigens and present them to T cells in a tolerizing fashion (Akbari *et al.*, 2001). Indeed, inhalation of the soluble protein ovalbumin (OVA) leads to the establishment of inhalation tolerance in animal models (Swirski *et al.*, 2002; Tsitoura *et al.*, 1999). Therefore, the failure of immature DC to become mature in response to inhaled allergens may be the primary reason most humans are tolerant to inhaled non-microbial antigens (Stumbles *et al.*, 1998).

A potential mechanism for the maintenance of DC immaturity may be the presence of inhibitory/regulatory activities. For example, alveolar macrophages (AM) can suppress local DC maturation by releasing nitric oxide (NO) (Holt *et al.*, 1993). Interestingly, the same mechanism is postulated to be responsible for the direct inhibition of T cell proliferation in the lung (Bilyk and Holt, 1993). The most compelling evidence of AM suppressive activity was generated in studies involving selective depletion of AM by local administration of toxic liposomes which resulted in great enhancement of responses to OVA (Bilyk and Holt, 1995; Holt *et al.*, 1993). Other studies have also implicated TGF-β, prostaglandins, and direct cell-cell contact in the suppression of lung DC activity by bronchoalveolar cells (Lipscomb *et al.*, 1993). Furthermore, autocrine production of IL-10 by immature DC can inhibit expression of MHCII and exert an inhibitory effect on T cell activation through the generation of regulatory T cell (T_R) (Akbari *et al.*, 2001; Stumbles *et al.*, 1998).

As stated before, DC immaturity can be easily overridden by inflammatory conditions in the lung or by delivering a "danger signal". Under such circumstances DC regain their ability to stimulate T cells to antigens that previously had been ignored. For example, transgenic expression of GM-CSF concurrently with OVA in the airways appears to very efficiently activate resident DC; such activation eventually leads to the generation of allergic airway inflammation (AAI) (Stampfli *et al.*, 1998). Interestingly, *in vitro* studies have documented that the suppressive function of AM can be effectively overridden by GM-CSF, and is amplified by the presence of TNF-α, due to interference with NO synthesis (Bilyk and Holt, 1993; Bilyk and Holt, 1995). Therefore, delivery of appropriate

stimuli can, in addition to the antigen *per se*, dramatically influence the nature of the ensuing immune response in the airways.

Taken together these observations support the notion that the lung environment plays a major role regulating DC function and, by extension, immune responses (reviewed in Appendix IV).

ALLERGY- DYSREGULATED Th2 RESPONSES

Allergic responses to common environmental antigens, such as those derived from house dust mite, plant pollens or animal proteins, cause clinical disorders such as asthma or allergic rhinitis (Erb, 1999). The hallmarks of allergic asthma include airway eosinophilic inflammation, airway eosinophilia, mucus hypersecretion and airway hyperreactivity (AHR) (Umetsu *et al.*, 2002). The development of allergy is related to the generation of a highly polarized T cell subset - T helper 2 (Th2) cells. T helper lymphocyte responses are generally divided on the basis of cytokine/chemokine production and the functions that they elicit after encounter with antigen. While Th1 cells make IFN-γ and TNF which promote inflammatory cellular immunity against intracellular pathogens, Th2 cells initiate immunity required for the elimination of extracellular pathogens and produce among others IL-4, IL-5 and IL-13 (Mosmann *et al.*, 1986). Allegedly, development of allergy or autoimmune diseases can reflect an aberrant development of Th2 or Th1 polarized responses, respectively.

Th2 cells activated by a relevant allergen secrete a specific set of cytokines (*e.g.* IL-4, IL-5, IL-13) with a variety of effects pertinent to the pathogenesis of allergic

inflammation. For example: IL-4 and IL-13 promote B cell immunoglobulin switch to IgE and IL-5 is an important cytokine for the growth, differentiation and activation of eosinophils (reviewed in (Alvarez *et al.*, 2001)). Many of the pathological features associated with allergic diseases result from the interplay between different cells and cytokines often acting in concert with each other.

The reasons why only some individuals develop allergies remain poorly understood. The simplified view is that genetic and environmental factors interact with each other to orchestrate the preferential development of Th2 cells. The *hygiene hypothesis* suggests that Th1 type infections during childhood can mitigate Th2 allergic responses and that, with improved hygiene, reduced levels of infections allow for the development of allergies (reviewed in (Wills-Karp *et al.*, 2001)). Another explanation proposes a deviation from the immunoinhibitory environment regulated by immunosuppressive cytokines such as IL-10 or TGFβ that, under normal circumstances, prevent the development of allergic responses (Umetsu *et al.*, 2002). Ultimately, both of these theories suggest that a key etiologic factor in allergic disease may be not the initial acquisition of allergen-specific Th2 immunity *per se* but, rather, a disturbance of protective/inhibitory mechanisms that permits immune-deviation towards a Th2 phenotype (Prescott *et al.*, 1998).

Our understanding of the steps involved in the generation of allergic responses has evolved substantially over the past decade. Generally, the development of such responses can be divided into two main steps: *sensitization and recall responses.* Although it has not been possible to study the fundamental events of primary sensitization

in children who become allergic, studies in mouse models have helped to broaden our perspective on this issue. It is believed that in genetically predisposed individuals the first encounter with allergen, sensitization, promotes the differentiation of Th2 cells. As a result of sensitization, allergic patients produce allergen-specific IgE antibodies and generate a pool of memory T cells that can, unlike naïve T cells, respond to allergen. Subsequent exposure to allergen (*recall response*) triggers an allergic reaction that is comprised of two major events: *immediate* and *late* reactions. The immediate reaction is initiated by allergen cross-linking of IgE bound to high affinity receptors (FcεRI) on mast cells and basophils resulting in the release of inflammatory mediators such as histamine and leukotrienes (Turner and Kinet, 1999) which leads to clinical symptoms as a consequence of vasodilation, bronchial smooth muscle bronchoconstriction and mucous secretion. The late phase response is mainly caused by the infiltration of allergen-specific memory CD4+ T cells and eosinophils that produce an array of cytokines, chemokines and other mediators that ultimately influence other inflammatory/structural cells in the lung eventually leading to chronic inflammation.

Constant exposure to allergens continues to promote inflammation and long-term damage to the airways (Muro *et al.*, 2000). Once established, the repetitive cycle of tissue damage and inflammatory cell recruitment becomes chronic leading to structural abnormalities and remodeling of the airway.

STEPS IN THE INITIATION OF ALLERGIC AIRWAYS INFLAMMATION

The initiation of immune responses in the mucosal airway surface is a very

complex process. It involves geographical transfer of information from the lung to the draining lymph nodes and consists of distinct phases including: 1) antigen uptake by lung DC, 2) DC migration to the draining lymph nodes, 2) antigen presentation and 3) T helper cell polarization. These steps eventually lead to the establishment of Th2 cell memory - a key component in recall responses to allergens.

Antigen uptake by lung DC. Inhaled antigen penetrates the respiratory epithelium to be captured by highly endocytic immature dendritic cells, clustered in dense networks directly beneath. Endocytosis promotes phenotypic and functional changes in dendritic cells, culminating in the complete transition from antigen-capture to antigen-presentation (maturation) (Banchereau *et al.*, 2000; Holt *et al.*, 1992). The process of DC maturation is firmly linked with the ability to migrate from peripheral tissues (lung) to the draining lymphoid organs. Under homeostatic conditions, spontaneously migrating DC retain a phenotype conducive to tolerance induction (Hawiger *et al.*, 2001; Steinman *et al.*, 2003). Therefore, unless contextualized by inflammatory mediators, allergen uptake does not lead to the development of allergic inflammation. That DC demonstrate exquisite sensitivity to signals delivered from the local environment is not without precedent. In animal models, activation of lung DC may be accomplished by delivering exogenous factors such as GM-CSF (Stampfli *et al.*, 1998), thereby leading to allergic sensitization. The interaction between structural cells (epithelial) and DC is likewise of major importance. Epithelial cells secrete an assortment of mediators among them, prostaglandin E2 (PGE2), thymic stromal lymphopoietin (TSLP), and TGFβ (Kauffman, 2003). While TGFβ promotes the generation

of "tolerogenic" DC, PGE2 and TSLP have been shown to produce Th2-promoting DC (Kalinski *et al.*, 1997a; Soumelis *et al.*, 2002). According to current findings, none of these are produced in response to allergens. It is known, however, that some allergens can modulate epithelial cell function by a protease-mediated mechanism. For example, serine and cysteine proteases of HDM have been shown to stimulate IL-6 production, a Th2-associated cytokine as well as other cytokines such as GM-CSF (Asokananthan *et al.*, 2002) (Fig. 1, p.13). It is reasonable to postulate that proteolytic activity of environmental allergens subverts, in susceptible individuals, lung immune homeostasis and promotes sensitization by influencing DC or epithelial cell function. Under such circumstances, one can envisage a scenario in which allergen-loaded DC acquire a specific Th2-inducing phenotype that, in presence of naïve T cells, leads to allergic inflammation.

DC migration to the draining lymph nodes. The differential distribution of inflammatory and lymphoid chemokines is vital in regulating DC migration from peripheral tissues to the draining lymph nodes. Accumulation of immature DC in non-lymphoid tissue during the early phase of inflammation is associated with local production of chemokines. Since immature DC express CCR6 (as well as CCR1 and CCR5), locally secreted chemokines such as MIP-3α, and defensins, for which CCR6 is a receptor, will attract more DC (Yang *et al.*, 1999a; Yang *et al.*, 1999b). After antigen uptake, DC are less responsive to MIP-3α and other chemokines specific for the immature DC (Dieu *et al.*, 1998; Sallusto *et al.*, 1998; Sozzani *et al.*, 1999). Upon maturation, DC up-regulate CCR7 and acquire responsiveness to MIP-3β (ECL, Exodus 3, CCL19) and 6Ckine (secondary lymphoid-

tissue chemokine (SLC), Exosdus 2, CCL21). Consequently, maturing DC leave the inflamed tissue, enter the lymph stream, and migrate toward the paracortical area where MIP-3β and/or 6Ckine are constitutively produced (Dieu *et al.*, 1998; Ngo *et al.*, 1999). The arriving DC may themselves become a source of MIP-3β and 6Ckine, thereby amplifying and/or maintaining the chemotactic signal (Dieu *et al.*, 1998). Because these chemokines attract both mature DC and naïve T cells, they likely play a key role in facilitating interaction between these cells (Campbell *et al.*, 1998; Gunn *et al.*, 1998). In this regard, it has been shown that SLC and CCR7-deficient mice exhibit specific deficiency in T cell and DC homing into draining lymph nodes (Forster *et al.*, 1999; Gunn *et al.*, 1999).

Events in the lymph nodes. There are two main outcomes of the immune response generated in organized lymphoid organs: development of B cells capable of producing antigen-specific immunoglobulins and differentiation of T cells capable of generating antigen-specific responses in non-lymphoid tissues.

Several lines of evidence indicate that DC are the most efficient APC for T cell priming (Banchereau *et al.*, 2000). First of all, MHC products and MHC-peptide complexes are expressed 10 to 100- fold higher on DC than on other APC like B cells or macrophages (Inaba *et al.*, 1997). In addition, that adoptive transfer of antigen pulsed DC, but not macrophages or naive B cells, can prime for both cellular and humoral immune responses *in vivo* supports the dominant immunogenecity of DC (Inaba *et al.*, 1990; Sornasse *et al.*, 1992). Also, the specific anatomical localization of DC in T cell areas in the lymph nodes is consistent with the importance of DC in the initiation of immune response (Steinman *et al.*,

1997). Thus, the anatomy and a wealth of *in vitro* and *in vivo* data strongly suggest that DC are the APC that initiate *in vivo* T cell responses.

Generally, T cells recognize the antigen in the context of major histocompatibility (MHC) molecules expressed by professional antigen presenting cells, mainly DC. Ligation of the TCR on naive antigen-specific T cells by the cognate peptide-MHC complex induces T cell activation and subsequent division provided that the duration of the contact and the strength of the interaction (i.e. avidity) between T cell and APC are sufficient for serial TCR triggering (Borovsky *et al.*, 2002). DC-T cell clustering is mediated by several adhesion molecules such as LFA-1/ICAM-1 (CD54) or DC-SIGN/ICAM-3 (Bachmann *et al.*, 1997; Geijtenbeek *et al.*, 2000). This specific clustering of complexes at the DC-T cell interface has been referred as the "immunological synapse" (reviewed in (Grakoui *et al.*, 1999)). These non-specific interactions serve to approximate two cells and allow for initial screening and serial triggering of the low affinity TCR for recognition of its specific peptide-MHC. An important part of this process involves the expression of costimulatory molecules (described in the next part). The repeated engagement between MHC and TCR results in calcium influx and activation, as measured by the increase in activation markers (*e.g.* CD25, CD69), finally followed by T cell proliferation (Gunzer *et al.*, 2000). The initial interaction between TCR and MHC-peptide complexes subsequently leads to TCR downregulation to presumably control the duration of T cell antigen engagement and optimize signal transduction.

After extensive proliferation in the T cell area, some of the allergen-specific CD4 T cells migrate into B cell-rich follicles to support antibody production (Garside *et al.*, 1998).

This recruitment correlates with the induction of CXCR5 on antigen-specific T cells that provide them with the ability to migrate in response to BCL (produced by follicular stromal cells) while simultaneously losing of responsiveness to SLE and ELC (Ansel *et al.*, 1999). Antigen-specific CD4 T cells recognize peptide-MHC complexes on antigen-specific B cells and provide help for antibody generation. B lymphocyte differentiation into IgE expressing cells is dependent upon three types of signals. The first signal is delivered through the B cell antigen receptor and is pivotal in determining the antigenic specificity of the response. The second signal is provided primarily by cytokines derived from T helper 2 (Th2) cells, IL-4 and IL-13. Finally, the third signal is provided via the interaction between the constitutively expressed CD40 molecule on B lymphocytes and CD154 (CD40 ligand), a molecule expressed on T lymphocytes following activation. In the presence of IL-4, IL-13 and CD40 crosslinking B cell undergoes class switching to produce IgE (Brady *et al.*, 2001). IgE is released into the blood and quickly binds to high-affinity IgE receptors (FcεRI) on the surface of both mast cells and peripheral blood basophils, and to the low-affinity IgE receptors (FCεRII or CD23) on the surface of lymphocytes, eosinophils, platelets, and macrophages . Once mast cells are coated with antigen-specific IgE, future encounter with antigen will lead to mast cell activation.

Polarization toward Th2. It is believed that the initial antigen recognition in the lymph nodes determines the fate of Th differentiation from precursor Th0 to Th2-polarized cells. The process by which uncommitted Th cells develop into mature Th1 or Th2 cell is very complex as it involves many different mechanisms including antigen dose, co-stimulators,

the cytokine milieu, DC subsets and certain genetic factors such as transcriptional factors. Two cytokines that critically control Th1 and Th2 differentiation are IL-12 and IL-4, respectively. These two cytokines concurrently induce the generation of their own T cell subsets and inhibit the production of the opposing subset. They exert their functions through tight regulation of subset-specific transcriptional factors. In this regard, IL-4 activates Stat-6 that translocates into the nucleus and induces expression of GATA-3, the master regulator of the Th2 differentiation pathway (Zhang *et al.*, 1999; Zheng and Flavell, 1997). Another important transcriptional factor, c-Maf, is also preferentially expressed in Th2 cells and serves as an IL-4 gene-specific activator (Kim *et al.*, 1999).

Although the cytokines that regulate T helper cell polarization are known, the initial source of these cytokines *in vivo* remains a matter of debate. Paradoxically, that already differentiated Th2 cells are the most potent source of IL-4 raises a conceptual problem and triggered a search for alternative cellular sources. While basophils, mast cells and NKT cells seem to be powerful producers of IL-4, none of them is indispensable (Zhang *et al.*, 1996). In addition, the finding that naïve human and mouse T cells can differentiate into Th2 cells in the absence of IL-4 argues that IL-4 may not be required to instruct the Th2 lineage (Kalinski *et al.*, 1995; Ritz *et al.*, 2002). Furthermore, that GATA-3 induces Th2 development in the absence of STAT-6 implies that this process is IL-4 independent (Ouyang *et al.*, 2000). Therefore, Th2 differentiation might involve mechanisms that are independent of instructions provided by IL-4.

Several lines of evidence suggest that DC can orchestrate the lineage commitment of naïve Th cells either through the expression of a certain pattern of

costimulatory molecules or the production of cytokines (Moser and Murphy, 2000). Since DC are a major source of IL-12, it has been postulated that Th2 development may merely reflect a "default" pathway in the absence of stimuli that would otherwise trigger the production of IL-12 and lead to Th1 differentiation (Maldonado-Lopez *et al.*, 2001). However, although IL-4 production by DC has not been demonstrated, DC can produce two Th2-associated cytokines such as IL-6 and IL-10 (Constant *et al.*, 2002; Stumbles *et al.*, 1998). Moreover, there is evidence that IL-10 is required for the development of Th2 responses by DC (Maldonado-Lopez *et al.*, 2001), and both cytokines have been proposed as major Th2-polarizing factors produced by DC in the lung (Constant *et al.*, 2002; Stumbles *et al.*, 1998). In addition, several costimulatory pathways have been implicated in T cell polarization and their role is discussed in the next part of the thesis. Therefore, the

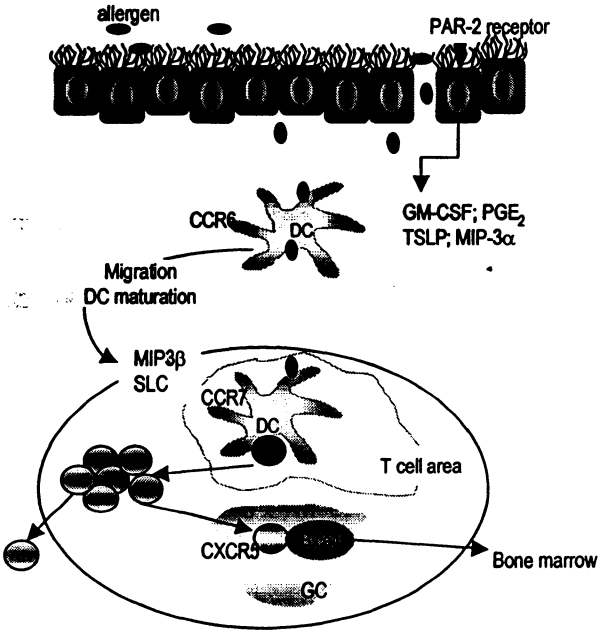

Figure 1. *Induction of allergic sensitization.* Allergens activate epithelial cells through interaction with PAR receptors. This lead to the release of DC-maturating factors, such as GM-CSF or chemokines that can attract immature DC expressing CCR6 (MIP-3α). Additional factors such as TSLP or PGE_2 can indirectly stimulate DC to preferentially induce Th2 cells in the lymph nodes. The uptake of allergen initiate the migration of DC to the T cell areas of lymph nodes in response to MIP-3β; DC express corresponding receptor-CCR7. In the T cell DC present allergen to naïve T cells resulting in T cell activation and proliferation. At this point, T cell acquire Th2 phenotype and either recirculate through the lymph nodes as T_{CM} or tissue as T_{EM}. Finally, some of T cells expressing CXCR5 migrate to the B cell follicles in response to BCL in order to support IgE production. Most high-affinity B cells go to the bone marrow to become long lived IgE-producing plasma cells. GC-germinal center,
● naive T cells ◉ T_{CM} ◉ T_{EM}

microenvironment and the type of inflammation present in the milieu in which DC reside during antigen uptake, influence the production of certain cytokines and costimulatory

molecules which have an impact on the polarization of Th cells induced by DC (Kalinski *et al.*, 1997a; Kalinski *et al.*, 1997b).

Formation of the memory T cell pool. T cells generated in response to antigen form the pool of immunological memory. These antigen-experienced T cells produce cytokines more rapidly than naïve T cells and at lower doses of antigen, thus enhancing the efficiency of the immune response (Jenkins *et al.*, 2001). Recent evidence indicates the existence of two functionally distinct memory T cell subsets: (1) "central memory" T cells (T_{CM}), which express the chemokine receptor CCR7 and CD62L and home to T cell areas of secondary lymphoid organs, and (2) "effector memory" T cells (T_{EM}), which have lost the expression of CCR7 and have acquired the capacity to migrate to nonlymphoid tissues (Sallusto *et al.*, 1999). Interestingly, whereas T_{CM} produce very low levels of IL-4 and IFN-γ, T_{EM} produce high levels of both cytokines indicating that both subsets might have different, but complementary, functions. Indeed, that T_{CM} can replenish the T_{EM} pool suggests that these cells might represent different steps in the differentiation pathway from naïve to terminally differentiated effector cells (Geginat *et al.*, 2001). Therefore, it is plausible that while T_{EM} cells are responsible for immediate reaction to allergen exposure, T_{CM} might be involved in the maintenance of the T_{EM} pool (Harris *et al.*, 2002). Thus, memory T cells provide both immediate as well as secondary protection to antigen, clearly a desirable feature during infectious diseases but highly detrimental in autoimmune or allergic diseases. Therefore, the development of strategies specifically targeting memory T cells appears extremely appealing in the context of Th2 mediated allergic diseases.

RESPONSE TO ALLERGEN UPON SUBSEQUENT ENCOUNTER

Allergic airway inflammation following allergen re-exposure in humans and animals results from reactivation of different cellular subsets, including mast cells, antigen-specific memory Th2 cells and eosinophils. The allergic response is generally divided into two phases: early and late. While the early phase is associated with IgE-dependent mast cell degranulation, the late phase involves activated memory Th2 cells and eosinophils. The release of chemokines from mast cells and structural cells during the early phase leads to the influx of late phase cells into the lung. Mast cells produce Th2-associated chemokines, notably CCL7 (MCP-3), CCL17 (TARC), and CCL22 (MDC), thus attracting memory Th2 cells expressing the corresponding receptors[1] (Wakahara *et al.*, 2001). Additionally, the airway epithelium is an important source of chemokines including CCL5 (RANTES), eotaxin/CCL1 and CCL13 (MCP-4), relevant to human allergy and asthma (Taha *et al.*, 1999). A similar set of chemokines attracts eosinophils to the airway. Eosinophils contribute to the pathogenesis of allergic disease by releasing preformed cytoplasmic granule mediators such as major basic protein, lipid mediators (LTC4, PAF), and a vast array of cytokines (Rothenberg, 1998). In the airways, these mediators cause direct tissue damage, smooth muscle contraction, and increased vascular permeability, ultimately leading to the recruitment of more eosinophils and mononuclear cells. Although eosinophils are indispensable to the development of allergic inflammation, allergen-specific memory Th2 cells are critical to the generation of the late phase response.

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[1] Th2 related chemokines receptor and their ligands (reviewed in (Lukacs, 2001 #150):
1/ CCR3: CCL5 (RANTES), CCL7 (MCP-3), CCL11 (eotaxin), CCL13 (MCP-4), CCL24 (eotaxin2) and CCL26 (eotaxin3)
2/ CCR4: CCL17 (TARC), CCL22 (MDC)
3/ CCR8: CCL1 (TCA3), CCL17 (TARC)

The involvement of Th2 cells in the pathophysiology of allergic asthma has been corroborated by studies in both humans and mice. Th2 cells are markedly expanded in asthmatic subjects and their presence correlates with AHR and airway eosinophilia (Azzawi *et al.*, 1990). This critical importance of Th2 cells has been substantiated in murine models of AAI where the depletion of CD4+ T cells abrogated eosinophilic infiltration and AHR (Nakajima *et al.*, 1992). The infiltrating effector Th2 cells produce Th2 cytokines/chemokines that collectively propagate the influx of cells, including eosinophils, to the lung. Additionally, Th2 cells express T1/ST2. Disrupting the interaction between T1/ST2 and its ligand has been shown to block Th2 development and effector function *in vivo* (Lohning *et al.*, 1998; Xu *et al.*, 1998). The precise role of this molecule is unknown, but it appears to be a specific marker of effector, rather than memory, Th2 cells.

The mechanisms that regulate memory Th2 cell activation at sites of tissue damage remain poorly understood and are subject of intensive research. Of particular interest are issues related to the regulation of local APC function and antigen presentation in the tissue. Although antigen presentation during secondary exposure does not differ from presentation during sensitization, Th2 memory cells nevertheless respond to antigenic stimulation more efficiently than naïve Th2 cells. This accelerated immune response may be a direct consequence of antigen presentation within the tissue; such presentation obviates the necessity for antigen to migrate to the draining lymph nodes. An increased number of lung DC with enhanced antigen presenting capacity would serve this purpose perfectly.

Indeed, elevated levels of pulmonary DC have been observed in both asthmatic

patients and experimental animals upon allergen exposure (Godthelp *et al.*, 1996; Hoogsteden *et al.*, 1999; Stampfli *et al.*, 1998; Tunon-De-Lara *et al.*, 1996). The importance of DC in this phase of the immune response is best supported by two findings: first, pulmonary DC isolated from atopic asthmatics activate allergen specific memory Th2 cells (Bellini *et al.*, 1993); and second, selective depletion of DC in mice prior to challenge but after sensitization leads to diminished eosinophilic inflammation, and decreased production of IL-4, IL-5 and IgE (Lambrecht *et al.*, 1998). Expectedly, elevated DC numbers in the lung tissue increase the chance of T cell encounter with antigen, as presented by DC in the context of MHCII. Additionally, some DC express the α chain of the high affinity IgE receptor (FcεRI), suggesting an alternative pathway by which allergens are internalized and targeted to MHC molecules inside the cell. This IgE-mediated pathway has been shown to enhance antigen-specific T cell activation *in vitro* by 100 to 1000-fold, as compared to conventional endocytosis (Maurer *et al.*, 1996; van der Heijden *et al.*, 1993). That FcεRI- bearing DC have been found in allergic lungs supports the notion that these cells function at tissue sites during secondary allergic responses (Tunon-De-Lara *et al.*, 1996). Locally produced IgE additionally contributes to the inflammatory response because it can influence antigen presentation (Chvatchko *et al.*, 1996).

Although enhanced accumulation of DC in allergic airways does not prove that antigen is presented in the lung, the recent observation that antigen-loaded pulmonary DC are retained within the tissue, as well as the concomitant preferential accumulation of memory T cells in the lung, compellingly supports this notion (Constant *et al.*, 2002; Julia *et al.*, 2002). It is also possible that, in addition to local antigen presentation, some DC

migrate to the draining lymph nodes and present antigen to recirculating central memory (T_{CM}) or naïve T cells. T_{CM} eventually become effector T cells (T_{EM}) and migrate to the effector organ. Such mechanisms would replenish peripheral tissues (lung) with a new pool of memory T cells poised to respond to allergen challenge. Thus, it is essential to further study events in lymphoid organs *and* lung tissue during secondary immune responses.

During recall challenge, the lung is a dynamic compartment that harbors multiple cellular interactions. Sensitization of new cohorts of naïve T cells to environmental allergens and reactivation of already existing Th2 memory cells represents two key processes that eventually contribute to the perpetuation of allergic inflammation.

COSTIMULATION IN THE AIRWAYS INFLAMMATION

It has been known for more than twenty years that optimal T cell activation requires two signals: one generated through the T cell receptor (TCR) signaling upon binding to the peptide-MHC expressed on APC, and a second antigen-independent signal called co-stimulation (Sharpe and Freeman, 2002). Indeed, T lymphocytes stimulated through the TCR alone fail to produce cytokines, are unable to sustain proliferation, and often undergo apoptosis or become non-responsive to subsequent stimulation (anergic) (Harding *et al.*, 1992). An ever increasing number of costimulatory molecules have been identified over the last few years. For simplicity they will be divided here into two major groups based on structural homology: (1) the B7 family of ligands and its receptors, and (2) members of the TNF receptor (TNFR) family (Carreno and Collins, 2002). Costimulatory molecules have also been grouped according to their biological function.

Although the major function of costimulatory molecules is the enhancement of T cell activation, some of them, such as CTLA-4 and PD-1, provide negative signals that attenuate the immune response and maintain peripheral tolerance, an important protective mechanism against autoimmune reactions (Carreno and Collins, 2002; Sharpe and Freeman, 2002). It seems, therefore, that the balance between negative and positive costimulatory signals together with TCR signaling sets the threshold for T cell activation and regulation. In addition, it has been postulated that some costimulatory pathways play an important role in Th cell polarization (Coyle and Gutierrez-Ramos, 2001). Since this thesis deals specifically with selected members of the B7 family, the involvement of TNF family members in Th2 polarization will be omitted.

CD28-B7.1/B7.2 pathway

Perhaps the best-studied costimulatory pathway is CTLA-4/CD28/B7. Whereas B7.1 (CD80) and B7.2 (CD86) are expressed on the surface of APC, both CD28 and CTLA-4 molecules are expressed on T cells with different kinetics reflecting their functions. Specifically, the CD28 receptor is expressed constitutively (Gross *et al.*, 1992), whereas CTLA-4 is induced after T cell activation (Linsley *et al.*, 1996). Engagement of CD28 by B7.1 or B7.2 results in the induction of IL-2 transcription, CD25 expression and cell cycle progression (Harding *et al.*, 1992; Jenkins *et al.*, 1991). CD28 also delivers critical survival signals to T cells thought the Bcl-X_L pathway (Boise *et al.*, 1995). Finally, CD28 costimulation decreases the threshold of T cell activation (Viola and Lanzavecchia, 1996). In contrast, CTLA-4 serves as a negative regulator of T cell activation as it is best

illustrated by the severe lymphoproliferative disorder observed in CTLA-4 deficient mice (Mandelbrot *et al.*, 1999).

The importance of the CD28 pathway in the generation of immune responses has been demonstrated in a multitude of *in vivo* systems. Blockade of the CD28/B7 interaction, either by genetic deletion of CD28 or by blocking agents (CTLA-4Ig[2]), can profoundly affect the development and progression of autoimmune disease and the course of certain viral and parasitic infections (reviewed in (McAdam *et al.*, 1998)). Furthermore, germinal centers are not formed in response to immunization in the absence of CD28 implying the importance of CD28 signaling in mediating B cell help (Ferguson *et al.*, 1996).

CD28 in Th2 responses. Although controversial, several studies have indicated that the CD28 pathway may also be important in T cell polarization. Early studies suggested that CD28 costimulation is important in differentiation of Th1 rather than Th2 cells (McKnight *et al.*, 1994). However, the initiation of Th1 differentiation appears to be less dependent on CD28 in CD28 deficient mice, or in mice in which CD28 costimulation was blocked by CTLA-4Ig (Green *et al.*, 1994; Shahinian *et al.*, 1993). In addition, CD28 costimulation has been shown to promote the production of Th2 cytokines, such as IL-4 and IL-5, which is consistent with differentiation toward a Th2 phenotype (Rulifson *et al.*, 1997). That IL-4, IgG1 and IgE production can be inhibited following infection of mice with a number of different parasites support the involvement of CD28 in Th2 differentiation (King *et al.*, 1996). At the intracellular level, signaling through CD28 augments GATA-3 expression, an

[2] chimeric protein consisting of extracellular domain of CTLA-4 fused to human or mouse Fc thus binding to B7 molecules and preventing interaction with CD28

important transcriptional factor for Th2 differentation (Rodriguez-Palmero *et al.*, 1999). Taken together, there is compelling evidence indicating that CD28 might be essential in the development of AAI.

B7.1 (CD80) and B7.2 (CD86) in Th2 responses. Although it is clear that blockade of the CD28 pathway inhibits T cell effector function during Th2 responses, the role of individual ligands, namely B7.1 and B7.2, in this process is unclear. Some reports have suggested that B7.1 and B7.2 can mediate equivalent costimulatory signals *in vitro* (Lanier *et al.*, 1995; Levine *et al.*, 1995), while other *in vitro* studies have suggested that IL-4 production by T cells is particularly dependent on B7.2 signaling (Freeman *et al.*, 1995; Ranger *et al.*, 1996). Administration of anti-B7.1 and/or anti-B7.2 during *in vivo* immune responses has yielded different results depending on the experimental system (Kuchroo *et al.*, 1995; Lenschow *et al.*, 1995). For example, in experimental encephalomyelitis (EAE), a Th1-mediated disease, administration of anti-B7.1 reduced, whereas anti-B7.2 increased, the incidence of disease. Conversely, in NOD mice that develop autoimmune diabetes (also Th1 in nature), anti-B7.2 abrogated, whereas anti-B7.1 significantly accelerated, disease development (Lenschow *et al.*, 1995). The apparent contradictory effects of blocking B7.1 or B7.2 costimulation might be explained by differences in the kinetics, level of expression and affinities of B7.1/B7.2 in their interaction with either CD28 or CTLA-4 (Bluestone, 1997). Thus, it remains unresolved whether the signals provided to T cells upon interaction of B7.1 and B7.2 with CD28 are qualitatively different and lead to the development of functionally distinct types of T cells.

The involvement of B7.1 *vs.* B7.2 in AAI also remains debatable. Blocking experiments in AAI models have provided conflicting results. For example, Harris *et al.* have postulated the requirement of B7.1 but not B7.2 in the induction of AAI (Harris *et al.*, 1997). At variance, two additional studies have reported that anti-B7.2 treatment of OVA-sensitized mice immediately before antigen challenge resulted in greatly diminished airway eosionophilia and airway hyperresponsiveness (Keane-Myers *et al.*, 1998; Tsuyuki *et al.*, 1997). However, eosinophilic recruitment into the airways was also significantly reduced in mice treated only with anti-B7.1 in both studies. These differences can also be explained by the differentepattern of expression. For example, both molecules are expressed by airway DC, whereas B cells preferentially express B7.2 (Masten *et al.*, 1997; Tsuyuki *et al.*, 1997). These potential differences in B7 expression might have significant implications for the development of treatment strategies. Therefore understanding the pattern of B7.1/B7.2 expression is important to clearly define therapeutic targets.

ICOS/B7RP-1 pathway

The B7 homologous protein (B7h) also termed B7-related protein (B7RP-1) (GL50, LICOS, B7H2) exhibits 20% homology to B7.1 and B7.2 and binds to the inducible costimulatory receptor (ICOS) on T cells. Although B7RP-1 is expressed in lymphoid organs (thymus, spleen and Peyer's patches), it is also found in a variety of non-lymphoid organs such as kidney, lung and testis. Resting B cells constitutively express B7RP-1, whereas B7RP-1 expression in monocytes is upregulated upon IFN-γ stimulation. CD34+ bone marrow precursors also express B7RP-1 following stimulation with TNF-α and GM-CSF. Mice

transgenic for B7RP-1-Fc develop T cell hyperplasia, plasmocytosis and hypergamma globulinemia. In addition, administration of B7RP-1-Fc augments the effector response during a cutaneous hypersensitivity reaction (Ling *et al.*, 2000; Swallow *et al.*, 1999; Wang *et al.*, 2000; Yoshinaga *et al.*, 1999).

Ligation of ICOS by B7RP-1 leads to T cell proliferation and production of IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN-γ and TNF-α. Interestingly, in contrast to CD28 ligation, ICOS signaling does not enhance IL-2 production. In addition, while CD28 is expressed constitutively on T cells, ICOS is expressed only upon T cell activation (Coyle *et al.*, 2000; Hutloff *et al.*, 1999). The differential expression of these two ligands suggests that ICOS is perhaps important in regulating effector rather than naïve T cell functions.

ICOS deficient mice fail to generate germinal centers and have impaired production of IgG1, IgG2a and IgE indicating that the ICOS-B7RP-1 pathway plays an essential role in T cell-dependent B cell activation (Dong *et al.*, 2001b; McAdam *et al.*, 2001; Tafuri *et al.*, 2001). Interestingly, this deficit can be overcome by CD40 costimulation demonstrating cross-regulation of the ICOS/B7RP-1 and CD40/CD40L pathways (Hutloff *et al.*, 1999; McAdam *et al.*, 2001).

ICOS/B7RP-1 in Th2 responses. Initial studies indicated that Th2 polarized cells express higher amounts of ICOS compared to Th1 cells (Coyle *et al.*, 2000). These observations correlate with *in vitro* studies showing that the ICOS-B7RP-1 interaction is important in Th2 polarization (McAdam *et al.*, 2000). In addition, interruption of this pathway in two typical Th1 mediated model disease- EAE - led to increased symptom severity correlated with

decreased production of IL-4 and increased production of IFN-γ (Dong *et al.*, 2001a; Rottman *et al.*, 2001). Interestingly, studies in ICOS deficient mice showed that these mice are susceptible to AAI but produce less IL-4 and IL-13 (Dong *et al.*, 2001a). These data implied that ICOS might be important for Th2 effector function rather than Th2 differentiation. Indeed, blocking this pathway in an AAI model during the effector phase but not during time of priming ameliorates inflammation (Gonzalo *et al.*, 2001). Therefore, CD28 and ICOS costimulation have distinct roles in regulating Th2 functions; whereas CD28 dominates in the priming phase, ICOS regulates effector functions of Th2 cells (Gonzalo *et al.*, 2001) (Figure 2).

It is currently unknown whether B7RP-1 constitutes the only ligand for ICOS or,

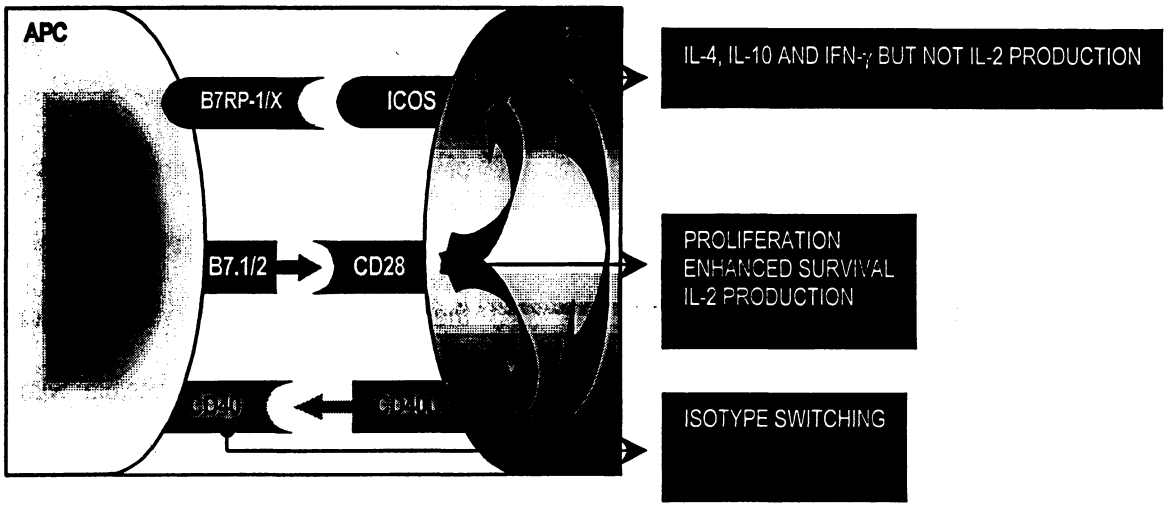


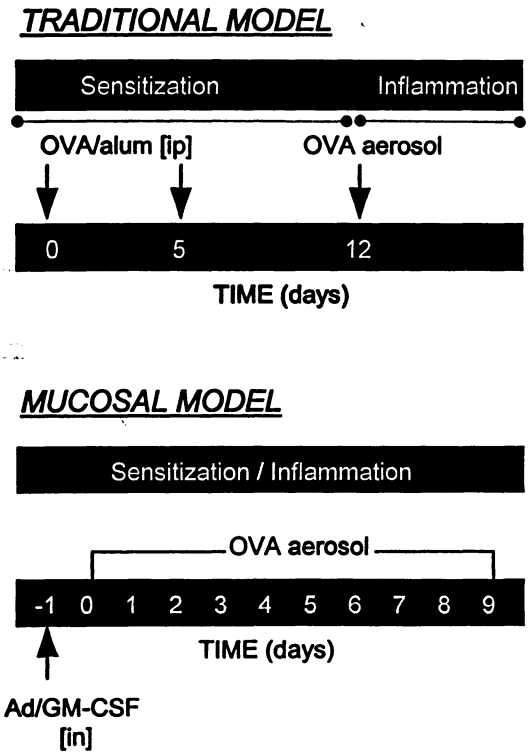
Figure 2. *Cross-regulation of costimulatory pathways.* T cell-APC interaction begins when TCR is stimulated by MHC/peptide complex (not shown). This signal is amplified by CD28/B7 interaction that induces upregulation of CD40 and ICOS on T cells; ICOS by itself can also regulate an expression of CD40. Signaling through CD40L leads to isotype switching (in the case of B cells as APC). CD28 promotes IL-2 production, T cell survival and entry into the cell cycle whereas ICOS engagement regulates T cell effector function through the enhancement of IL-10, IL-4 and IFN-γ production.

similarly to B7.1/B7.2, represents one of many. In addition, the role of B7RP-1 specifically

in the development of Th2 responses in AAI has not been adequately examined.

ANIMAL MODELS

Although our knowledge of allergy has greatly benefited from studies of allergic patients, our understanding of the complex cellular interactions underlying allergic inflammation originates mainly from studies in experimental animal models. Development of animal models aiming to recapitulate the human disease, including asthma, allow several issues to be addressed that, due to practical and ethical considerations, cannot be pursued in human subjects (Isenberg-Feig *et al.*, 2003). Murine models of allergic airways inflammation are especially attractive due to the availability of genetically altered mice. Even though these models do not replicate human disease perfectly, much of what is currently known about the pathogenesis of asthma, including the therapeutic potential of certain agents, would not have been possible without them.



The majority of current animal models of asthma utilize ovalbumin (OVA) as a surrogate antigen. Sensitization is accomplished by systemic, intraperitoneal (i.p.) administration of OVA emulsified to adjuvant-alum

[TRADITIONAL MODEL] or by repeated injection of adjuvant-free OVA (Hessel *et al.*, 1995; Kung *et al.*, 1994; Ohkawara *et al.*, 1997). This particular mode of antigen delivery has been chosen so as to prevent the induction of inhalation tolerance, a consequence of repeated inhalation of OVA (Swirski *et al.*, 2002; Tsitoura *et al.*, 1999). In order to generate airways inflammation, the sensitization phase is followed by aerosol challenge with OVA. The resulting immune response exhibits the cardinal features of asthma including eosinophilic inflammation, Th2 polarization and airways hyperresponssiveness. One of the advantages of this model is the ability to dissect two distinct processes: sensitization (primary response) and recall (secondary response). This allows events occurring in the lymph nodes, such as the interaction between a naïve T cell and an antigen-bearing APC, to be discreetly examined. Interestingly, the peritoneal cavity of the mouse preferentially drains to the thoracic lymph nodes, thus directly mimicking the drainage of the lungs.

Even though traditional models are suitable to address multiple immunological questions (*e.g.* lymph node activation) and are valuable for certain applications (*e.g.* therapeutic interventions), they are limited in that they underestimate the contribution of the airway microenvironment during sensitization. To address this, several strategies have been devised. For example, the intratracheal administration of bone-marrow derived OVA-pulsed DC prior to OVA aerosol challenge leads to symptoms similar to those observed in mice subjected to the traditional i.p. model (Lambrecht *et al.*, 2000; Sung *et al.*, 2001). These studies demonstrate that DC manipulation *in vitro* efficiently induces sensitization to inhaled antigens, possibly because it elicits DC maturation.

The endogenous activation of DC provides an alternative approach. Our laboratory has developed a mucosal model involving the concurrent delivery of OVA aerosol and an adenoviral vector encoding a physiologically relevant adjuvant, GM-CSF (Stampfli *et al.*, 1998). An extensive characterization of this model demonstrated the expansion OVA-specific IgE, Th2 cytokines, eosinophilic airway inflammation and long-term antigen-specific memory. Interestingly, delivery of adenoviral vectors coding for other cytokines, such as TNF-α, IL-6, or IL-4, did not generate an inflammatory response, likely because GM-CSF is a powerful DC activating factor.

Although these studies have succeeded in mimicking the route by which humans are exposed and sensitized to aeroallergens, they have failed to address the impact of "real life" allergens in asthma. While OVA, the surrogate allergen employed, is innocuous, many allergens are proteases that activate and impact a variety of biological functions, including DC activity. For this reason, the establishment of novel models utilizing mucosal delivery of allergens such as ragweed or house dust mite is absolutely necessary.

In summary, currently available animal models allow to the study of several aspects of generation of allergic sensitization, aspects which otherwise would be impossible to investigate, given the difficulty in establishing initial allergen encounter, in humans. Therefore, animal models have been remarkably useful to understanding sensitization to allergens.

AIMS AND OUTLINE OF THE THESIS

The preceding chapter has already indicated that the understanding of processes governing the generation of allergic airways inflammation (AAI) is incomplete. An important aspect of AAI is the initial interaction between naïve T cells and antigen presenting cells (APC), an interaction that eventually determines the fate of the ensuing immune response. In this respect, several issues can be addressed, including activation status of APC and T cells, localization of the APC-T cell interaction, and the role of costimulation in establishing/maintaining AAI. In this thesis we examined some of the basic mechanisms behind antigen presentation in AAI.

The major purpose of the studies demonstrated in ***Chapter 2*** was to gain an understanding of the geographic localization of primary and secondary immune responses in a model of AAI. I focused on the sequence of events following administration of antigen in a traditional and mucosal model (*Appendix I*) of antigen-induced airway inflammation. Specifically, we examined T cell and antigen presenting cell (APC) activation[1], cytokine production and expression of Th2-related transcription factors (GATA-3 and STAT-6) following priming and recall phases at two major sites: lung tissue and the draining lymph nodes[2]. The pattern of expression of costimulatory molecules, namely B7.1 and B7.2, in the process of Th2 phenotype acquisition, was also investigated[1].

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[1] investigated in both traditional and mucosal model (Chapter 2 and Appendix I)
[2] investigated only in traditional model (Chapter 2)

Considering the multitude of changes occurring in the draining lymph nodes following the initial exposure to antigen, the importance of lymph nodes during sensitization was investigated in ***Chapter 3***. First, the role of the draining lymph nodes in T cell priming was studied in lymphotoxin-α knockout (LTKO), lymph node deficient mice. Subsequently, the importance of all lymphoid organs was addressed in splenectomized LTKO mice. In a separate set of studies (*Appendix II*), we examined the maintenance of secondary immune responses in mice devoid of all lymphoid organs. This approach allowed us to evaluate the importance of local (lung) antigen presentation in the reactivation of an already established effector T cell population.

Finally, in ***Chapter 4*** I explored the contribution of the ICOS/B7RP-1 costimulatory pathway to the development of Th2 responses in AAI. For this purpose, we utilized B7RP-1 deficient mice and examined cellular and humoral responses directed against antigen. The involvement of a different costimulatory pathway, namely CD28/B7, in generating of AAI was also studied in CD28 deficient mice (*Appendix III*).

All findings have been summarized and discussed in light of the currently prevailing paradigms of asthma pathogenesis in ***Chapter 5***: Discussion.

Appendix IV and *Appendix V* include two papers. The former one (*Appendix IV*) is a review that I have written on the role of dendritic cells and GM-CSF in AAI, whereas the latter

(*Appendix V*), on which I am a second author, evaluates the importance of the costimulatory molecule B7-H3 in airway inflammation.

CHAPTER 2

TEMPORAL-SPATIAL ANALYSIS OF THE IMMUNE RESPONSE IN A MURINE MODEL OF OVALBUMIN-INDUCED AIRWAYS INFLAMMATION

Gajewska B.U., Świrski F.K., Alvarez D., Ritz S.A., Goncharova S., Cundall M., Snider D.P., Coyle A.J., Gutierrez-Ramos J.-C., Stämpfli M.R. and Jordana M. *Am. J. Respir. Cell Mol. Biol.* 2001, 25:326-334

This publication describes the cellular changes in two major anatomical compartments, the draining lymph nodes and lungs, during the establishment of allergic airway inflammation.

The work presented in this study was performed by the author of the thesis. Dr. Coyle and Dr. Gutierrez-Ramos provided T1/ST2 antibodies used in the study. Dr. Stämpfli provided useful discussion on the results of this work. Supervision was provided by Dr. Manel Jordana resulting in the multiple authorship of this paper.

Temporal–Spatial Analysis of the Immune Response in a Murine Model of Ovalbumin-Induced Airways Inflammation

Beata U. Gajewska, Filip K. Swirski, David Alvarez, Stacey A. Ritz, Susanna Goncharova, Meghan Cundall, Denis P. Snider, Anthony J. Coyle, José-Carlos Gutierrez-Ramos, Martin R. Stämpfli, and Manel Jordana

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The objective of this study was to define phenotypic changes of antigen-presenting cells (APCs) and T cells in a murine model of antigen-induced airways inflammation that involves intraperitoneal sensitization with ovalbumin (OVA)/adjuvant followed by antigen aerosolization. We investigated the APC and T-cell compartments both after sensitization (primary immune response) and after challenge (secondary immune response) at the thoracic lymph nodes (initiation site) and the lung (effector site). Our findings document a major cellular expansion in the lymph nodes after both sensitization and challenge. After sensitization, this expansion was comprised mainly of B cells, a considerable proportion of which expressed B7.2. At this time, T cells were markedly expanded and activated as assessed by CD69 expression; further, although GATA-3 and signal transducer and activator of transcription-6 were expressed at this time point, expression of interleukin (IL)-4, IL-5, and IL-13 messenger RNA (mRNA) levels were marginal. However, *in vitro* stimulation of lymph-node cells with OVA led to cytokine production. In contrast, 24 h after challenge, but not after sensitization, there was a major expansion of dendritic cells and macrophages in the lungs. This expansion was associated with enhanced expression of both B7.1 and B7.2. We also observed expansion of activated $CD3^+/CD4^+$ T cells expressing the T helper-2–associated marker T1/ST2 in the lung, most notably 5 d after challenge. Further, IL-4, IL-5, and IL-13, but not interferon-γ mRNA were expressed at high levels 3 h after challenge. This study helps to elucidate the "geography" of immune responses generated in a conventional murine model of allergic airways inflammation.

Airway eosinophilia is a characteristic feature of asthmatic inflammation in humans, as well as in murine experimental models that recapitulate this process (1, 2). However, the accumulation of eosinophils in the airway may be viewed as a terminal step in a sequence that involves complex cellular interactions not only in the airway but also in other immunologically important sites, such as the thoracic lymph nodes. Although a critical role of T lymphocytes in the development of experimental allergic inflammation is well established (3), T cells cannot respond effectively to antigen unless it is adequately presented to them by antigen-presenting cells (APCs) (4, 5). Dendritic cells (DCs), the most potent APCs, are believed to be indispensable to the initiation of primary T-cell responses (6, 7). In addition to their ability to capture and process antigen, DCs have a considerable migratory capacity. Because both T cells and DCs display discrete migration patterns, the temporal and spatial interplay between antigen, APCs, and T cells is of central importance to our understanding of specific immunity. The objective of this study was to investigate spatial and temporal changes in the phenotype of APCs and T cells in a model of antigen-induced airways inflammation.

We used a murine model of antigen-induced airways inflammation that involves sensitization with ovalbumin (OVA) delivered intraperitoneally together with an adjuvant, followed by antigen aerosol challenge. Our model and other similar models have been characterized in a number of parameters (8, 9); these studies, however, have largely focused on events that take place in the lung after aerosol challenge. Our objective was to define, after both sensitization and challenge, cellular subsets within the APC and lymphocyte compartments at two sites, namely the lungs and the thoracic lymph nodes.

The pattern that emerged from these studies is that there is a cellular expansion, mainly of B and T cells, in the thoracic lymph nodes shortly after the second intraperitoneal (i.p.) injection. A considerable number of APCs expressed the costimulatory molecule B7.2, with no significant changes (compared with naive mice) in B7.1 expression. Although we observed increased expression of CD69 on T cells, T1/ST2 expression remained at the level detected in naive mice. Although these cells expressed GATA-3 and signal transducer and activator of transcription (STAT)-6 and were capable of cytokine production *in vitro* after stimulation with OVA, expression of several cytokine messenger RNA (mRNA) species *in vivo* was marginal. Throughout the process of antigen sensitization, the lung remained immunologically silent. However, shortly after aerosol challenge there was an expansion of APCs in the lung that was different in character from that observed in the lymph nodes. That is, whereas APC expansion in the lymph nodes was mainly due to B cells, APC expansion in the lung largely consisted of macrophages and DCs. This expansion was accompanied by enhanced expression of both B7 molecules. We also observed a marked expansion of $CD69^+$ T cells in the lung after aerosol challenge. In contrast to our findings in the lymph nodes, however, we documented a remarkable increase in the number of $CD4^+$ T cells expressing the T helper (Th)-2–associated marker T1/ST2. In addition, we observed a dramatic increase in effector ac-

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Abbreviations: antigen-presenting cell, APC; dendritic cell, DC; fluorescein isothiocyanate, FITC; Hanks' balanced salt solution, HBSS; intraperitoneal, i.p.; interleukin, IL; major histocompatibility complex, MHC; messenger RNA, mRNA; ovalbumin, OVA; polymerase chain reaction, PCR; ribonuclease, RNase; standard error of the mean, SEM; signal transducer and activator of transcription, STAT; T helper, Th.

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tivity, as detected by expression of interleukin (IL)-4, IL-5, and IL-13 mRNA after aerosol challenge. These Th2-associated cytokines were expressed at a considerably greater level than interferon (IFN)-γ and IL-15, whereas IL-2 and IL-10 were expressed at marginal levels. Our study indicates that whereas the major events, including expansion of activated T cells and APCs, occur in the lymph nodes after sensitization, the effector functions are executed within tissue presumably upon the encounter of antigen.

Materials and Methods

Animals

Female Balb/c mice (6 to 8 wk old) were purchased from Harlan (Indianapolis, IN). Mice were maintained under a 12-h light–dark cycle in an access-restricted area. Cages, food, and bedding were autoclaved, and the handling of mice was carried out in a laminar flow hood only by gloved and masked personnel. The Animal Research Ethics Board of McMaster University approved all the experiments described here.

Sensitization and Antigen Challenge Protocol

The sensitization and challenge protocol has been described previously (8). In brief, mice were sensitized at Days 0 and 5 by i.p. injection of 8 μg OVA (Sigma Chemical Co., St. Louis, MO) adsorbed to 4 mg of aluminum hydroxide (Aldrich Chemicals Co., Milwaukee, WI) overnight at 4°C in a total volume of 0.5 ml of phosphate-buffered saline (PBS). At 7 d after the second sensitization, mice were placed in a Plexiglas chamber (10 × 15 × 25 cm) and exposed to aerosolized OVA (10 mg/ml in 0.9% saline) for 1 h on two occasions 4 h apart. The aerosolized OVA was produced by a Bennet nebulizer at a flow rate of 10 liters/min.

Lymph Nodes and Lung Cell Isolation

Thoracic lymph nodes, including the hilar, mediastinal, and tracheobronchial nodes, were removed and adjacent connective tissue was dissected away. The nodes were immediately placed in cold (4°C) Hanks' balanced salt solution (HBSS) (GIBCO BRL, Grand Island, NY). The nodes were ground between frosted slides and filtered through nylon mesh (BSH Thompson, Scarborough, ON, Canada). The cell suspension was centrifuged at 1,200 rpm for 10 min at 4°C and resuspended again in PBS. After this washing step, the cells were resuspended in flow cytometric analysis buffer (PBS supplemented with 0.2% bovine serum albumin or in RPMI (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin. Cells were cultured in medium or alone with 40 μg OVA/well at 8 × 10^5 cells/well in a 96-well flat-bottom plate (Becton Dickinson, Lincoln Park, NJ). After 5 d of culture, supernatants were harvested for cytokine measurement.

For isolation of lung cells, lungs were flushed via the right ventricle of the heart with 10 ml of warm (37°C) HBSS (calcium- and magnesium-free) containing 5% FBS (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). The lungs were then cut into small pieces (approximately 2 mm in diameter) and shaken at 37°C for 1 h in 15 ml of 150 U/ml collagenase III (Worthington Biochemical, Freehold, NJ) in HBSS. Using a plunger from a 5-ml syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon mesh. After lysing red blood cells with ACK lysis buffer (0.5M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2–ethylenediaminetetraacetic acid at pH 7.2 to 7.4), cells were washed twice and mononuclear cells were isolated by density centrifugation in 30% Percoll (Pharmacia, Uppsala, Sweden). With this enzymatic digestion protocol approximately 60% of cells constituted mononuclear cells, and these were gated for flow cytometric analysis.

Flow Cytometric Analysis

Panels of monoclonal antibodies (mAbs) were selected to study the phenotype of APCs and T lymphocytes in lung and lymph-node cells. To minimize nonspecific binding, 10^6 cells were preincubated with FcBlock (CD16/CD32; Pharmingen, Mississauga, ON, Canada). For each antibody combination, 10^6 cells were incubated with mAbs at 0 to 4°C for 30 min; the cells were then washed and treated with second-stage reagents. The following antibodies were purchased from Pharmingen: anti-B7.1 (biotin-conjugated 16-10AI), anti-B7.2 (biotin-conjugated GLI), anti-CD11b (Mac1) (phycoerythrin [PE]-conjugated MI/70), anti-CD11c (PE-conjugated HL3), anti-CD3 (biotin-conjugated 145-2CII), anti-CD4 (fluorescein isothiocyanate [FITC]-conjugated L3T4), anti-CD8 (FITC-conjugated Ly-2), and anti-CD69 (PE-conjugated H1 2F3); the anti–major histocompatibility complex (MHC) class II antibody (FITC-conjugated M5/114.152) was prepared by Dr. D. P. Snider (Department of Pathology and Molecular Medicine, McMaster University). T1/ST2 (3E10) antibody was provided by Millenium Pharmaceuticals, Inc. (Cambridge, MA), and FITC-labeled in-house according to a standard protocol (10). Streptavidin PerCP (Becton Dickinson, San Jose, CA) was used as a second-step reagent for detection of biotin-labeled antibodies. Titration was used to determine the optimal concentration for each antibody. Cells were fixed in 1% paraformaldehyde and counted on a FACScan, and analyses were performed using PC-LYSIS software (Becton Dickinson, San Jose, CA). A total of 50,000 to 100,000 events was acquired.

Collection, Extraction, Separation, and Isolation of mRNA from Tissues

Thoracic lymph nodes, spleens, and lungs (typically the left lobe and one right lobe) were collected and placed in 1 ml TriPure Isolation Reagent, a monophasic solution of phenol and guanidine thiocyanate (Boehringer Mannheim Canada, Laval, PQ, Canada). Tissues were then homogenized with a Polytron 7-mm power homogenizer (Kinematica, Lucerne, Switzerland) and RNA was isolated according to the TriPure Isolation Reagent protocol. The RNA pellet was resuspended in 20 μl of diethypyrocarbonate-treated ribonuclease (RNase)-free water. To determine the concentration of total RNA collected, the optical density was calculated using an Ultraspec 1000 UV/Visible spectrophotometer (Pharmacia Biotech [Biochrom] Ltd., Cambridge, UK). The RNA was stored in a −70°C freezer until analysis.

RNase Protection Assay

The RiboQuant Multi-Probe RNase Protection Assay (Pharmingen) was used to detect and quantify mRNA species from lungs and lymph-node tissues. Briefly, on Day 1 of the assay, an [α-^{32}P]uridine triphosphate (30,00 Ci/mmol, 10 mCi/ml)–labeled antisense RNA probe set was synthesized using the mCK-1 Multi-Probe Template Sets. The probe (~ 1 × 10^6 counts per min [cpm]/μl) was then hybridized with the desired amount of target RNA, typically 15 μg, overnight at 56°C. PharMingen control RNA (2 μg) and yeast transfer RNA (2 μg) were used as positive and negative controls, respectively. On Day 2, the unhybridized RNA and protein were digested and the protected probes were purified, precipitated, and resuspended in loading buffer according to the manufacturer's protocol. The samples were loaded at approximately 2,000 cpm/lane and electrophoresed at 60 W constant power on a denaturing 5% polyacrylamide gel to resolve the RNase-protected probes. The gel was dried for ~ 1 h at 80°C under a vacuum gel drier (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada) and placed on a phosphor storage screen

(Molecular Dynamics, Sunnyvale, CA) overnight. The gel was visualized and analyzed using ImageQuant software (Molecular Dynamics). To quantify, each test and housekeeping band, as well as the background of each lane, was captured by first drawing a rectangle around it and then integrating the volume of intensity inside the rectangle. Background volumes were subtracted, and the ratio of the test band volume to the average of the housekeeping band volume was generated and expressed as RNase protection assay (RPA) units ($\times 10^3$).

Real-Time Polymerase Chain Reaction (Taqman)

STAT-6 and GATA-3 expression was evaluated using real-time quantitative polymerase chain reaction (PCR) analysis. In brief, an oligonucleotide probe was designed to anneal to the STAT-6/GATA-3 genes between two PCR primers. The probe was then fluorescently labeled with 6-carboxyl-fluorescein (reporter gene) on the 5′ end and with 6-carboxyl-tetramethyl rhodamine (quencher dye) on the 3′ end. A similar probe and PCR primers were purchased for rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The probe for this gene incorporated VIC as the reporter dye. PCR reactions were run that included the primers and probes for these two genes as well as complementary DNA (cDNA) made from cells isolated from lymph nodes. As the polymerase moved across the gene during the reaction, it cleaved the dye from one end of each probe, which caused a fluorescent emission that was measured by the Sequence Detector 7700. The emissions recorded for each cDNA were then converted to determine the level of expression for STAT-6/GATA-3 normalized to the expression of mGAPDH. Expression of STAT-6 and GATA-3 was determined on cells isolated from lymph nodes after a second i.p. sensitization.

Measurement of Cytokines

Enzyme-linked immunosorbent assay (ELISA) kits for murine IL-4 and IL-5 were purchased from R&D Systems (Minneapolis, MN) and Amersham (Buckinghamshire, UK), respectively; each of these systems has a threshold of detection of 1.5 to 5 pg/mL.

Data Analysis

Data are expressed as means ± standard error of the mean (SEM). Whenever suitable, results were interpreted using analysis of variance (ANOVA) test with Tukey's *post hoc* test or Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

Results

Phenotype of APCs in the Thoracic Lymph Nodes and Lungs

Mice were killed at Days 0 (naive mice), 6 (24 h after sensitization), and 13 (24 h after challenge). We observed an enlargement of mediastinal, bronchial, and tracheobronchial lymph nodes after the second i.p. injection with OVA/alum. Numerically, this translated into a significant cellular increase from $1.2 \pm 0.4 \times 10^6$/mouse at Day 0 to $6.4 \pm 0.7 \times 10^6$/mouse at Day 6, as assessed by counting with hemocytometer; this enlargement was also observed at Day 13 after challenge ($4.9 \pm 0.8 \times 10^6$/mouse). In the lungs, the total cell numbers at Days 0 and 6 were not significantly different from one another. However, they increased more than 3-fold from $2.2 \pm 0.4 \times 10^6$/mouse at Day 0 to $6.9 \pm 1.6 \times 10^6$/mouse at Day 13.

Having documented an expansion in total cell number at Day 6 in the lymph nodes and at Day 13 in the lungs, we then examined the types of APCs contributing to this expansion. To this end, B cells, macrophages, and DCs were identified on the basis of $B220^+$ (11), MHC II^+/$Mac\text{-}1^+$ (12), and MHC II^+/$CD11c^+$ (13) expression, respectively. As shown in Figure 1, it is apparent that the increase in APCs in the lymph nodes at Day 6 was due largely to B cells (40.9% compared with 18.8% in naive mice). The same distribution of APCs in the lymph nodes was observed at Day 13 (data not shown). In contrast, the increase in APCs observed in the lungs at Day 13 was due to increases in macrophages (16.6 ± 2.3 versus 3.8 ± 0.2) and DCs (9.1 ± 1.8 versus 2.2 ± 0.2).

Expression of B7.1 and B7.2 on APCs

Next, we examined the expression of the costimulatory molecules B7.1 and B7.2 on APCs in lymph nodes and lung. As shown in Figure 2, MHC II^+/$B7.2^+$ cells increased considerably at Day 6, from 14.7% to 38.4%, and remained at a similar level at Day 13 (45.5%) in the lymph nodes. In contrast, the percentage of MHC II^+ cells expressing B7.1 did not increase at any time point tested.

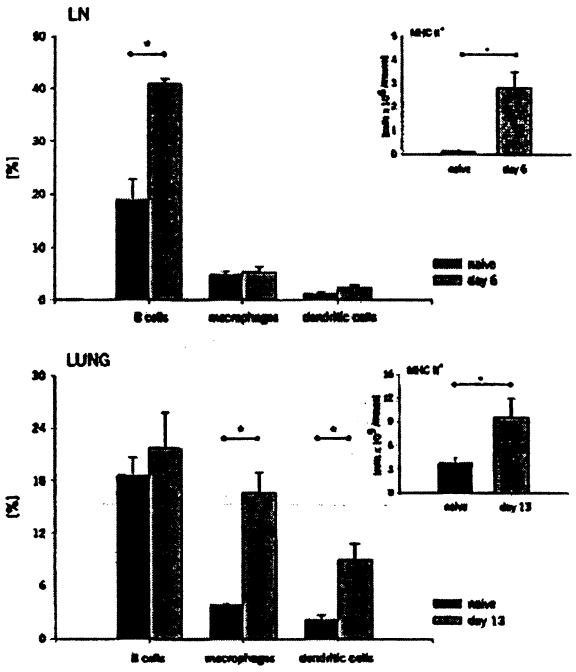

Figure 1. Proportion of B cells, macrophages, and DCs in the lung and lymph nodes at different time points after sensitization and challenge with OVA. Totals of 50,000 and 100,000 events were collected for lungs and lymph-node cells, respectively, and analyzed for MHC/CD11c (DCs), MHC/Mac-1 (macrophages), and B220 (B cells). *Inserts* indicate changes in the number of APCs (MHC II^+) at the time of major expansion. Lymph nodes and lungs from five to 10 mice were pooled for each group. Values represent means ± SEM of three to five experiments. Statistical analysis was performed by *t* test; $^*P < 0.05$.

Further, Table 1 shows that a large proportion of the increase in B7.2$^+$ cells was contributed by B cells (from 12.3 ± 3.4% to 28.1 ± 7.1%). This preferential expression of B7.2 on B cells was also detected at Day 13 after challenge (40.3 ± 6.3%) (data not shown). We also observed a trend for increased B7.2 expression on DCs from 3.4 ± 1.6% in naive to 8.3 ± 2.1% at Day 6 and 14.8 ± 4.1% at Day 13. The proportion of B cells, macrophages, and DCs expressing B7.1 did not change at any time point.

In the lung, we observed an increase in the proportion of APCs expressing either B7.1 or B7.2 (Figure 2). Table 1 shows that there was a large increase in the proportion of macrophages expressing both B7 molecules. Interestingly, although the proportion of DCs expressing B7.1 did not change significantly, we observed an increase in the proportion of DCs expressing B7.2 at Day 13.

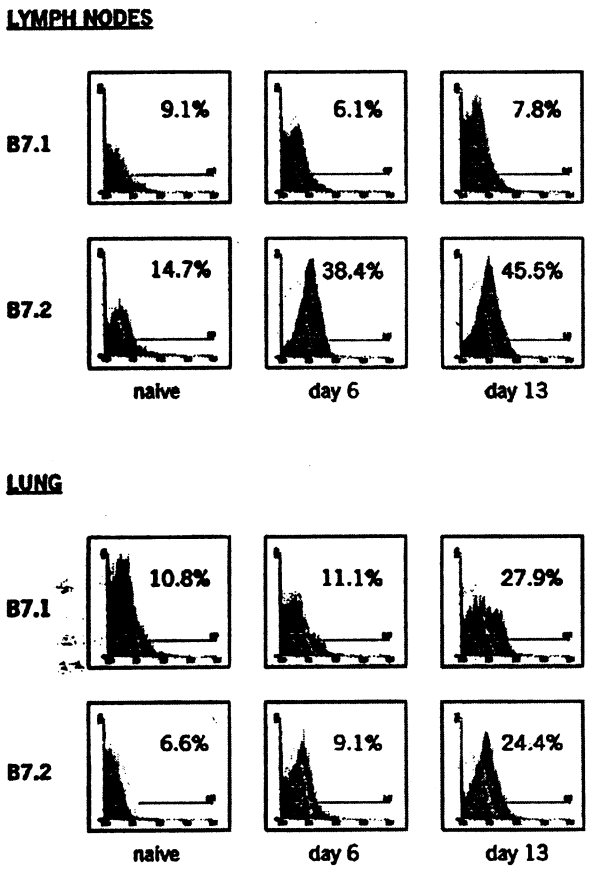

Figure 2. B7.1 and B7.2 expression on MHC II$^+$ cells isolated from lymph nodes and lung tissue at the indicated time points after sensitization and challenge. Cells were gated on MHC II$^+$ cells and then evaluated for the distribution of B7 markers on the cell surface. Representative histograms from one experiment are shown; *unfilled histograms* represent cells stained with isotype-matched control mAbs. Lymph nodes and lungs from five to 10 mice were pooled for each group. One of four representative experiments is shown.

Activation Status of T Cells in the Thoracic Lymph Nodes and Lungs

Figure 3 depicts the content and distribution of activated (CD69$^+$) T-cell subsets. In the lymph nodes, the absolute number of activated CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ T cells significantly increased after sensitization (Day 6). Our findings show that 5 to 6% of CD4$^+$ or CD8$^+$ cells expressed CD69 in the lymph nodes of naive mice. Intraperitoneal sensitization resulted in a doubling of the proportion of activated cells, which remained at a similar level at Day 13 of the protocol.

In the lung, there were no statistically significant differences in the numbers of activated CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ cells at Days 6 and 13 compared with naive mice. Interestingly, at Day 17 (i.e., 5 d after challenge) the number of CD3$^+$/CD4$^+$ T cells that were CD69$^+$ increased significantly, whereas the increase in CD8$^+$/CD69$^+$ cells was comparatively marginal. In terms of percentages, the proportion of CD3$^+$/CD4$^+$ cells expressing CD69$^+$ reached 19 ± 5.4%.

Cytokine Expression

Cytokines are a defining component of an effector immune response. To examine expression of a broad range of cytokines in both the thoracic lymph nodes and the lung *in vivo* we chose to evaluate mRNA expression using an RPA. The mCK-1 template, which includes cytokines that are particularly relevant to allergic airways inflammation, was used. mRNA was obtained from lung and lymph nodes at 3, 6, 12, and 18 h after sensitization and after challenge. The kinetics of mRNA expression for all cytokines in both compartments followed a similar pattern: peak expression at 3 h after either sensitization or challenge, with a return to baseline levels between 12 and 18 h (data not shown). Consequently, only the data at 3 h are shown in Figure 4. Cytokine mRNA expression in naive lungs and spleens was minimal for the eight cytokines examined in this template. Likewise, very low mRNA expression was detected in the lung and lymph nodes at Day 5. In contrast, in the challenged lung (Day 12) we observed robust expression of mRNA for IL-4, IL-13, IL-5, and IL-6; comparatively modest expression of IFN-γ and IL-15; and minimal expression of IL-2 and IL-10. Given the unaltered expression of cytokine mRNA in the lymph nodes at the time of major cellular expansion (Day 6), we sought evidence for Th2 polarization by examining the expression of STAT-6 and GATA-3 in the thoracic lymph nodes. Figure 5 shows that, compared with naive lymph nodes, expression of both genes was upregulated after i.p. sensitization (Day 5). Hence, we next examined the ability of lymph-node cells to produce Th2 cytokines upon antigen recall. To this end, cells were cultured with OVA or medium alone for 5 d, and IL-5 and IL-4 production in culture supernatants was measured by ELISA. IL-5 (5,050 ± 2,730 pg/ml) and IL-4 (628 ± 230 pg/ml) were detected only in supernatants from cells stimulated with OVA (Figure 6).

T1/ST2 Expression in the Lymph Nodes and Lungs

Table 2 shows the percentage of CD4$^+$ T cells that expressed T1/ST2. We did not observe statistically significant differences at Days 6 or 13 in either the lung or the lymph

TABLE 1

Expression of B7.1 and B7.2 on different APCs in lungs and lymph nodes

	B Cells		Macrophages		DCs	
	B7.1	B7.2	B7.1	B7.2	B7.1	B7.2
Lymph nodes						
Naive	4.5 ± 2.0	12.3 ± 3.4	11.8 ± 4.3	17.2 ± 4.8	3.0 ± 1.3	3.4 ± 1.6
Day 6	2.9 ± 0.9	28.1 ± 7.1*	5.9 ± 0.5	17.1 ± 2.1	2.7 ± 0.7	8.3 ± 2.1
Lungs						
Naive	6.2 ± 0.4	8.9 ± 1.1	5.8 ± 4.6	7.7 ± 1.3	18.9 ± 8.2	5.4 ± 1.7
Day 13	5.1 ± 0.7	14.3 ± 2.6	25.9 ± 3.0*	18.8 ± 2.5*	24.6 ± 7.0	29.2 ± 4.8*

Mice were sensitized with OVA/aluminum hydroxide on Days 0 and 5 and exposed to aerosolized OVA on Day 12 of protocol. Levels of B7.1 or B7.2 were evaluated within the gates for separate subpopulations (B cells, macrophages, and DCs). Lymph nodes and lungs were pooled from five to ten mice for each time point. Data shown are %; means ± SEM.

*$P \leq 0.05$ compared with naive.

nodes compared with naive mice. However, there was a significant increase in the proportion of $CD4^+$ T cells expressing $T1/ST2^+$ in the lung at Day 17 of the protocol (18.5 ± 1.5% compared with 3.6 ± 1.2% in naive animals).

Discussion

The primary objective of this study was to define, spatially and temporally, immune activity in a conventional murine model of allergic airways inflammation: one that involves the introduction of antigen (OVA) together with adjuvant (aluminum hydroxide) into the peritoneal cavity followed by respiratory exposure to aerosolized antigen. We have previously documented cell and cytokine changes in the bronchoalveolar lavage fluid (BALF), peripheral blood, and bone marrow of the model described in this study (8). Here, we focused our analysis on mononuclear cells, particularly APCs and T cells, because together with antigen they constitute the tripartite interaction that determines the nature of the immune–inflammatory response.

Our data document a remarkable expansion of cells in the lymph nodes after sensitization. The increase in the APC compartment was largely due to B cells (Figure 1) which translates into the establishment of humoral responses (immunoglobulin [Ig] E production; ref. 8) and may implicate B cells as major APCs. However, recent studies have demonstrated that T cells can still be primed, and airways inflammation established, in B cell–deficient mice (14). This indicates the involvement of different cell types in the process of antigen presentation, possibly DCs, which among all APCs represent the subset with greater antigen-presenting capacity crucial for the activation of naive T cells both *in vitro* and *in vivo* (15, 16). Thus, the functional significance of the comparatively small changes in DCs that we observed in the lymph nodes after sensitization may be much greater than what the quantitative changes would imply.

In the lung, robust APC accumulation was observed at Day 13 (24 h after aerosol challenge). In sharp contrast to our findings in the lymph nodes, DC and macrophage expansion predominated in the lung, with no significant changes in B cells (Figure 1). These findings are consistent with the notion that an increase in DCs is a universal feature of the response after mucosal exposure to bacteria and to viral and soluble protein antigens (17, 18). The importance of lung DCs as APCs and, specifically, in the induction of secondary responses to surrogate allergens has recently been demonstrated (19). With respect to macrophages, the functional significance of the rather remarkable increase that we observed is unclear. This expanded macrophage population expressed the costimulatory molecules B7.1 and B7.2. Hence, antigen challenge might alter the functional phenotype of lung macraphages from poor APCs (20, 21), as has been described previously, to more effective APCs. We cannot exclude the possibilty that our observed increases in macrophages are due to DC precursors in the lung vasculature, particularly because DCs have been shown to share certain phenotypic characteristics with monocytes/macrophages (22). Alternatively, the major role of macrophages may be, as proposed by Gong and colleagues (23), to degrade soluble proteins into antigenic peptides that are then captured by DCs to be presented to T cells. Our current understanding of the interactions between the innate and adaptive immune systems in general and, particularly, among macrophages, DCs, and T cells is incomplete, especially as it relates to responses to aeroallergens.

Costimulatory molecule expression is clearly a central requirement for the generation of a productive immune response (24). Specifically, the importance of the CD28/CTLA4/B7 pathway in the elicitation of immune responses in models of allergic airways inflammation has recently been demonstrated (25–29). Our data document an increase in $B7.2^+$ APCs, particularly B cells, in the thoracic lymph nodes 24 h after sensitization (Day 6 of the protocol) (Figure 2 and Table 1). In contrast to B7.2, we did not detect significant changes in the number of APCs expressing B7.1. Thus, our data demonstrate that the dominant B7 costimulatory molecule expressed in the thoracic lymph nodes at the time of sensitization is B7.2, a finding that is consistent with the notion that expression of B7.1 or B7.2 will privilege CD4 T-cell differentiation toward the Th1 or Th2 phenotypes, respectively (30, 31).

In the lung, however, our data demonstrate that expression of both B7.1 and B7.2 molecules increases considerably after challenge. Although B7.2 is considered the dominant B7 costimulatory molecule in this model on the basis of evidence that treatment with anti-B7.2 antibodies prevents pulmonary eosinophilia, secretion of Th2-type cyto-

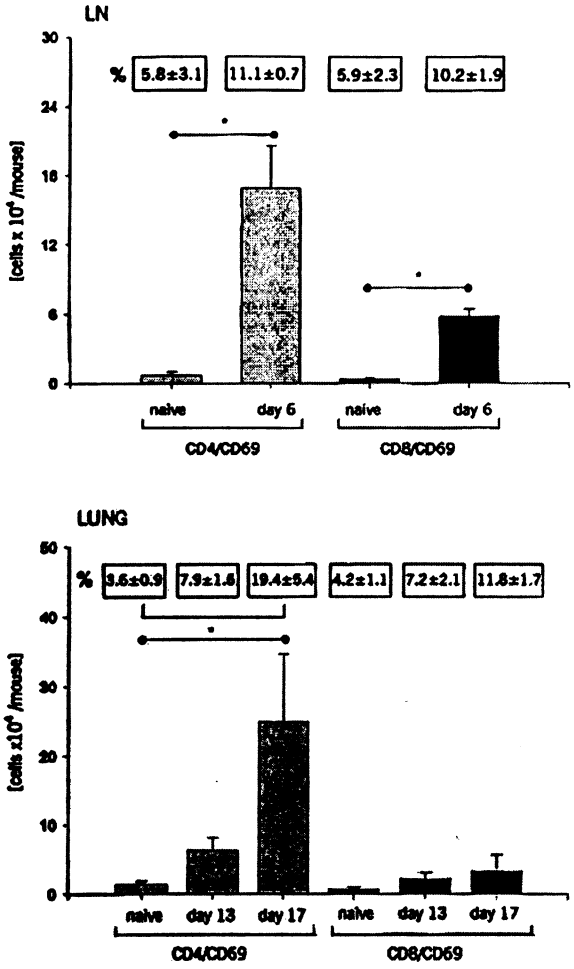

Figure 3. Activated T-lymphocyte subsets in lung and lymph nodes at different time points after sensitization and challenge with OVA. Values represent means ± SEM of three to five experiments. For each time point, cells were pooled from five to 10 mice. Statistical analysis was performed by *t* test (lymph nodes) or ANOVA with Tukey's *post hoc* test (lung); $*P < 0.05$.

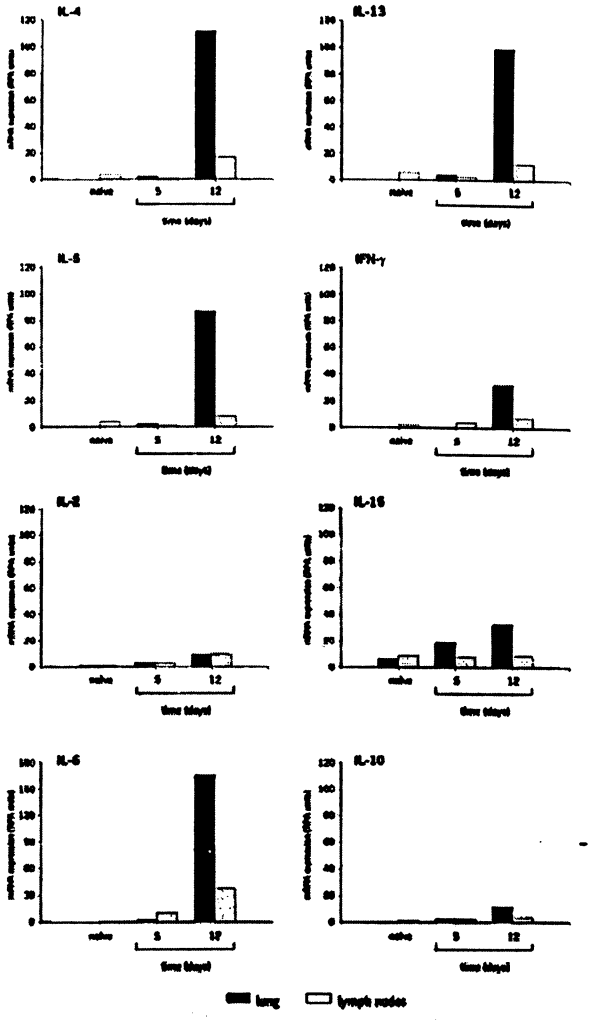

Figure 4. RPA. Detection of mRNA expression for selected cytokines in lung and lymph nodes after sensitization and challenge. Mice were sensitized or sensitized and challenged and total RNA was extracted from lymph nodes and lungs at the indicated times after the second sensitization (Day 5; 3 h after second i.p. sensitization) or challenge (Day 12; 3 h after challenge). For naive mice, RNA was extracted from spleen. Data are representative of two independent experiments.

kines, IgE production, and bronchial hyperreactivity, it is likely that B7.1 also plays a meaningful, perhaps somewhat unappreciated, role in this process. In this regard, several studies have shown that administration of anti-B7.1 antibodies significantly decrease airways eosinophilia (29, 32). Particularly informative is the finding by Harris and associates (32), who showed that although treatment with anti-B7.1 antibodies significantly decreased airways eosinophilia, it did not decrease peripheral blood eosinophilia. Moreover, Masten and coworkers (33) demonstrated that B7.1 signaling by lung DCs plays an important role in costimulation. The concept that B7.1. and B7.2 likely play complementary roles has recently been reinforced by studies using B7.1 and/or B7.2 knockout mice (34). Our data support the hypothesis that whereas B7.2 has a predominant role in the sensitization event that takes place in the lymph nodes, B7.1 plays an important role in the lung after secondary antigen exposure. For example, B7.2 may be essential in the process leading to the generation of peripheral blood eosinophilia, whereas the influx of eosinophils into the tissue may require additional signals mediated by B7.1 in the lung/airway. Thus, both B7.1 and B7.2 are probably required for the full expression of the allergic phenotype, with discrete requirements for both molecules depending on the time, site, and context of their expression.

The APC increase in the lymph nodes after the second i.p. sensitization is concomitant with the expansion of activated ($CD69^+$) T lymphocytes (Figure 3), a phenomenon that is sustained after OVA challenge. There is very lim-

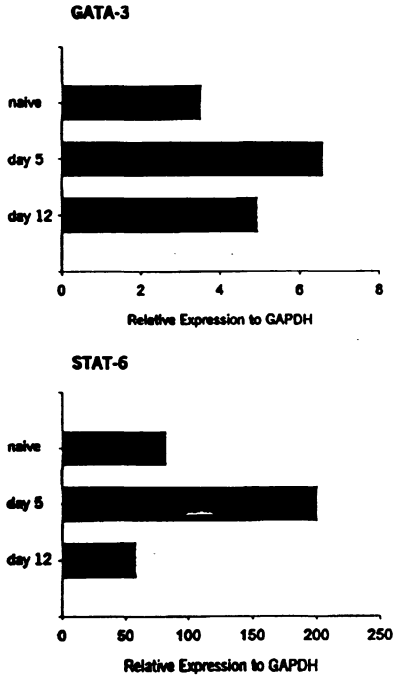

Figure 5. Effect of sensitization on the levels of GATA-3 and STAT-6 in lymph nodes. Mice were sensitized twice by i.p injection of OVA/aluminum hydroxide, the lymph nodes were removed, and total RNA was extracted. Real-time quantitative PCR (TaqMan) was run. Data are representative of two experiments.

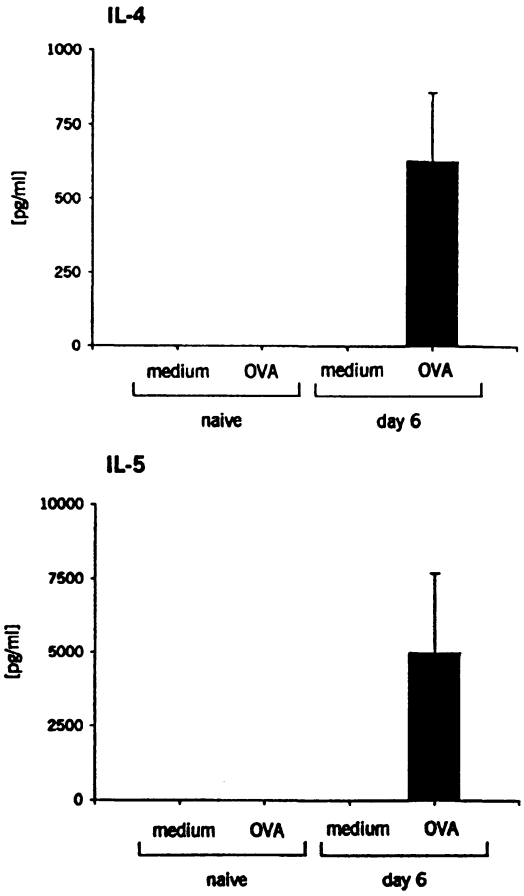

Figure 6. Th2 cytokine production from lymph nodes cultured *in vitro* with or without OVA. At the indicated time points, lymph nodes were removed, pooled, and placed in culture for 5 d in either medium or OVA. Cytokines were measured by ELISA (n = 3; means ± SEM). Three independent experiments yielded similar results.

ited information with respect to events in the thoracic lymph nodes in this type of experimental model. Krinzman and colleagues (35) reported an increase of $CD4^+$ T cells in the thoracic lymph nodes of sensitized mice after challenge. Our findings extend this observation because we demonstrate that such changes are already established after sensitization. This pattern reflects the general model in which primary T-cell activation and expansion, facilitated by APCs, occur in lymphoid tissues.

In the lung, we observed an initial increase in activated T cells 24 h after challenge. However, the major expansion of $CD4^+/CD69^+$ cells took place at Day 17 (5 d after challenge), in accordance with our earlier studies describing massive influx of lymphocytes into BALF (8). It is of interest to note that whereas the ratio of $CD4^+/CD69^+$ to $CD8^+/CD69^+$ cells in the thoracic lymph nodes at Day 6 (24 h after sensitization) was 2.9, this ratio was 7.5 in the lung 5 d after challenge, indicating preferential participation of $CD4^+$ T cells in airways inflammation. Indeed, the involvement of $CD4^+$ T cells in the development of airway inflammatory responses to allergens is well established (36, 37). In fact, the role of $CD4^+$ T cells is pivotal: CD4 knockout mice cannot be sensitized, and depletion of CD4 cells in wild-type animals prevents antigen-induced airway hyperreactivity and airways eosinophilia (38).

To investigate whether expansion in the lymph nodes and lungs reflects the establishment of effector activity, we evaluated cytokine mRNA expression. As shown in Figure 4, expression of the prototypic Th2 cytokines IL-4, IL-5, and IL-13 was minimal and virtually identical to that observed in naive mice (in the thoracic lymph nodes) at all time points examined. We think that it is very unlikely that we missed cytokine upregulation because mRNA was obtained at 3, 6, 12, and 18 h after the second i.p. injection. We find it informative that despite clear evidence of T-cell expansion and preferential activation, as assessed by CD69 expression especially on $CD4^+$ cells, actual effector activity, as assessed by cytokine mRNA expression, was not apparent. Interestingly, OVA sensitization led to increased levels of STAT-6 and GATA-3, which are implicated in the differentiation of naive T cells into Th2 cells (Figure 5) (39, 40). In our view, these findings convey a functional logic: there is no immunologic advantage of producing effector molecules in the regional lymph nodes, whereas the production of effector molecules in the target organ (in our case, the lung) is essential. Indeed, *in vitro* stimulation of lymph-node cells with OVA induced the

TABLE 2
T1/ST2 expression on CD4 cells in lung and lymph nodes

	CD4 T1/ST2	
	Lung	Lymph Node
Naive	3.6 ± 1.2	4.0 ± 1.1
Day 6	5.1 ± 2.4	5.3 ± 0.9
Day 12	7.7 ± 0.7	5.0 ± 0.8
Day 17	18.5 ± 1.5*	6.3 ± 1.0

Mice were sensitized with OVA/aluminum hydroxide on Days 0 and 5 and exposed to aerosolized OVA on Day 12 of protocol. Expression of T1/ST2 within the CD3/CD4 subpopulation was examined by flow cytometry at the indicated time points. Lymph nodes and lungs were pooled from five to ten mice for each time point. Data shown are %; means ± SEM.

*$P \leq 0.05$ compared with naive.

production of typical Th2 cytokines such as IL-5 and IL-4 (Figure 6). The ability of lymph-node cells to respond to antigen *in vitro* can be explained within the model proposed by Sallusto and associates in which immunologic memory is displayed by distinct T-cell subsets: lymph node–homing cells lacking inflammatory functions and tissue-homing cells endowed with various effector functions such as cytokine production (41). Because *in vitro* conditions simulate the *in vivo* tissue environment, we observed the transition from nonproducers to cytokine producers.

The findings discussed earlier argue that for Th cells to execute their effector program they need to encounter antigen in the right environment in the tissue. Indeed, in agreement with observations by Krzesicki and coworkers (42), we detected substantial effector activity in the lung after challenge as indicated by considerable upregulation of IL-4, IL-13, IL-5, and IL-6, but not IFN-γ, mRNA. However, we also found that mRNA expression for the cytokines IL-2 and IL-15 was minimal and, in fact, very similar to that observed in naive mice. It is interesting that, in the face of a major expansion of mononuclear cells, expression of cytokines with well-established proliferative activity remains so modest. This suggests that recruitment/relocalization, rather than *in situ* proliferation, may play a prominent role in the mononuclear cell expansion that we observed in the lung.

Further, and in sharp contrast to our findings in the thoracic lymph nodes, the proportion of $CD3^+/CD4^+$ cells expressing T1/ST2 increased as early as 72 h after challenge, with a considerable increase 5 d after challenge. T1/ST2 has significant homology to the IL-1 receptor, but does not bind IL-1α or IL-1β (43). More recently, T1/ST2 has been identified as a marker of advanced differentiation to the Th2 phenotype in Th cells polarized *in vitro* toward Th2 but not Th1 (44, 45). Interestingly, expression of T1/ST2 in the lymph nodes during sensitization remained at the level detected in naive mice (approximately 5%). Together, these data might argue for preferential recruitment of activated T1/ST2 $CD4^+$ T cells to the lung after antigen aerosol challenge. Alternatively, T cells may acquire this phenotype in the lung as a consequence of the Th2-polarized immune response. The geography of T1/ST2 expression (i.e., in the lung) is consistent with this logic and suggests that T1/ST2 may best be characterized as a marker of effector Th2 cells rather than simply of Th2 cells.

In summary, our studies provide an elaborate description of immunologic activity in the thoracic lymph nodes and lung during a response to the soluble antigen OVA. Our data identify the main sites of the primary and secondary immune responses, elucidate distribution and phenotype of APCs and T cells, and provide evidence that may prompt the revisiting of accepted concepts of activation and effector activity, particularly in allergic inflammation.

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CHAPTER 3

GENERATION OF ALLERGIC AIRWAYS INFLAMMATION IN THE ABSENCE OF DRAINING LYMPH NODES

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This publication assessed the ability of the mice devoid of the lymph nodes to generate Th2 inflammation in the lungs.

The work presented in this study was performed by the author of the thesis. David Alvarez contributed equally to this paper. Dr. Coyle and Dr. Gutierrez-Ramos provided T1/ST2 antibodies used in the study. Dr. Stämpfli provided useful discussion on the results of this work. Supervision was provided by Dr. Manel Jordana resulting in the multiple authorship of this paper.

Generation of experimental allergic airways inflammation in the absence of draining lymph nodes

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The objective of this study was to investigate the contribution of secondary lymphoid organs in the generation and maintenance of experimental allergic airway inflammation. We employed a previously reported murine model of respiratory mucosal allergic sensitization, induced by repeated aerosolizations of ovalbumin in the context of a GM-CSF airway environmentoWe executed this protocol in wild-type (WT) and lymphotoxin-α–deficient mice (LTα-KO) mice, which are devoid of lymph nodes (LNs) and possess rudimentary spleen structures. Despite the lack of pulmonary LNs draining the airway compartment, LTα-KO mice were fully capable of mounting a robust inflammatory response in the airways, consisting of Th2 polarized CD4⁺ T cells and eosinophils. This was accompanied by IL-5, IL-13, and IFN-γ production by splenocytes and generation of ovalbumin-specific serum IgE. Exposure to the same antigen 7 weeks after complete resolution of airway inflammation once again induced a Th2 polarized infiltrate, demonstrating intact immunological memory. To investigate inherent plasticity in establishing antigen-specific immunity, mice were splenectomized before sensitization. Allergic sensitization was completely abrogated in splenectomized LTα-KO mice, compared with eusplenic LTα-KO controls. These data demonstrate that secondary lymphoid organs, either LN or spleen, are essential for the generation of allergic airway responses.

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Introduction

Current understanding is that antigens that penetrate the mucosae are taken up by antigen presenting cells (APCs), mainly dendritic cells (DCs), and transported to the regional lymph nodes (LNs). In the LNs, these antigens are presented in the context of MHC molecules to naive T cells that will, then, undergo differentiation (1). An extension of this notion with reference to allergic asthma would assume that allergen-specific T cells are generated in the LN compartment, undergo Th2 differentiation, and traffic to the airway, where they execute their effector function including, most notably, production of a distinct cytokine and chemokine program that results in eosinophilic inflammation, goblet cell hyperplasia, and bronchial hyperreactivity (2–4). Evidence that intranasal delivery of antigen leads to activation events in the cervical LNs (5, 6), and a recent elegant demonstration of DC migration from the lung to the thoracic LNs in response to allergen aerosolization (7), support a role of the regional LNs in the generation of immune responses to aeroallergens. Yet the functional requirement of the thoracic LNs in the generation of allergic responses has not been directly investigated.

Lymphotoxin-α–deficient mice (LTα-KO) are born without morphologically detectable LNs or Peyer's patches; in addition, they have an altered splenic architecture, with the loss of the typical T and B cell areas and deficient germinal center formation (8–10). Hence, LTα-KO mice provide a particularly suitable model to investigate the role of secondary lymphoid structures in the generation of mucosal immune responses. To investigate the role of LNs in this process, we used a murine model in which allergic sensitization is achieved by repeated aerosolizations of ovalbumin (OVA) in the context of a GM-CSF airway environment, which is established by gene transfer of a replication-deficient adenovirus encoding murine GM-CSF (11).

Our data show that, despite a complete deficiency of all peripheral LNs, LTα-KO mice subjected to this experimental protocol mount a robust airway inflammatory response that is qualitatively comparable and quantitatively greater than that observed in wild-type (WT) littermates. In vivo antigen recall responses, Th2-associated IgE synthesis, and in vitro cytokine production confirmed the establishment of antigen-specific immunity and memory. To investigate the site of allergic sen-

sitization in LTα-KO mice, we surgically removed the spleen. Splenectomy of LTα-KO mice before the initiation of our protocol prevented sensitization and airway inflammation, whereas splenectomy of WT littermates did not affect the generation of allergic sensitization. These data establish a critical requirement of secondary lymphoid organs in the generation of Th2 responses to aeroallergens and intimate the plasticity of the immune system in the generation of mucosal immunity.

Methods

Animals. C57BL/6 and LTα-KO mice (5–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in a specific pathogen-free environment after a 12-hour light-dark cycle. All experiments performed were approved by the Animal Research Ethics Board of McMaster University.

Model of respiratory mucosal allergic sensitization. Replication-deficient human type 5 adenoviral construct encoding murine GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF) was delivered intranasally 24 hours before the first OVA exposure. Ad/GM-CSF was administered intranasally at a dose of 3×10^7 pfu in 30 μl of PBS vehicle (two 15-μl administrations, 5 minutes apart) into anesthetized animals. Over a period of 10 consecutive days (days 0–9), mice were placed in a Plexiglas chamber (10 cm × 15 cm × 25 cm) and exposed for 20 minutes daily to aerosolized OVA (1% wt/vol in 0.9% saline). OVA aerosol was generated by a Bennet nebulizer (Puritan-Bennet Corp., Carlsbad, California, USA) at a flow rate of 10 l/min. For in vivo rechallenge with OVA, mice were reexposed to a 1% OVA aerosol for 20 minutes daily for 3 consecutive days, after complete resolution of initial airways inflammation.

Collection and measurement of specimens. At various time points, mice were sacrificed and bronchoalveolar lavage (BAL) was performed according to standard protocol (11). Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson and Co., Sparks, Maryland, USA). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml); approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. Cell pellets were resuspended in PBS, and smears were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, Pennsylvania, USA) at 10.12 *g* for 2 minutes. Hema 3 (Biochemical Sciences Inc., Swedesboro, New Jersey, USA) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 minutes at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. Sections (3-μm-thick) were stained with hematoxylin and eosin (H&E).

Splenocyte culture. Spleens were harvested into sterile tubes containing sterile HBSS (Life Technologies Inc., Burlington, Ontario, Canada). Tissue was triturated between the ends of sterile frosted slides, and the resulting cell suspension was filtered through nylon mesh (BSH Thompson, Scarborough, Ontario, Canada). Red blood cells were lysed with ACK lysis buffer (0.5 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2EDTA [pH 7.2–7.4]). Remaining splenocytes were washed twice with HBSS and then resuspended in RPMI supplemented with 10% FBS (Life Technologies Inc.), 1% L-glutamine, 1% penicillin/streptomycin. Cells were

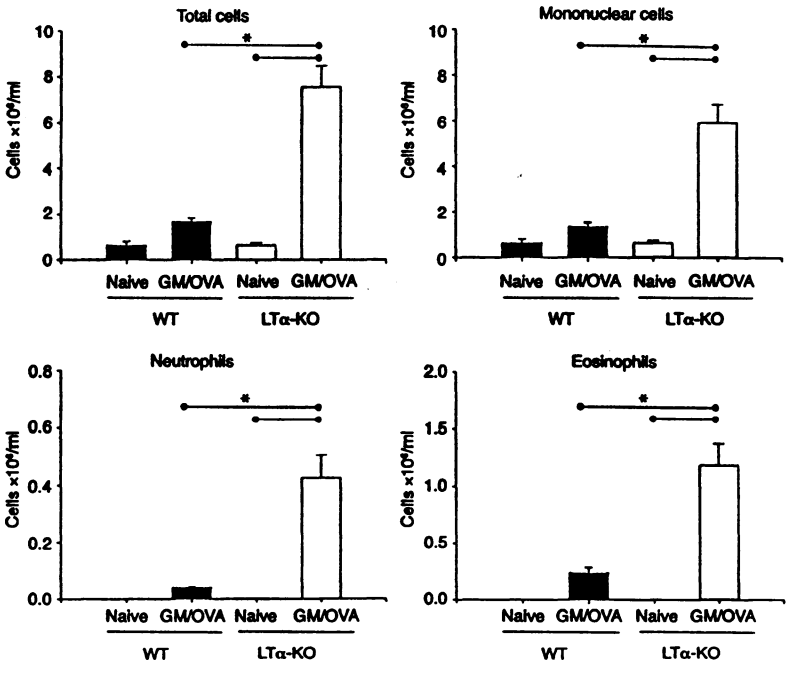

Figure 1
Airway eosinophilia in BAL of C57BL/6 (WT) and LT-α-deficient (LTα-KO) mice exposed to aerosolized OVA in the context of GM-CSF expression. Over a period of 10 consecutive days, mice were exposed daily to OVA. Twenty-four hours before first exposure, mice were infected intranasally with adenoviral construct expressing GM-CSF. Data were obtained 48 hours after last exposure to OVA. (Mean ± SEM; $n = 4–6$; statistical analysis was performed using one-way ANOVA with Fisher's post hoc test; $^*P < 0.05$.)

cultured in medium alone or with 40 μg OVA per well at 8×10^5 cells per well in a flat-bottom, 96-well plate (Becton Dickinson, Franklin Lakes, New Jersey, USA). After 5 days of culture, supernatants were harvested for cytokine measurements.

Cytokine and Ig measurement. ELISA kits for murine IL-13, and IFN-γ, were purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA), whereas the kit for murine IL-5 was obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Each of these assays has a threshold of detection of 3–5 pg/ml. Levels of OVA-specific IgE were measured using an antigen-capture ELISA method described previously (11).

Flow cytometric analysis. Flow cytometric analysis was performed on lung cells isolated as described previously with slight modifications (11). Briefly, total lung mononuclear cells were isolated by collagenase digestion (Collagenase type III; Life Technologies Inc., Rockville, Maryland, USA) followed by discontinuing gradient centrifugation in 30% and 60% Percoll (Pharmacia Biotech AB, Uppsala, Sweden). Interface containing mononuclear cells was collected, washed twice with PBS, and stained with a panel of antibodies. In addition, we performed flow cytometric analyses on white blood cells. To this end, blood was collected from the abdominal vein into 1-ml syringes coated with heparin. Red blood cells were lysed with lysing buffer (150 mM NH_4Cl, 0.1 mM disodium EDTA, and 10 nm $NaHCO_3$), and samples were subjected to three washes with PBS. Total white cell number in peripheral blood was counted with a hemocytometer, and cells were stained with appropriate antibodies. The following antibodies were purchased from PharMingen (Mississauga, Ontario, Canada): anti-CD3 (PE-conjugated 145-2CII), anti-CD4 (biotin-conjugated L3T4), and anti-MHC class II (FITC-conjugated 25-9-17). The T1/ST2 (3E10) antibody was provided by Millennium Pharmaceuticals Inc. (Cambridge, Massachusetts, USA), and FITC was labeled in-house according to a standard protocol (12). To minimize nonspecific binding, 10^6 cells were preincubated with FcBlock (CD16/CD32; PharMingen). For each antibody combination, 10^6 cells were incubated with mAb's at 0–4°C for 30 minutes; the cells were then washed and treated with second-stage reagents. Streptavidin PerCP (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was used as a second-step reagent for detection of biotin-labeled antibodies. Titration was used to determine the optimal concentration for each antibody. Cells were fixed in 1% paraformaldehyde and counted on a FACScan (Becton Dickinson and Co.), and analyses were performed using WinMDI software (The Scripps Research Institute, La Jolla, California, USA). A total of 20,000–30,000 events were acquired.

Surgical removal of spleen. Mice were anesthetized with isoflurane, and buprenorphine was administered subcutaneously. Splenectomy was preformed as described previously (13). After surgical skin preparation, the spleen was exteriorized through a 1-cm left subcostal incision. The splenic artery and vein were double ligated and the spleen was removed. The peritoneum and skin were closed in separate layers using 4.0 absorbable suture. Mice were rested for 10 days before commencement of our allergic sensitization protocol.

Data analysis. Data are expressed as mean ± SEM. Statistical interpretation was performed using ANOVA with Fisher's post hoc test or Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

Results

Immune-inflammatory responses in the airway. LTα-KO mice and WT littermates subjected to our protocol of respiratory mucosal allergic sensitization were sacrificed 48 hours after the last OVA aerosolization, and the BAL cellular profile was assessed (Figure 1). The BAL cellular content in naive mice from either strain was very similar. Compared with naive controls, both strains mounted an inflammatory response after sensitization. The extent of the response in WT mice was similar to that reported previously (11). Interestingly, the inflammatory response detected in the BAL of LTα-KO mice was approximately five times greater than that observed in sensitized WT mice. Owing to the unequal variance between strains,

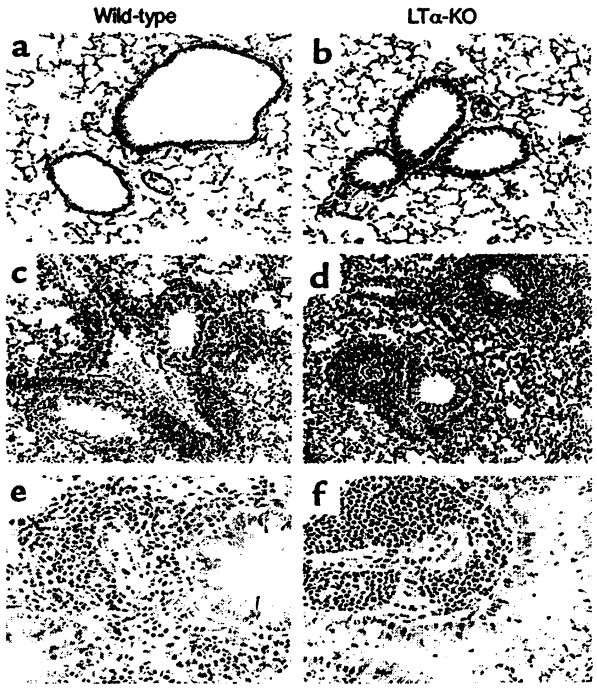

Figure 2
Light photomicrograph of paraffin-embedded sections of lung tissues. Over a period of 10 consecutive days, mice were exposed to OVA, and tissues were obtained 48 hours after last exposure. Sections were stained with H&E. Panels represent naive mice (a and b) and mice exposed to OVA in the context of GM-CSF expression (c–f). ×50 (a–d); ×200 (e and f).

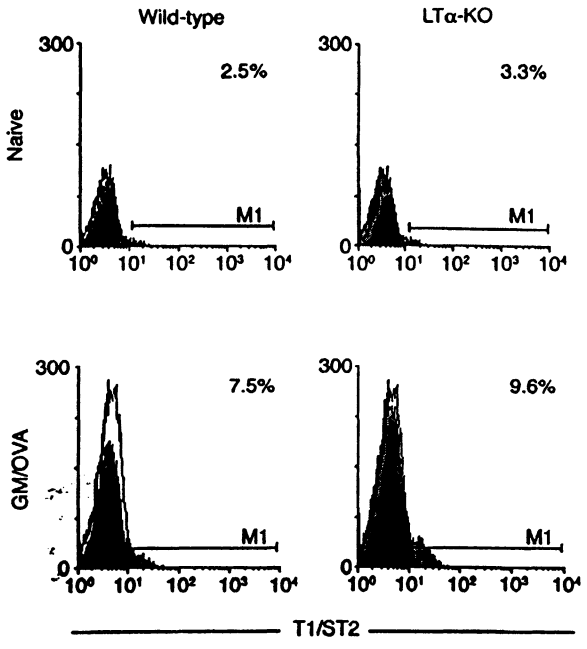

Figure 3
T1/ST2 expression on T cells obtained from lung tissues after exposure to OVA in the context of GM-CSF. Lungs were collected 48 hours after last exposure and subjected to enzymatic digestion. Cells were stained with mAb's against CD3, CD4, and T1/ST2 and analyzed by flow cytometry. Histograms were gated on $CD3^+/CD4^+$ T lymphocytes. Open histograms represent the background staining with the isotype control antibody. Data are representative of two independent experiments that yielded similar results.

statistical significance between naive and OVA-treated WT mice was not reached, as assessed by one-way ANOVA. However, a *t* test conducted only on WT mice showed statistical significance. Proportionately, the percentage of eosinophils in LTα-KO and WT mice was similar (15.5 ± 2.0% vs. 13.8 ± 2.5%, respectively); in contrast, the percentage of lymphocytes in LTα-KO mice was considerably greater, 37 ± 5% vs. 5 ± 3% in WT mice.

Histological examination of lung tissues paralleled the findings observed in the BAL (Figure 2). In agreement with our previous reports, sensitized WT mice showed a marked accumulation of inflammatory cells that was both perivascular and peribronchial; evidence of goblet cell hyperplasia and mucus production was readily apparent. In naive LTα-KO mice, there was no indication of peribronchial inflammation, but we observed scattered mild mononuclear perivascular infiltrates. The response to allergic sensitization was qualitatively similar in both strains, with extensive peribronchial inflammation characterized primarily by eosinophils and mononuclear cells. Of note, the perivascular infiltrate of LTα-KO mice was rather massive and consisted almost exclusively of mononuclear cells.

To determine whether the considerable infiltration of mononuclear cells reflected an accumulation of Th2 cells, we examined the percentage of $CD4^+$ T cells expressing T1/ST2, a putative marker of Th2 polarized cells (14). As indicated in the histograms shown in Figure 3, the percentage of $CD3^+/CD4^+$ cells expressing this marker was similar in naive mice of both strains. This percentage increased approximately threefold in both WT and LTα-KO mice after OVA sensitization.

Flow cytometric analysis of lung cells from naive C57BL/6 and LTα-KO mice. Given that the airway inflammatory response observed in LTα-KO mice was considerably greater than that observed in WT controls, we used flow cytometric analysis to investigate aspects of the immunological status of lungs from naive mice of both strains. Figure 4a shows the absolute number of lung

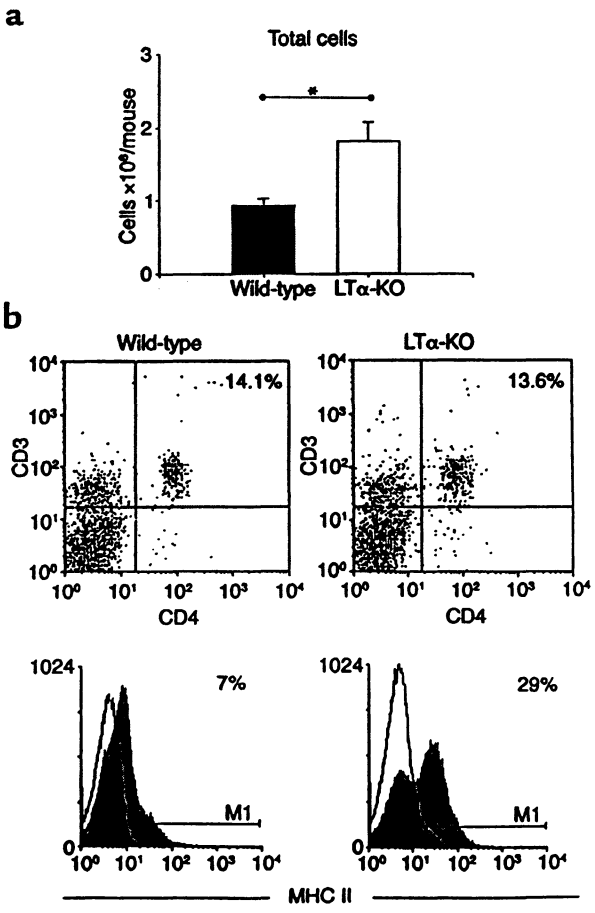

Figure 4
Flow cytometric analysis of T cells ($CD3^+/CD4^+$) and APC ($MHC\ II^+$) obtained from lungs of naive C57BL/6 and LTα-KO. (a) Lung mononuclear cell fraction was obtained by enzymatic digestion of whole lung. Lung cells from three to four mice were pooled for each group. Bars represent data obtained from three independent experiments. (Mean ± SEM; statistical analysis was performed using Student's *t* test; $^*P < 0.05$.) (b) Cells were stained with indicated antibodies or alternatively with isotype controls, and 30,000 events were collected. Data are representative of three independent experiments that yielded similar results.

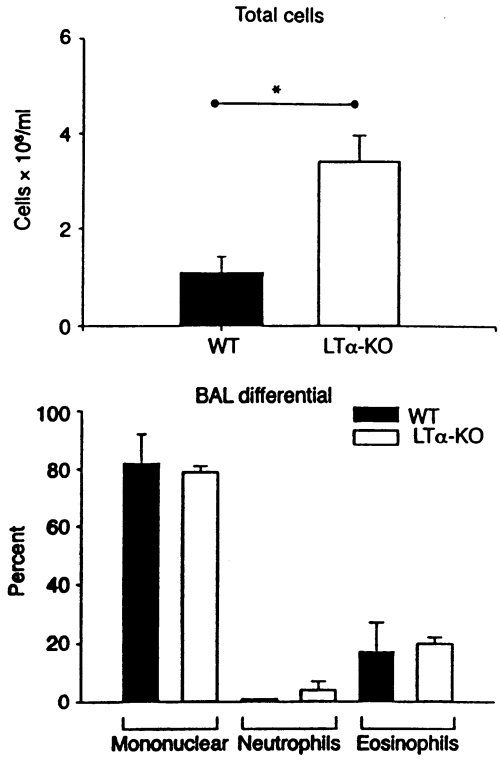

Figure 5
In vivo rechallenge of C57BL/6 (WT) and LT-α-deficient (LTα-KO) mice exposed to OVA in the context of GM-CSF. At day 60, after complete resolution of the airway inflammation, mice were re-exposed to aerosolized OVA for 3 consecutive days. Data show total cell number and differential of cells obtained 72 hours after last challenge from BAL (mean ± SEM; n = 4–6; statistical analysis was performed using Student's t test; $^*P < 0.05$).

mononuclear cells isolated. In agreement with our histological observations, the number of mononuclear cells in LTα-KO mice was approximately 40% greater, although the percentage of $CD3^+/CD4^+$ cells was similar in both strains, at approximately 14% (Figure 4b). In contrast, the percentage of cells expressing MHC II was greater in LTα-KO mice (29%) than in WT mice (7%), suggesting that LTα-KO mice may have intrinsically enhanced antigen-presenting capability.

Long term in vivo OVA recall responses. Next, we examined the impact of LN deficiency on the generation of long-term antigen-specific memory. After complete resolution of the acute airway inflammatory response, mice were reexposed to OVA (day 60). Seventy-two hours after the last exposure, they were sacrificed and the BAL cellular response assessed. As shown in Figure 5, LTα-KO mice mounted a robust mononuclear and eosinophilic airway inflammatory response upon OVA reexposure.

Cytokine production of cultured splenocytes and OVA-specific serum IgE. Effector cytokines were measured in the supernatant of splenocyte cultures. This was done for two reasons: first, our experience is that it is generally difficult to detect cytokines in the BAL of mice with a C57BL/6 background in our model; second, it was particularly important in these studies to obtain evidence of systemic Th effector activity. As shown in Table 1, the levels of IFN-γ, IL-5, and IL-13 detected in the supernatant of unstimulated splenocytes from either strain was very low. Splenocytes from sensitized mice of both strains produced considerably greater levels of these three cytokines. This response was three to four times greater in LTα-KO mice.

Table 1 provides additional evidence with respect to levels of OVA-specific IgE in serum, indicating that the response was quantitatively similar in WT as in LTα-KO mice subjected to the protocol of allergic sensitization.

Impact of splenectomy in the generation of allergic sensitization. We addressed the role of the spleen in the generation of allergic airway inflammation. To this end, we surgically removed spleens of both WT and LTα-KO mice and, after full recovery, subjected the mice to the respiratory mucosal allergic sensitization protocol. The airway inflammatory response in splenectomized WT mice was not significantly different from that observed in eusplenic sensitized control mice (Figure 6a). In contrast, splenectomized LTα-KO mice were unable to generate an airway inflammatory response. In fact, total and differential cell counts in the BAL were identical to those of naive LTα-KO mice (Figure 6b). The histological evaluation of lungs from these mice agreed with the BAL data (Figure 6c).

Peripheral blood changes before and after splenectomy. To assess the impact of splenectomy on number and distribution of white blood cells (specifically T cells and B cells) in the peripheral blood, blood samples were collected before and 10 days after surgical removal of the spleen and subjected to flow cytometric analyses. Total white blood cell counts were greater in LTα-KO than in WT mice preceding and following splenectomy (Table 2). However, removal of the spleen did not result in significant changes in cell numbers in either WT or LTα-KO mice. In addition, although splenectomy in WT mice did not have an impact on T and B cells distribution, we observed an increase in both $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells in LTα-KO mice.

Table 1
Antigen-specific cytokine and IgE production

	WT		KO	
	Medium	OVA	Medium	OVA
IFN-γ	14 ± 5	2732 ± 777	10 ± 2	9978 ± 4967
IL-5	0 ± 0	82 ± 44	0 ± 0	363 ± 121
IL-13	129 ± 19	1928 ± 541	95 ± 32	3757 ± 1037
	Naive	GM/OVA	Naive	GM/OVA
IgE	2 ± 0.5	176 ± 61	0 ± 0	127 ± 32

For cytokine measurement, splenocytes were obtained from C57BL/6 (WT) or LTα-KO (KO) mice after nine OVA exposures. Cells were cultured for 5 days in medium alone or stimulated with OVA. Data represent picograms per milliliter, mean ± SEM; n = 4 per group. Levels of OVA-specific IgE were measured in serum of WT and KO mice obtained 48 hours after last OVA exposure. Data represent units per milliliter, mean ± SEM; n = 4 per group.

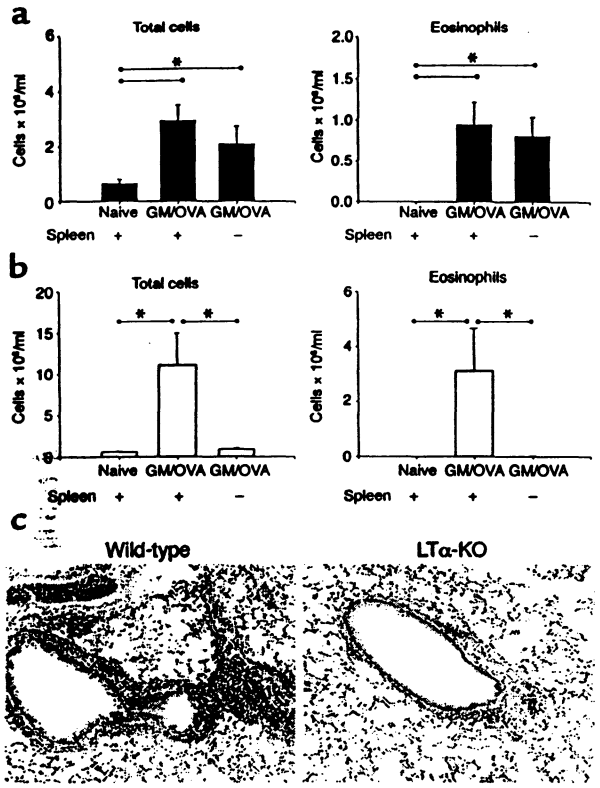

Figure 6
Impact of splenectomy on the generation of inflammation in airways of C57BL/6 and LTα-KO mice. Mice were splenectomized as described in Methods. Ten days after surgery, mice were infected intranasally with Ad/GM-CSF and subsequently exposed to OVA. BAL samples were obtained 48 hours after last OVA exposure from C57BL/6 (a) and LTα-KO (b) mice. (Mean ± SEM; $n = 4$; statistical analysis was performed using ANOVA with Fisher's post hoc test; $^*P < 0.05$.) (c) Light photomicrograph of lung sections stained with H&E. ×50.

Discussion

To our knowledge, this is the first demonstration of intact antigen-specific Th2 immunity upon mucosal sensitization of LTα-KO mice. Indeed, compared with WT mice, LTα-KO mice responded to OVA aerosolization in the context of GM-CSF with a similar pattern of T1/ST2 expression on Th2 cells and a robust Th2-associated inflammatory response characterized primarily by eosinophilia in the lung tissue. Moreover, after resolution of the initial response, OVA reexposure readily reconstituted airway eosinophilic inflammation, indicating antigen specificity and the persistence of immunological memory. As further evidence of Th2 immunity, LTα-KO mice were able to generate OVA-specific IgE responses. This is of particular interest in the light of previous data indicating the compromised ability of LTα-KO mice to generate antibody responses against KLH, HSV-1, and SRBC (9, 15). It is also of interest because germinal centers, which are considered to be essential for Ig isotype switching, are absent in these mutant mice. However, Matsumoto et al. (16) recently demonstrated near-normal affinity maturation and isotype switching in LTα-KO mice after repeated challenge with high doses of antigen (NP-OVA) together with an adjuvant. As suggested by Wang et al. (17) and illustrated by our experimental system, persistence of antigen in the context of adjuvant plausibly explains Ig affinity maturation of B cells outside germinal centers.

The magnitude of the airway inflammatory response in LTα-KO mice exposed to aerosolized OVA in the context of GM-CSF was rather striking. Indeed, whether in terms of total number of cells or of differential cell counts, the degree of the inflammatory response in the mutant mice was four- to sixfold greater than that observed in WT controls. As documented histologically (Figure 2), and in agreement with Banks et al. (9), part of the reason may be that there is an increased number of mononuclear cells in the perivascular areas of naive mutant mice. However, this difference (Figure 4a) appears quantitatively insufficient to explain the changes documented after exposure to our allergic sensitization protocol. A contributing factor may be that, as a result of lacking lymphoid structures (other than the spleen), there is an increased number of immune cells circulating in LTα-KO mice, as described previously by De Togni et al. (8) and confirmed in our studies (Table 2). We provide evidence of an additional, potentially important mechanism underlying the enhanced immune responsiveness observed in the mutant mice: a dramatic increase in the number of $MHCII^+$ cells in the lung of naive mice: 29% in LTα-KO versus 7% in WT mice. That LTα-KO mice have an increased capacity for presenting antigen may also explain the very recent observation by Lee et al. (18) of enhanced immune responses of LTα-KO mice to murine gammaherpes virus 68.

Compelled by the observation that mice lacking LN were able to generate Th2 immunity and airway eosinophilic inflammation, we investigated whether the spleen, albeit architecturally aberrant in LTα-KO mice, was able to compensate as the site of the primary immune response. The ability of splenocytes from LTα-KO mice subjected to repeated OVA aerosolizations in the context of GM-CSF to produce cytokines upon OVA recall in vitro argues for the role of the spleen in this

Table 2
Changes in peripheral blood lymphocytes following splenectomy

	WBC (cells × 10^6/ml)	$CD3^+/CD4^+$ (%)	$CD3^+/CD8^+$ (%)	$B220^+$ (%)
WT/+SP	5.8 ± 1.6	12.9 ± 1.5	6.7 ± 1.6	70.5 ± 3.3
WT/–SP	8.6 ± 2.9	20.8 ± 2.3	10.1 ± 1.5	63.1 ± 5.5
KO/+SP	33.2 ± 8.8[A]	17.5 ± 1.1	7.1 ± 0.6	73.2 ± 2.0
KO/–SP	24.7 ± 3.6[B]	27.7 ± 0.1[C]	17.8 ± 1.4[C]	59.8 ± 4.8

Changes in peripheral blood were collected from naive C57BL/6 (WT) and LTα-KO (KO) mice before (+SP) or after (–SP) splenectomy as described in Methods. Cells were stained with antibodies against indicated markers and subjected to flow cytometric analysis. A gate was set up for lymphocytes and 30,000 events were collected. [A]Statistically different from WT/+SP. [B]Statistically different from WT/–SP. [C]Statistically different from KO/+SP.

process. That cytokine content in splenocyte cultures supernatant was, in fact, two to three times greater in LTα-KO mice than in WT control mice suggests an immune response of greater strength in the mutant mice. A decisive functional role for the spleen in allergic sensitization was provided by the splenectomy experiments. Indeed, splenectomy of LTα-KO mice fully prevented the development of airway inflammation. Our data are different from those of Davis et al. (13), who demonstrated that removal of the spleen in LTα-KO mice decreased, but did not abrogate, humoral responses to *Salmonella* in intestines. In our experiments, mice were subjected to allergic sensitization protocol 10 days after splenectomy; at this time, the peripheral cell counts of LTα-KO mice before and after splenectomy were not significantly different, indicating that the absence of sensitization and airway inflammation was not due to depletion of circulatory leukocytes and, specifically, of T cells ($CD3^+/CD4^+$ and $CD3^+/CD8^+$) or B cells (Table 2). Although these data demonstrate that the spleen indeed supplanted the role of the regional LN in LTα-KO mice, splenectomy of WT mice had no impact on the ability of asplenic mice to generate allergic sensitization and airway inflammation.

Our current understanding of the generation of mucosal immunity is that antigens penetrating mucosae are captured by DCs and transported to the draining LNs to initiate immune responses (7). The data presented here indicate that the spleen can facilitate allergic sensitization to antigens introduced into the respiratory mucosae. This observation evokes an important question: How does aerosolized OVA reach the spleen to initiates allergic immunity? Wolvers et al. (5) have demonstrated that after OVA delivery to the nasal mucosae, there is evidence of APCs transporting this antigen to the spleen. This DC migration may occur via the bloodstream or, presumably, through the lymphatic system, which, importantly, remains intact in LTα-KO mice. In our experimental system, OVA aerosolization leads to eosinophilic airway inflammation only in the context of GM-CSF transgene expression. Such expression is compartmentalized within the lung/airway, as GM-CSF cannot be detected in the circulation. Therefore, we surmise that it is very unlikely that exogenous GM-CSF expression in the spleen contributed to the ability of LTα-KO mice to generate allergic sensitization. Thus, our data intimate that the generation of allergic priming in the spleen of mice lacking LNs may be the result of mobilization of OVA-carrying DC from the respiratory mucosae.

In short, our data show that allergic sensitization and airway inflammation take place in the absence of thoracic (and other) LNs. That these responses are abrogated in splenectomized mice establishes the essential requirement of secondary lymphoid organs and implies that the lung does not possess the intrinsic capability to generate allergic sensitization. That the spleen can supplant the role of the draining LNs illustrates the plasticity of the immune system and highlights the systemic nature of allergic sensitization. What is the potential clinical significance of these findings? The current emphasis of the pharmacological management of asthma rests on local treatment, which effectively inhibits inflammatory processes that take place in the airway itself. However, one would argue that processes that are generated, and probably maintained, outside the airway will likely evade this treatment strategy. Thus, attempts to ultimately cure, rather than control, allergic airway inflammation may need to incorporate a systemic dimension.

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CHAPTER 4

GENERATION OF Th2 RESPONSES AND AIRWAYS INFLAMMATION IN B7RP-1 DEFICIENT MICE

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This publication examined the importance of B7RP-1 costimulatory molecule in the generation of allergic airways inflammation.

The work presented in this study was performed by the author of the thesis. B7RP-1 deficient mice were generated by Dr. Tafuri in Dr. Mak's laboratory. Supervision was provided by Dr. Manel Jordana resulting in the multiple authorship of this paper. This paper has been submitted to the *Journal of Immunology.*

GENERATION OF Th2 RESPONSES AND AIRWAYS INFLAMMATION IN B7RP-1 DEFICIENT MICE

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ABSTRACT

The recently described ICOS-B7RP1 co-stimulatory pathway has been implicated in the generation of effector Th2 responses since ICOS stimulation leads to the production of Th2-related cytokines, and disruption of this pathway results in the attenuation of Th2 responses. In the present study, we used B7RP1 deficient mice to further investigate the role of the ICOS-B7RP1 pathway in the generation and maintenance of Th2 responses. We found that exposure of B7RP1 KO mice to OVA in the context of GM-CSF leads to airway eosinophilic inflammation that was of a degree comparable to that observed in control littermate mice. This response was long lasting since re-challenge of mice with the same antigen, after resolution of the acute phase, recapituled airway eosinophilia. Moreover, significant expression of T1/ST2, a marker of effector Th2 cells on lung T cells, and evidence of cytokine production (IL-5, IL-4, IL-13) by splenocytes from sensitized mice further supported the establishment of Th2 responses. Finally, flow cytometric studies on lung cells demonstrated expression of ICOS on T cells, and suggested the existence of a novel second ligand for ICOS, other than B7RP1, on APC. These findings could provide an explanation for the ability of T cells to differentiate toward Th2 phenotype in the absence of B7RP-1. In summary, our results indicate that B7RP1 by itself, or the ICOS-B7RP1 pathway, is redundant for the generation of allergic airway inflammation.

INTRODUCTION

Optimal T cell activation requires T cell receptor (TCR) engagement with peptide-MHC on antigen presenting cells (APC) and a second antigen-independent signal referred to as a co-stimulatory signal [1, 2]. The importance of co-stimulatory pathways in both the generation and control of immune responses is undisputed. In particular, various co-stimulatory pathways such as CD28-B7, OX40-OX40L and CD40-CD40L pathways appear to play important roles in different aspects of the generation of Th2 responses [2, 3]. Recently, new co-stimulatory pathways have been discovered including ICOS-ICOSL [4, 5]. ICOS interaction with its ligand - ICOSL (B7RP1, LICOS, B7h, B7H2, GL50) has been implicated in the development of Th2 effector activity since disruption of this interaction results in the attenuation of Th2 responses both *in vitro* and *in vivo* [6, 7]. The most persuasive evidence has been obtained in mice with a genetically disrupted ICOS gene. Indeed, ICOS-deficient mice are characterized by impaired germinal formation, have a profound defect in isotype class switching in T cell dependent B cell responses, and are defective in IL-4 and IL-13 production [8, 9]. Furthermore, blockade of ICOS in a conventional model of allergic sensitization attenuated eosinophilic accumulation in the lungs [10]. However, it has become increasingly clear that ICOS is primarily involved in Th2-mediated effector functions rather than in Th2 differentiation *per se* [11].

To further investigate the function of this newly described co-stimulatory pathway, we used mice deficient in B7RP1 in a model of respiratory mucosal sensitization that involves repeated respiratory exposure to antigen (OVA) in the context of a GM-CSF-rich airway

environment achieved by the intranasal delivery of a replication-deficient adenoviral vector carrying the GM-CSF transgene [12]. Our data demonstrate that the absence of B7RP-1 does not impede the generation of T cells expressing the Th2-associated marker T1/ST2 and able to produce Th2-affiliated cytokines. Moreover, the development of Th2-type inflammatory responses in the airway either in the primary or secondary phase is intact in B7RP1 deficient mice. Our data also intimate the presence of a second ligand for ICOS that might be involved in the generation of Th2 responses in the absence of B7RP1.

MATERIAL AND METHODS

Animals. B7RP-1 deficient mice and control littermates were obtained from Dr. Tak Mak (University of Toronto, ON). Mice were housed in a specific pathogen-free environment following a 12h light-dark cycle. All experiments performed were approved by the Animal Research Ethics Board of McMaster University.

Model of respiratory mucosal allergic sensitization. As previously described, a replication-deficient human type 5 adenoviral construct encoding murine GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF) was delivered intranasally (i.n.) 24 h prior to the first OVA exposure. Ad/GM-CSF was administered i.n. at a dose of $3x10^7$ pfu in 30μl of phosphate-buffered saline (PBS) vehicle (two 15μl administrations, 5 minutes apart) into anaesthetized animals. Over a period of 10 consecutive days (days 0-9), mice were placed in a Plexiglass chamber (10 cm x 15 cm x 25 cm) and exposed for 20 min daily to aerosolized OVA (1% wt/vol in 0.9% saline). OVA aerosol was generated by a Bennet nebulizer at a flow rate of 10 litres/min. For *in vivo* rechallenge with OVA, mice were re-exposed to a 1% OVA aerosol for 20 min daily for three consecutive days, following complete resolution of initial airways inflammation.

Collection and Measurement of Specimens. At various time points mice were killed and bronchoalveolar lavage (BAL) was performed according to a standard protocol [12]. Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2

ml); approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hæmocytometer. Cell pellets were resuspended in PBS and smears were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA) at 300 rpm for 2 min. Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hæmocytological criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. 3-μm-thick sections were stained with hematoxylin and eosin.

Splenocyte culture. Spleens were harvested into sterile tubes containing sterile HBSS (Gibco, Burlington, ON, Canada). Tissue was triturated between the ends of sterile frosted slides and the resulting cell suspension was filtered through nylon mesh (BSH Thompson, Scarborough, ON). Red blood cells were lysed with ACK lysis buffer (0.5M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2EDTA at pH 7.2-7.4). Remaining splenocytes were washed twice with HBSS and then resuspended in RPMI supplemented with 10% FBS (Gibco), 1% L-glutamine, 1% penicillin/streptomycin. Cells were cultured in medium alone or with 40 μg OVA/well at $8x10^5$ cells/well in a flat-bottom, 96-well plate (Becton Dickinson, Lincoln Park, NJ). After 5 days of culture, supernatants were harvested for cytokine measurements.

Cytokine and Immunoglobulin measurement. ELISA kits for murine IL-13, IL-4, IFN-γ and IL-5 were purchased from R&D Systems (Minneapolis, MN). Each of these assays has a threshold of detection of 3-5 pg/ml. Levels of OVA-specific IgE and IgG1 were measured using a previously described antigen-capture ELISA method [12].

Flow cytometric analysis. Flow cytometric analysis was performed on lung cells isolated as previously described with slight modifications [12]. Briefly, total lung mononuclear cells were obtained by collagenase digestion (Collagenase type III; Life Technologies, Rockville, MD) followed by discontinuing gradient centrifugation in 30 and 60% Percoll (Pharmacia, Uppsala, Sweden). The interface containing mononuclear cells was collected, washed twice with PBS and stained with a panel of antibodies. The following antibodies were purchased from Pharmingen (Mississauga, ON, Canada): anti-CD3 (PE-conjugated 145-2CII), anti-CD4 (biotin-conjugated L3T4), anti-MHC Class II (FITC-conjugated 25-9-17); T1/ST2 (3E10) antibody was provided by Millennium Pharmaceuticals Inc. (Cambridge, MA, USA) and FITC-labeled in-house; ICOS-Fc was furnished by the Ontario Cancer Institute and detected by human anti-Fc FITC-labeled (Jackson Laboratories, US). To minimize nonspecific binding, 10^6 cells were preincubated with FcBlock (CD16/CD32; Pharmingen). For each antibody combination, 10^6 cells were incubated with monoclonal antibodies at 0-4°C for 30 min; the cells were then washed and treated with second stage reagents. Streptavidin-cy5 (BD Pharmingen, San José, CA) was used as a second step reagent for detection of biotin-labeled antibodies. Titration was used to determine the optimal concentration for each antibody. Cells were fixed in 1% paraformaldehyde,

counted on a FACScan and analyses were performed using WinMDI software (The Scripps Research Institute). 20,000-30,000 events were acquired.

Data Analysis. Data are expressed as mean ± SEM. Statistical interpretation was performed using ANOVA with Fisher *post hoc* test or Student t-test. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Impact of the B7RP1 deficiency on the generation of primary Th2 responses

It has been postulated that ICOS is important for the generation of Th2 cells since ICOS mRNA is expressed at high levels on *in vitro*-differentiated Th2 cells [6], ICOS ligation mediates IL-4 expression [7] and IL-4 expression is deficient in ICOS-KO mice [8] [9]. Thus, the prediction was that the absence of ICOS ligand, namely B7RP1 and, therefore, the disruption of the ICOS-ICOSL costimulatory pathway would lead to a similar outcome. However, our experiments with mice genetically engineered for the absence of B7RP1 (B7RP1-KO), demonstrate otherwise. B7RP1-KO mice and control littermates subjected to our protocol of respiratory mucosal sensitization were sacrificed 48h after the last OVA aerosol challenge and the BAL content was assessed. Both strains mounted a substantial inflammatory response in the lungs. The percentage of eosinophils in the BAL was 43±6% and 27±4% in B7RP1-KO and WT, respectively and was statistically different between groups (Fig 1A and B). Upon histopathological examination, the extent of the lung inflammatory infiltrate was, in agreement with the BAL findings, higher in B7RP-1KO than in control littermate mice (Fig 1B). The accumulation of inflammatory cells, primarily eosinophils and mononuclear cells, was apparent in both perivascular and peribronchial areas.

To further determine the ability of B7RP1-KO mice to develop Th2 responses, we re-stimulated splenocytes collected 48h after last OVA exposure with OVA antigen. Effector Th2-related cytokines were measured in the supernatants of these cultures. As shown in

Figure 2A the levels of IL-4, IL-5 and IL-13 detected were in fact higher in B7RP1-O mice as compared to control littermates. Thus, the absence of the ICOS-B7RP1 interaction in mice mucosally sensitized not only does not preclude Th2 differentiation but insinuates even greater Th2 polarization. A compromised IFN-γ production that would counterbalance Th2 responses [13], could potentially explain the particularly elevated production of Th2 cytokines. However, as demonstrated in Figure 2A, the levels of IFN-γ were similar in B7RP1-KO and control littermates indicating that another, yet unidentified mechanism, is responsible for the increase production of Th2 cytokines in B7RP1-KO.

The ICOS-B7RP1 pathway appears to be important in humoral immunity as ICOS deficient mice subjected to different immunization protocols revealed deficits in IgG1, IgG2a and IgE levels [8, 14-16]. In order to examine the ability of B7RP1-KO to generate humoral responses when mucosally sensitized to OVA, we measured serum OVA-specific IgE and IgG1 48h after last OVA aerosol challenge. Figure 2B shows that IgE and IgG1 production in B7RP1-KO mice is comparable to that observed in control littermate mice. While apparently at variance with the results observed in ICOS KO mice, it must be noted that the impairment in antibody production observed in ICOS-deficient mice can be overcome by the utilization of a strong adjuvant such as CFA [16]. The presence of intact humoral responses in B7RP1-KO can be explained by the nature of our protocol that involves repeated exposure to antigen (OVA) in the context of a GM-CSF rich airway microenvironment.

Impact of the B7RP1 deficiency on the generation of memory responses

The importance of costimulatory pathways in the generation of memory T cell responses is controversial [17]. The fact that ICOS is expressed on T cells upon stimulation would suggest that every exposure to antigen leads to ICOS upregulation and interaction with its ligand. Therefore, while not playing a major role in Th2 differentiation, the ICOS-ICOSL interaction could be important for the generation of Th2 effector memory. To investigate this aspect, mice sensitized to OVA were left for 35 days to allow a complete resolution of the acute inflammatory response and were, then, re-exposed to aerosolized OVA on three consecutive days. Seventy two hours after the last exposure, mice were sacrificed and the BAL cellular response assessed. As shown in Figure 3A, B7RP1-KO and control littermate mice mounted an eosinophilic airway inflammatory response that was quantitatively similar. To determine whether the infiltration of T cells reflected a preferential accumulation of Th2 cells, lung mononuclear cells were subjected to flow cytometric analysis. T1/ST2, a putative marker of Th2 effector cells [18], was expressed on CD3/CD4 cells to the same degree in both B7RP1-KO and control mice (Fig. 3B).

Expression of costimulatory molecules in lung cells

Since the generation of primary and memory Th2 responses is intact in B7RP1-KO mice, the importance of the ICOS costimulatory pathway seems to be marginal. It is known that expression of ICOS on T cells is dependent on TCR and CD28 signals and, that absence of CD28 results in diminished levels of ICOS [7]. To this end, we examined the expression of CD28 and ICOS on CD3/CD4 cells isolated from the lungs in our recall protocol. As

shown on Figure 4A, both molecules are expressed in B7RP1 KO implying the ability of T cells to interact with B7 and ICOS-L. Two potential explanations can emerge from our studies: either the CD28-B7 pathway can substitute for the absence of a secondary signal, namely ICOS-ICOSL, or there is a second ligand for ICOS, distinct than B7RP1. Previous studies using either CD28KO or B7.1/2 antagonists have shown that the CD28-B7 pathway is absolutely necessary for the generation of Th2 responses supporting the former notion [19]. In addition, current literature postulates that the blockade of ICOS-ICOSL pathway with ICOS-Ig does not prevent Th2 differentation, but can reduce acute airways inflammation [11, 20]. In addition, evidence of eosinophilic infiltration in a model of allergic airway inflammation in ICOS KO [9] supports the notion that CD28/B7 is the primary pathway for Th2 responses, whereas ICOS-ICOSL serves rather as an enhancing arm. However, we utilized ICOS-Fc for flow cytometry analysis in order to determine whether there is a second ligand for ICOS that, in the absence of B7RP1, can interact with T cells. Indeed, even though B7RP1 molecule was absent in B7RP1KO mice, we detected with ICOS-Fc the presence of a molecule that is expressed in B7RP1-KO lung cells. The extent of ICOS-Fc staining was similar in B7RP-1KO and control mice. Thus, the presence of a second ligand could explain unexpected results in our initial hypothesis claiming the importance of B7RP1 in the generation of Th2 responses. The issue that remains unresolved is the potential contribution of B7RP1 to T cell priming. Whereas, B7RP1 Is expressed on resting B cells and macrophages, the presence of this molecule on murine DC is questionable. Human DCs expressed B7RP1, which is downregulated upon stimulation with LPS or TNF-α [3]. Since mice transgenic for B7RP-1Fc develop T

cell hyperplasia, plasmocytosis and hypergammaglobulinemia the potential role of B7RP-1 could result from a direct interaction of B cells rather than DC with ICOS [15]. However, the presence of antigen-specific antibodies in our model supports the notion that stimulation through CD40-CD40L can compensate for the lack of *in vivo* ICOS stimulation [16]. The potential preferential expression of B7RP1, as opposed to the second ICOS ligand, on B cells awaits, however, further examination. In summary, our study demonstrates that B7RP1 is redundant for the generation of effective Th2 responses and suggests the presence of the second ligand can substitute for the absence of B7RP1.

ACKNOWLEDGEMENTS

This research has been supported by grants from the Canadian Institute of Health Research (CIHR). We also acknowledge the support of the Hamilton Health Science Corp. and the St. Joseph Hospital Foundation. We are grateful to Susanna Goncharova for technical assistance, Jill Johnson for initial help with genotyping, Mary Kiriakopoulos for secretarial assistance, and all the members from Dr. Manel Jordana's lab for helpful discussions.

FIGURE LEGENDS

Figure 1: Airway eosinophilia in BAL of control littermates (WT) and B7RP1 deficient (KO) mice exposed to aerosolized OVA in the context of GM-CSF expression. Over a period of 10 consecutive days mice were exposed daily to OVA. 24h before first exposure mice were infected i.n. with adenoviral construct expressing GM-CSF. Data were obtained 48 h after last exposure to OVA. ***A*** Total cell number acquired in BAL fluid and differential cell count expressed as % (mean±SEM;n=7-8; statistical analysis was performed using Student's t-test; * $p<0.05$). ***B*** Light photomicrograph of paraffin-embedded sections of lung tissues. Sections were stained with hematoxylin and eosin. Panels represent WT (a,b) and KO (c,d) mice exposed to OVA in the context of GM-CSF expression; magnification of panels: a and c *x 50*, b and d *x 200.*

Figure 2: Cytokine and immunoglobulin production by control littermates (WT) and B7RP1 deficient (B7RP1KO) mice. ***A*** For cytokine measurement, splenocytes were obtained from both stains 48h after last OVA exposure. Cells were cultured for 5 days in medium alone or stimulated with OVA. (mean±SEM; n=7-8; statistical analysis was performed using one-way ANOVA with Fisher's post-hoc test; * $p<0.05$). ***B*** Levels of OVA-specific IgE and IgG1 were measured in serum of WT and KO mice obtained 48h after last OVA exposure. (mean±SEM; n=7-8; statistical analysis was performed using using Student's t-test; * $p<0.05$).

Figure 3: *In vivo* rechallenge of control littermates (WT) and B7RP1 deficient (KO) mice exposed to OVA in the context of GM-CSF. At day 60, after complete resolution of the airway inflammation, mice were re-exposed to aerosolized OVA for 3 consecutive days. ***A*** Data show total cell number and differential of cells obtained 72h after last challenge from BAL (mean±SEM; n=4; statistical analysis was performed using Student's t-test; * $p<0.05$). ***B*** T1/ST2 expression on T cells obtained from lung tissues. Lungs were collected 72h after last

exposure and subjected to enzymatic digestion. Cells were stained with mAbs against CD3, CD4 and T1/ST2. Histograms were gated on $CD3^+/CD4^+$ T lymphocytes. Open histograms represent the background staining with the isotype control antibody. Histograms are representative of four independent measurements (n=4/group) for individual lungs. Data in table shows mean±SEM for WT and KO; n=4.

Figure 4: Flow cytometric analysis of T cells ($CD3^+/CD4^+$) and APC ($MHC\ II^+$) obtained from lungs control littermates (WT) and B7RP1 deficient (KO). Lung mononuclear cell fraction was obtained by enzymatic digestion of whole lung. Lung cells from individual mice were were tested for the presence of specific surface marker. Cells were stained with indicated antibodies or alternatively with isotype controls and 30,000 events were collected. ***A*** Expression of CD28 and ICOS on T cells. Histograms were gated on $CD3^+/CD4^+$ T lymphocytes. Open histograms represent the background staining with the isotype control antibody. Histograms are representative of four independent measurements (n=4 mice/group) for individual lungs. Data in table shows mean±SEM for WT and KO. ***B*** Expression of ICOSL on APC as measured by ICOS-Fc staining. Histograms were gated on $MHCII^+$ cells. Open histograms represent the background staining with the isotype control antibody. Histograms are representative of four independent measurements (n=4 mice/group) for individual lungs. Data in table shows mean±SEM for WT and KO.

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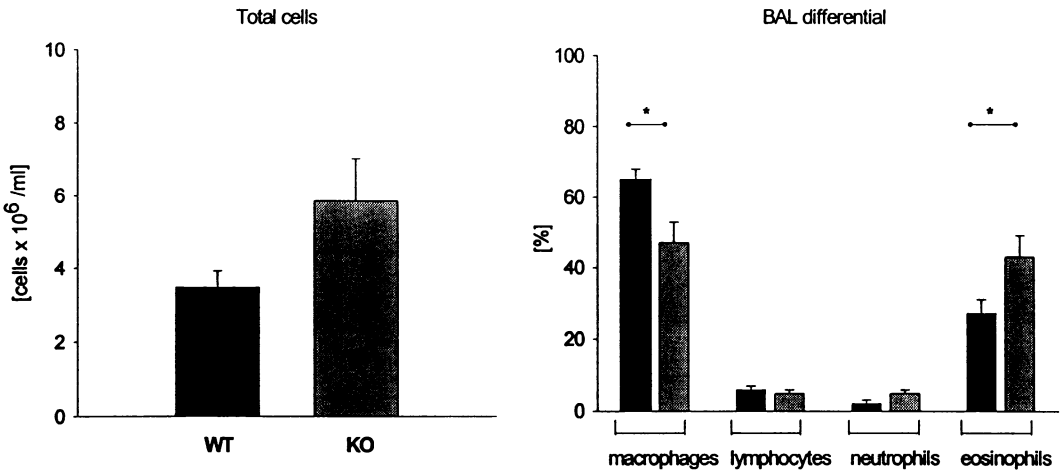
Figure 1

A.


Total cells
[cells x 10^6 /ml]
10
8
6
4
2
0
WT
KO
BAL differential
[%]
100
80
60
40
20
0
*
*
macrophages
lymphocytes
neutrophils
eosinophils

B.

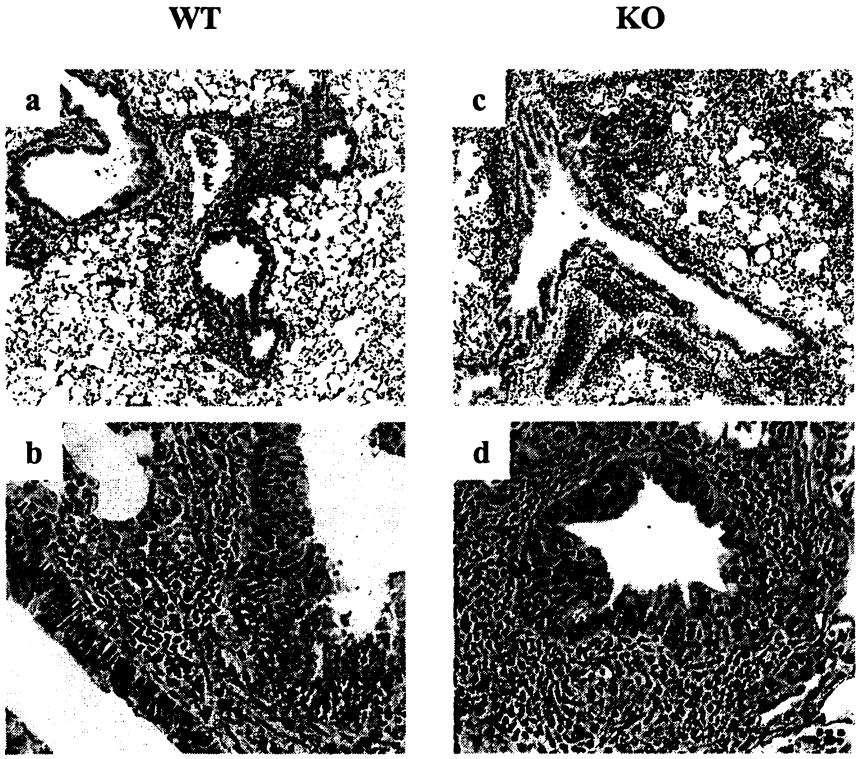

WT
KO
a
b
c
d

Figure 2

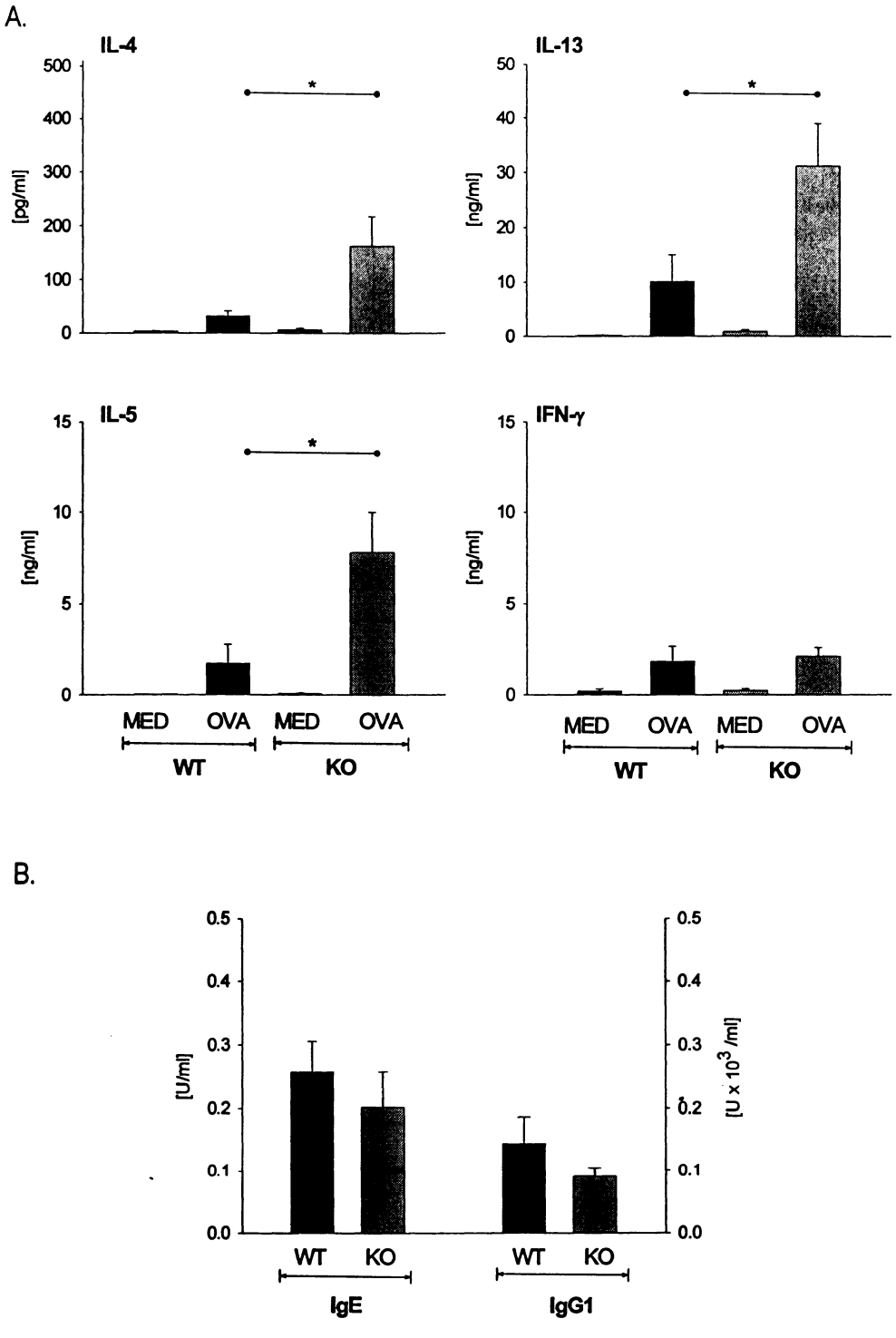
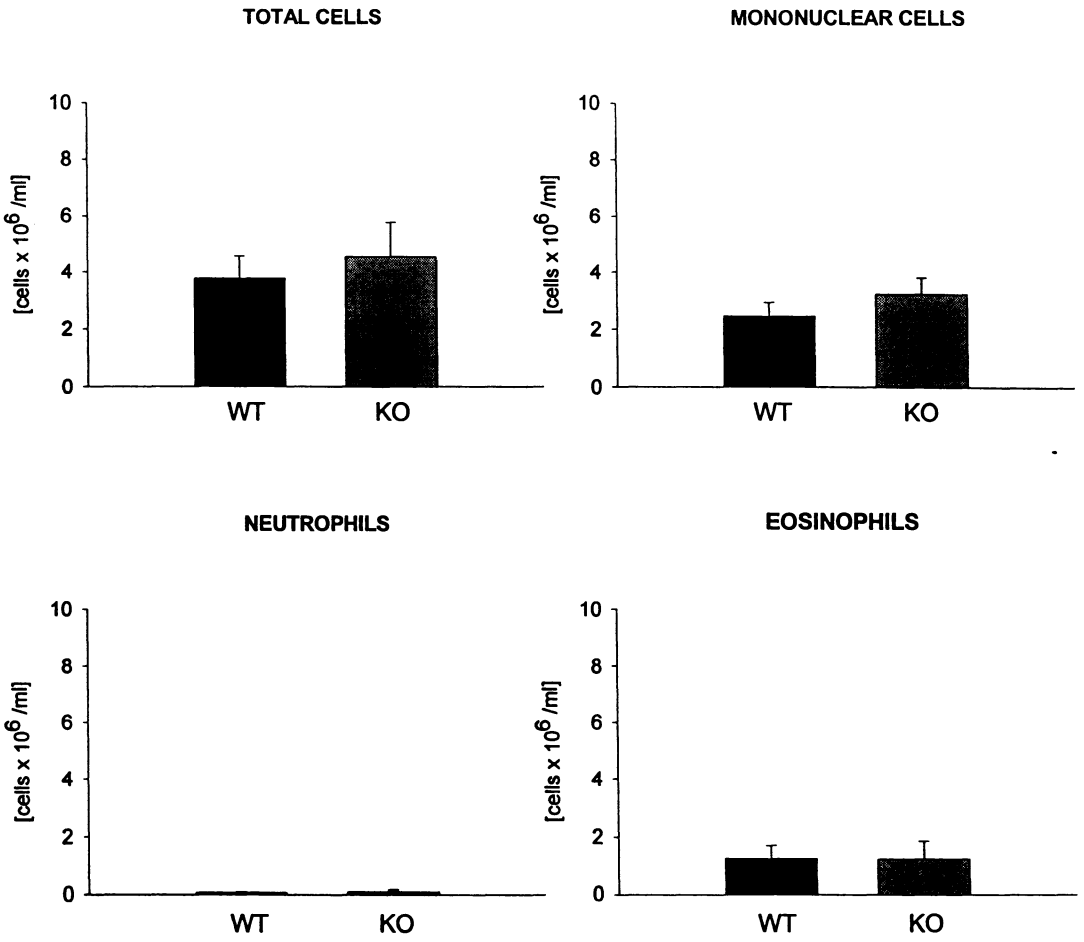
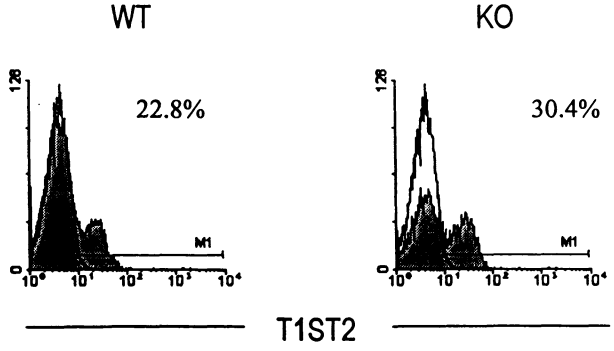

A.
IL-4
[pg/ml]
500
400
300
200
100
0
*
IL-13
[ng/ml]
50
40
30
20
10
0
*
IL-5
[ng/ml]
15
10
5
0
*
IFN-γ
[ng/ml]
15
10
5
0
MED
OVA
MED
OVA
WT
KO
MED
OVA
MED
OVA
WT
KO
B.
[U/ml]
0.5
0.4
0.3
0.2
0.1
0.0
[U x 10³ /ml]
0.5
0.4
0.3
0.2
0.1
0.0
WT
KO
WT
KO
IgE
IgG1

Figure 3

A.



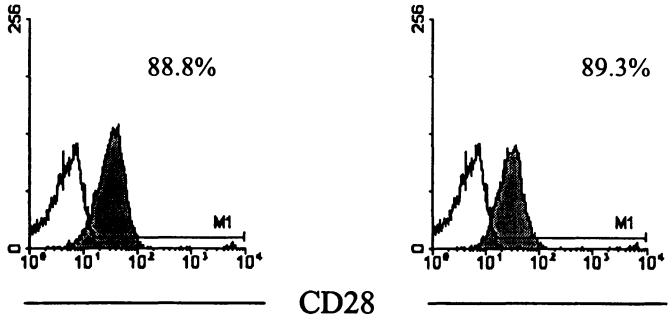
B.



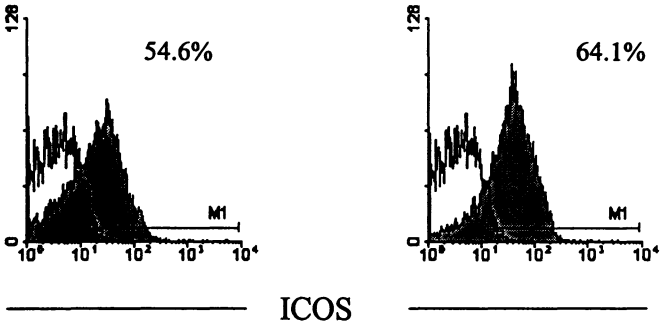
WT (%)	KO (%)
21.5 ± 1.0	34.4 ± 2.4

Figure 4

A.

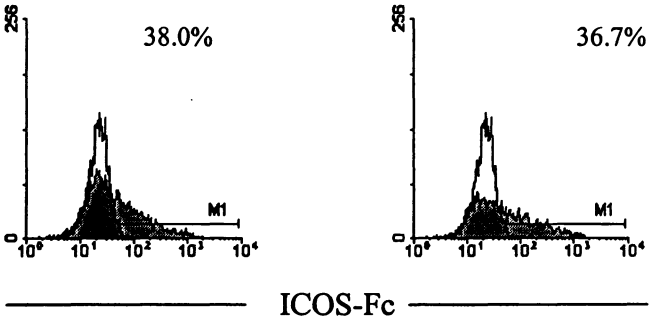


WT (%)	KO (%)
90.5 ± 0.6	90.2 ± 2.9



WT (%)	KO (%)
55.5 ± 1.9	68.8 ± 3.8

B.



WT (%)	KO (%)
32.0 ± 4.0	24.8 ± 8.1

CHAPTER 5 : DISCUSSION

Our current understanding of the processes underlying the development of allergic airways inflammation (AAI) is largely the result of research in animal models. Indeed, several inflammatory features present in allergic human subjects have been successfully replicated in animals, including eosinophilia, Th2-polarized responses in the lung, and airway hyperresponsiveness (Ohkawara *et al.*, 1997; Stampfli *et al.*, 1998). However, the major advantage of studies of animal models is the opportunity to examine intricate *initial* events that are necessary for the establishment of AAI.

This thesis utilized animal models of AAI in order to understand what are considered to be important issues directly relevant to the generation of Th2 responses. We have focused on three major aspects of AAI: (1) the geography of allergic immune responses, (2) the importance of lymphoid organs in the establishment *vs.* maintenance of this response, and (3) the role of selected costimulatory molecules in the development of the allergic response.

EVENTS IN THE DRAINING LYMPH NODES AND LUNGS FOLLOWING ANTIGEN ENCOUNTER

In order to understand the geography of allergic immune responses we utilized a conventional model of AAI (Gajewska *et al.*, 2001). We had previously characterized cellular changes in the BAL, lung tissue, peripheral blood and bone marrow, as well as serum levels of immunoglobulins and cytokine profiles in serum and BAL (Ohkawara *et al.*, 1997). The ability to dissect two distinct phases of the immune response, namely sensitization and recall, is the major strength of this model.

Our data indicate that initial contact with antigen is associated with the induction of immune responses in the draining thoracic lymph nodes. We documented a considerable expansion of both T cells and APC, particularly B cells. Notably, these expanded cell subsets showed signs of activation, as indicated by the expression of the early activation marker CD69 on T cells, and preferential up-regulation of B7.2 (CD86) on B cells. These phenotypic changes correlated with an up-regulation of the Th2-affiliated transcription factors GATA-3 and STAT-6 (Kaplan *et al.*, 1996; Zheng and Flavell, 1997). Although these distinct transcriptional instructions for the development of Th2 responses were present, the levels of cytokine mRNA and T1/ST2 expression (a putative marker of Th2 effector T cells) (Townsend *et al.*, 2000) remained at naïve levels in the draining lymph nodes. However, *in vitro* recall with OVA triggered production of the Th2 associated cytokines IL-4 and IL-5, demonstrating the ability of lymph nodes cells, predominantly T cells, to respond to antigen under appropriate conditions. Therefore, it appears that the lymph nodes do not represent a suitable environment for the execution of T cell effector function. These findings are consistent with the current paradigm that memory T cells are divided into two independent subsets: T_{CM} (T central memory), which posseses lymph-node homing ability but lack inflammatory functions; and T_{EM} (T effector memory), which exhibits effector function and preferential homing to inflamed tissue (Sallusto *et al.*, 1999). That we observed an accumulation in the lung of effector T cells expressing T1/ST2 and mRNA for Th2 cytokines following OVA aerosol challenge supports this postulate.

The expansion of B cells in the lymph nodes correlates with the establishment of humoral responses and the production of Th2-affiliated immunoglobulins IgE and IgG1.

Activated B cells in the lymph nodes preferentially express B7.2 (CD86), which can be linked to the specific functions that this costimulatory molecule plays. Indeed, it has been recently demonstrated that whereas B7.1 (CD80) up-regulates a plethora of pro-apoptotic signals, B7.2 favors proliferation and the production of IgG1 and IgG2a (Suvas *et al.*, 2002). Intriguingly, B cells are the predominant APC subpopulation expanded in the lymph nodes during the priming phase of the immune response. This might suggest that B cells play a major role in T cell priming upon initial encounter with antigen. However, data obtained in B cell-deficient mice prove otherwise, as these mice are perfectly able to generate Th2 responses (Hamelmann *et al.*, 1999). Interestingly, the DC subset, the most efficient APC in *in vitro* and *in vivo* systems (Banchereau *et al.*, 2000), comprises a rather marginal, quantitatively, pool of cells in the lymph nodes. Therefore, even the minimal changes in the number of DC that we observed in the lymph nodes at the time of sensitization appear sufficient to initiate a productive immune response.

These initial events in the lymph nodes lead to the generation of an effective Th2 mediated inflammatory response in the lung upon antigen re-challenge. In the lung tissue, we observed an initial massive expansion of macrophages and DC followed by an accumulation of effector T cells. Such a prompt response in the effector organ likely relies on preexisting antigen-specific IgE that can directly stimulate mast cells to release preformed cytokines and chemokines in response to antigen exposure. The collective action of multiple immune mediators results in the infiltration and activation of other inflammatory cells such as DC or T cells. In addition to changes in the lung, the lymph nodes are also actively involved in the recall response. That antigen presentation might

also occur specifically in lymphoid organs during recall responses provides an explanation for the observed expansion of cells in the lymph nodes at this time. This hypothesis has been tested in this thesis and is discussed below. Alternatively, changes in the lymph nodes might result from new-sensitization that serves to replenish the antigen-specific T cell pool.

In summary, this part of the thesis provides a detailed analysis of events following primary and secondary exposure to antigen in a model of AAI. Our data have delineated the phenotypic changes in T cell and APC populations at different time points of the protocol and have demonstrated the functional significance of such changes in terms of Th2-associated cytokines and transcription factors.

IMPORTANCE OF LYMPHOID ORGANS IN THE GENERATION OF AAI

The changes observed in the draining lymph nodes in our conventional model of AAI prompted us to investigate the sequence of events in our mucosal sensitization model, principally because this system more accurately recapitulates the route of allergic sensitization in humans (Stampfli *et al.*, 1998).

The pattern of responses that we observed in the mucosal model is quite similar to that described in the conventional model. Specifically, we detected an expansion of $MHCII^+$ cells and activated T cells (Appendix 1, figure 2A,B) in the thoracic lymph nodes upon allergen challenge. The striking difference, however, lies in the dramatic changes observed in the lung during the course of ten OVA aerosol exposures. Since sensitization

and recall are not temporally distinct in the mucosal model, processes appear almost simultaneously in both initiation and effector sites. The expansion of the APC compartment in the lung is quite dramatic early in the protocol, specifically at day 3 (Appendix 1, figure 1 A-C). At day 7, the peak point for GM-CSF transgene expression, APC expansion reaches its maximum and subsequently subsides concomitantly with antigen withdrawal. Accumulation of APC in the lung and tracheas of mice undergoing bacterial or viral infection has been previously documented (McWilliam *et al.*, 1996). In our model, involving exposure to an innocuous antigen, the increase in the APC compartment can be attributed to local production of GM-CSF introduced either as a transgene in an adenoviral vector or as a recombinant protein [unpublished observation]. Indeed, GM-CSF is commonly recognized as an activation factor for DC, up-regulating antigen-presenting functions and costimulatory molecule expression, while down-regulating the immunosuppressive functions of alveolar macrophages (Bilyk and Holt, 1993; McKenna, 2001). Therefore, it is conceivable to envision a scenario in which lung DC activated by GM-CSF sample antigen (OVA), migrate to the regional lymph nodes and present the antigen to naïve T cells, thereby triggering a sequence of events that results in the generation of effective Th2 responses. Indeed, the migration of antigen-loaded DC to the thoracic lymph nodes upon administration of antigen alone or antigen-pulsed DC to the airway has been recently demonstrated (Lambrecht *et al.*, 2000; Vermaelen *et al.*, 2001).

Whereas the involvement of the lymph nodes in the establishment of Th2 responses is apparent in both models, the possibility of antigen presentation and sensitization locally in the lung was considered in the context of the mucosal model.

Therefore, we decided to examine this concept directly in mice congenitally devoid of lymph nodes: lymphotoxin α knockout (LTαKO) mice. Exposure of LTαKO mice to OVA in the context of GM-CSF resulted in the generation of cellular (presence of effector T cells) and humoral (ability to produce IgE) Th2 responses. Similar findings were reported in studies with viruses, demonstrating the establishment of antiviral immunity in the absence of lymph nodes (Lee *et al.*, 2000; Lund *et al.*, 2002). Our data point to the redundancy of lymph nodes in the process of sensitization, although the role of other lymphoid organs, such as the spleen, remained vague. Surgical removal of the spleen preceding the sensitization phase fully precluded the generation of a Th2 response thus underscoring the necessity of lymphoid tissue during the priming of immune response. The fact that the spleen can supplant the role of the draining lymph nodes clearly illustrates the plasticity of the immune system. Moreover, these findings indicate that sensitization cannot occur in the lung, despite the massive expansion of the APC compartment.

It is thought that, following sensitization, re-exposure to antigen initiates a rapid response carried out by the preexisting pool of memory T cells (Zinkernagel, 2002). However, it remains to be elucidated whether antigen presentation occurs locally, i.e. in the lung, during the secondary phase of the immune response. The inflamed lung is perfectly equipped for handling antigen presentation *in situ* due to the increased number of inflammatory cells, including DC (Gajewska *et al.*, 2001; Jahnsen *et al.*, 2001; Upham *et al.*, 2002). In addition, experimental evidence suggests that chronic inflammation results in ectopic formation of *de novo* lymphoid structures whose function can overlap with those of the draining lymph nodes (Hjelmstrom, 2001). Indeed, bronchus-associated lymphoid

tissue (BALT) which appears in grossly inflamed lungs, contains all the necessary components for T cell-DC interaction and the production of IgE (Chvatchko *et al.*, 1996; Pabst, 1992). In order to examine whether the lung provides an environment for local antigen presentation, we splenectomized LTαKO mice after sensitization but before antigen re-challenge. As expected, the generation of secondary responses did not require the presence of lymphoid organs (Appendix II). Therefore, this study supports the notion that the environment in the lung at the time of allergen recall is permissive to restimulation of memory T cells. However, it is conceivable that under homeostatic conditions lymph nodes provide the environment were T_{CM} expand and replenish peripheral tissues with new effector cells (T_{EM}). Therefore, re-activation of different memory T cell subsets in lymphoid and non-lymphoid tissues might occur simultaneously upon encounter with antigen. In addition, the process of neo-sensitization that specifically takes place in the draining lymph nodes cannot be excluded.

Our observations in LTKO mice illustrate the differential requirements for lymphoid organs at different stages of the immune response (Figure 3). Whereas the induction of immune response depends upon antigen reaching lymphoid organs, memory T cells can be activated by relevant antigen in the target organ without the participation of lymphoid organs. In this context, it would be of considerable interest to investigate whether chronic OVA exposure in mice devoid of lymphoid organs could lead to the exhaustion of the memory T cell pool due to the inability to replace T_{EM} with T_{CM}.

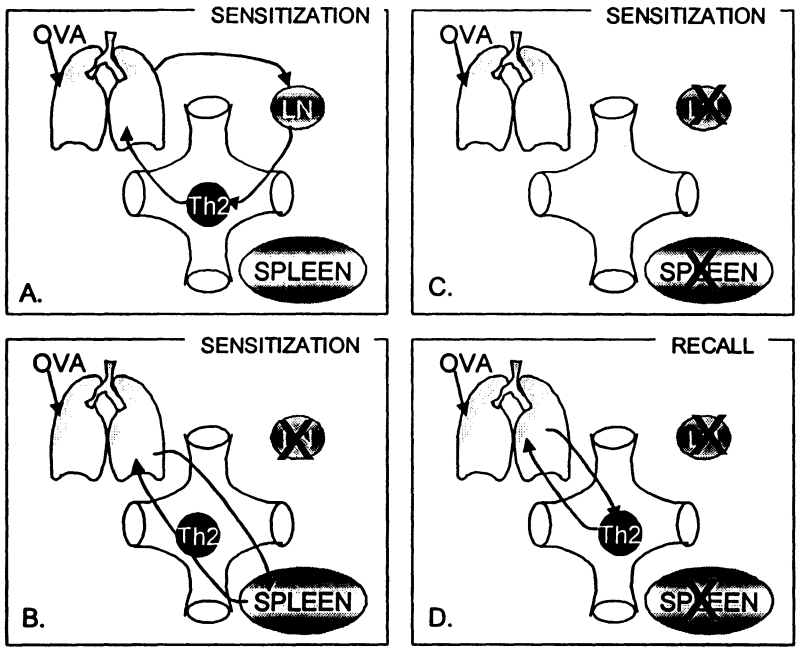


Figure 3. ***Importance of lymphoid organs in different phases of immune responses.*** A. In the sensitization phase antigen (OVA) is transported to the lymph nodes (LN) draining the airways. Differentiated Th2 cells circulate through blood stream and migrate to the lung upon subsequent exposure to antigen. Under these circumstances, spleen does not represent a major site for antigen presentation. B. In the absence of lymph nodes (lymphotoxin α deficient mice-LTKO), spleen is necessary for the initiation of immune responses. Removal of spleen from LTKO mice prior to initial exposure to antigen results in abrogation of Th2 cell generation (C) indicating the requirement of lymphoid organs for sensitization. However, when the spleen is removed post sensitization (D) recall responses are intact suggesting that effector responses are not dependent on lymphoid organs.

ROLE OF COSTIMULATORY MOLECULES IN THE GENERATION OF AAI

Costimulatory molecule expression is clearly a central requirement for the generation of a productive immune response. During the past 20 years the list of costimulatory molecules has grown considerably with new members being continuously discovered. This thesis specifically focuses on the role of B7RP-1 in the generation of Th2 responses; however, the importance of other members of the B7 family, namely B7.1/B7.2, and their ligand CD28, have also been studied in other parts of the work presented here ((Gajewska *et al.*, 2001)(Appendix I, Table1)(Appendix III).

CD28 has received significant attention due to its ability to regulate immune responses. The absence of signaling through CD28 impairs T cell proliferation, rendering T cells anergic. In addition, while T cells isolated from CD28-deficient mice exhibit impaired T helper responses, induction of CD8+ cytotoxic T lymphocytes (CTL) appears to be intact (Shahinian *et al.*, 1993). Moreover, it has been demonstrated that the interaction between B7 molecules on APCs and CD28 on T cells is essential for IL-4 production and Th2 differentiation (Rulifson *et al.*, 1997) (Schweitzer and Sharpe, 1998). In order to evaluate the involvement of this molecule in the generation of AAI, we used CD28-deficient mice (CD28KO). As indicated in Appendix III, CD28KO mice were unable to mount an eosinophilic inflammatory response in BAL, produce Th2-associated cytokines and generate IgE. These data complement results from different models of AAI utilizing CTLA-4Ig which blocks the CD28 pathway by binding to CD28 ligands (B7.1/B7.2) (Keane-Myers *et al.*, 1997; Padrid *et al.*, 1998; Van Oosterhout *et al.*, 1997). In summary, these findings emphasize the importance of the CD28 pathway in the generation of Th2 immunity and imply that disruption of the CD28/B7 signaling pathway represents a potential therapeutic strategy for the effective abrogation of allergen-induced airway dysfunction.

Several studies have suggested that the B7/CD28 interaction may be important not only in T cell activation and IL-2 production, but in T cell differentiation into Th1 or Th2 cells (Freeman *et al.*, 1995; Kuchroo *et al.*, 1995). Specifically, two groups postulated simultaneously that there are differential requirements for B7.1 *vs.* B7.2 in the development of Th1 and Th2 responses (Freeman *et al.*, 1995; Lenschow *et al.*, 1995). In order to evaluate the differential expression of B7.1 and B7.2 in AAI, we investigated the

expression of these molecules on APC in the thoracic lymph nodes and lung at different time points in both the conventional and mucosal models of AAI. Our data demonstrated an increase in B7.2 expression, particularly by B cells, in the thoracic lymph nodes in both models (Chapter 2; Appendix I, Table1). The predominant expression of B7.2 on B cells could be explained by its potential involvement in isotype switching and/or establishment of the B cell memory pool (Harris *et al.*, 1997; Mathur *et al.*, 1999; Suvas *et al.*, 2002). Since we did not detect any changes in the expression of B7.1 on APC, the data imply that the predominant costimulatory molecule expressed in the lymph nodes at the time of priming is B7.2. These findings are consistent with the notion that B7.2 privileges CD4 T cell differentiation toward a Th2 phenotype. In the lung, however, we demonstrated that expression of both B7.1 and B7.2 increased considerably after challenge, arguing against preferential involvement of either molecule in the establishment of AAI. The apparently conflicting results following blockade of B7.1 and B7.2 in AAI models, where treatment with either anti-B7.2 or anti-B7.1 before antigen challenge results in diminished airway eosinophilia reinforced this ambiguity (Harris *et al.*, 1997; Keane-Myers *et al.*, 1998; Mathur *et al.*, 1999; Tsuyuki *et al.*, 1997). Additionally, recent studies in B7.1- and/or B7.2-deficient mice indicate that whereas B7.2 is quantitatively more significant in the induction of the response, both B7 molecules may play complementary roles in the development of AAI (Mark *et al.*, 2000). Taken together, our data support the notion that the relative importance of these molecules is geographically dependent; whereas B7.2 plays a predominant role in the lymph nodes, B7.1 has an important, although in many studies underappreciated, role in the lung. Moreover, as suggested by others, B7.2 could provide

the dominant costimulatory signals in T cell activation while B7.1 might play a more significant role in sustaining T cell costimulation at distant inflammatory sites (Mathur *et al.*, 1999).

The last part of this thesis addresses the role of B7RP-1 in the establishment of AAI. The interest in this particular molecule directly stems from the demonstration that the B7RP-1 ligand ICOS is crucial for the development of Th2 effector activity. This concept is supported by the finding that ICOS-deficient mice develop enhanced EAE which is Th1-mediated and reduced Th2-dependent responses (Dong *et al.*, 2001a). In addition, blockade of ICOS in a model of AAI attenuated the accumulation of eosinophils in the lung (Tesciuba *et al.*, 2001). Our data demonstrate that the generation of Th2 responses in our mucosal model is intact in B7RP-1-deficient mice indicating the redundancy of the ICOS-B7RP-1 pathway. Not only did we observe airway eosinophilia accompanied by Th2 effector cells, but we were also able to detect IgE and IgG1. These findings are interesting in the light of data generated in ICOS-deficient mice, which indicate that isotype switching is impaired in these mice under most conditions (Dong *et al.*, 2001b; Tafuri *et al.*, 2001). However, this deficit can be overcome by CD40 costimulation (McAdam *et al.*, 2001). Since the CD40 pathway is intact in our model, CD40-CD40L ligation may be substituting for the absence of B7RP-1/ICOS signaling. Alternatively, the presence of a second, compensatory ligand for ICOS could explain the efficiency of B7RP-1-deficient mice in generating not only an isotype switch but also an effector Th2 response. In this regard, we have presented supportive evidence for the existence of a second ligand for ICOS in B7RP-1 KO mice. Therefore, either ICOS/ICOS ligand X is important for the establishment

of Th2 immune responses or, as suggested by others, the ICOS pathway is not essential for Th2 differentiation but rather for Th2 effector functions (Tesciuba *et al.*, 2001; Wiley *et al.*, 2003).

Historically, costimulatory molecules have been described as attractive targets for therapeutic manipulation. At present, glucocorticoids are the most effective drugs in the treatment of asthma. Unfortunately, glucocorticoids are not immunologically selective and more discriminating inhibitor may be desirable. Interruption of costimulatory pathways on T lymphocytes may represent a good alternative. Since the CD28 and ICOS pathways seem to have some overlapping effects in Th2 development, combination therapy involving simultaneous blockade of both pathways might be advantageous, especially in established diseases such as allergic asthma.

Taken together, the data presented in this thesis demonstrate different facets of the generation of Th2 immune responses to a model antigen (OVA). We believe that these findings have contributed to our understanding of AAI, and specifically to our appreciation of the events controlling the development of Th2 responses. This knowledge, we hope, provides a foundation for the development of novel therapies directed at optimal costimulatory pathways in specific target organs depending on the stage of the immune response.

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APPENDICES: I - V

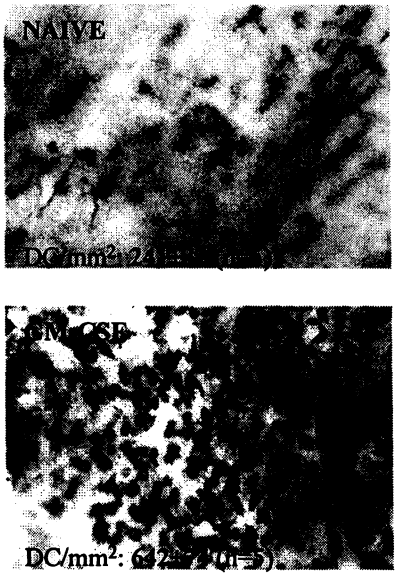
APPENDIX I

ANALYSIS OF CELL ACTIVATION IN A MUCOSAL MODEL OF ANTIGEN-INDUCED AIRWAY INFLAMMATION

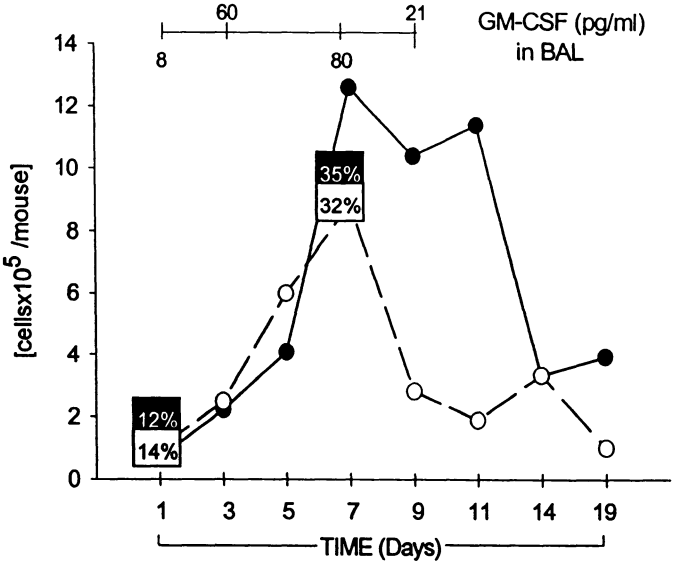
Figure 1: ***Impact of OVA delivery in the context of GM-CSF on the expansion of APC (MHCII) and activated T cells (CD3/CD69) in the airway.***

[A]. Tissues were obtained from tracheas of either naïve mice or mice undergoing GM-CSF/OVA treatment (day 7). Sections were pre-blocked with rabbit serum, incubated with anti-MHCII (clone M5/114) followed by incubation with rabbit anti-rat $F(ab')_2$ fragments conjugated to horseradish peroxidase (Serotec, Oxford, UK). Signal was developed by incubation with 0.05% diamionobenzidine (DAB). The cellular density of the airway DC network (cells/mm^2) was calculated using the Impact image analysis system (Alcetel, TITN Answare, Oberkochen, Germany). This experiment was done in collaboration with Dr. Bart Lambrecht (Erasmus University, Rotterdam, Netherlands). **[B,C].** Mononuclear cells from lung tissues were obtained at different time points of the sensitization (Ad/GM-CSF + OVA) or control protocols (Ad/GM-CSF) by enzymatic digestion (as described before) and stained with antibodies against either MHCII [B] or CD3 [C]. T cells activation was evaluated based on the expression of CD69. A total of 50,000 events were acquired by FACScan (Becton Dickinson, USA) and analyzed using WinMDI software (Scripps Research Institute, La Jolla, California, USA). Blocks indicate the % of each subset at selected time point. ■ Ad/GM-CSF+OVA ☐ Ad/GM-CSF

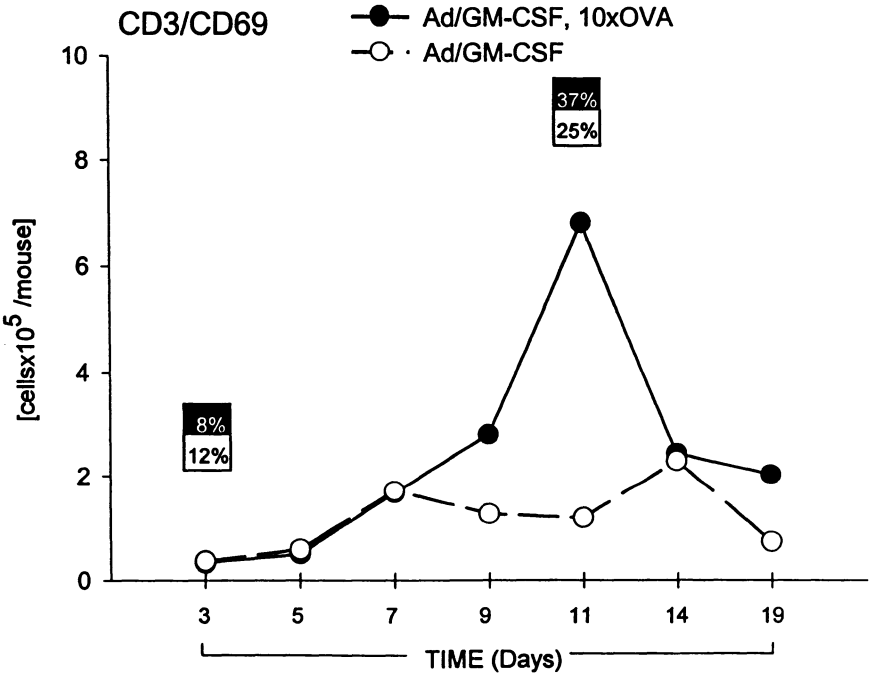
A.


NAIVE
GM-CSF

B.


GM-CSF (pg/ml)
in BAL
60
21
8
80
35%
32%
12%
14%
[cellsx10^5 /mouse]
TIME (Days)

C.


CD3/CD69
Ad/GM-CSF, 10xOVA
Ad/GM-CSF
37%
25%
8%
12%
[cellsx10^5 /mouse]
TIME (Days)

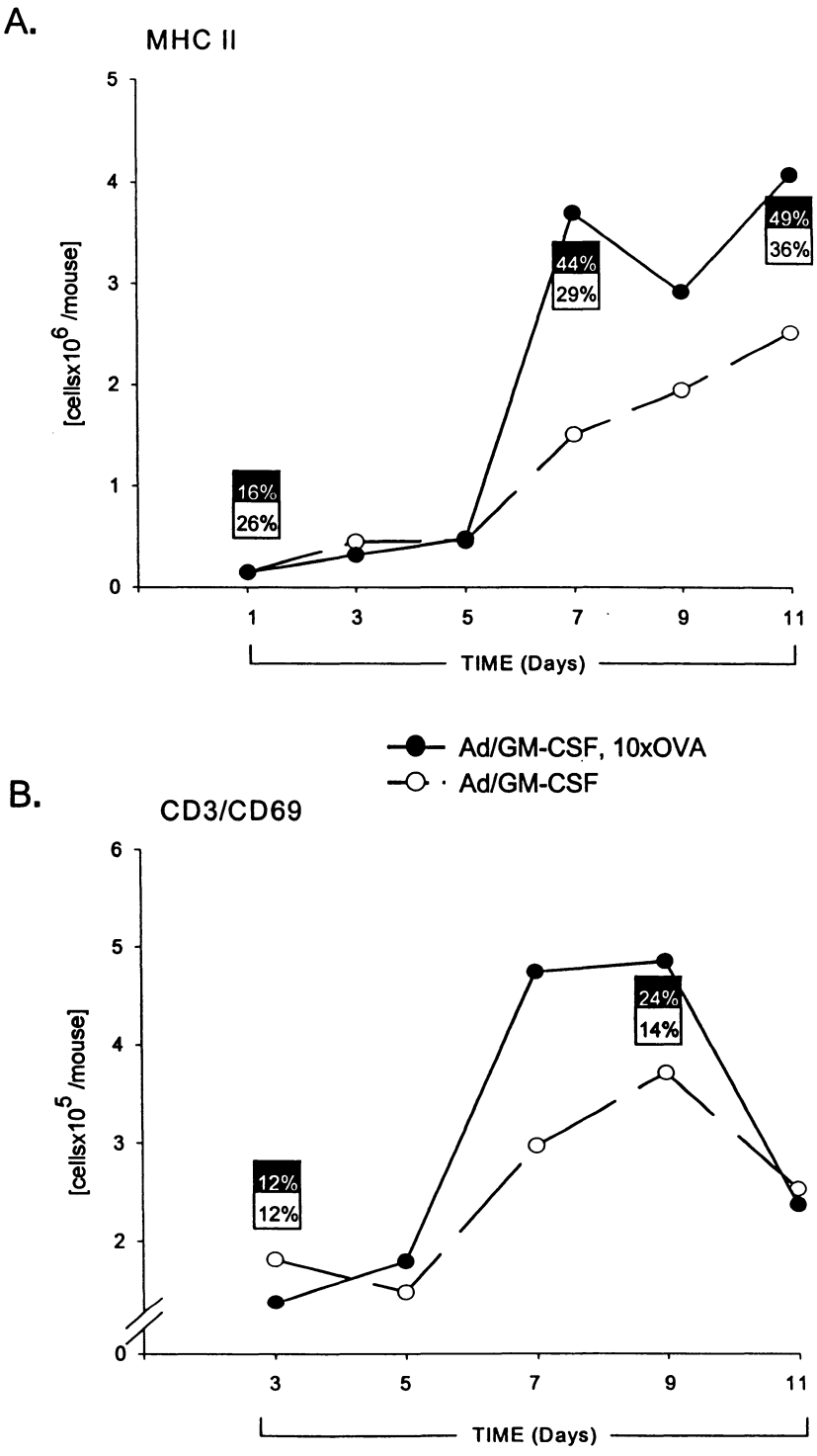
Continuation of Appendix I:

ANALYSIS OF CELL ACTIVATION IN A MUCOSAL MODEL OF ANTIGEN-INDUCED AIRWAY INFLAMMATION

Figure 2: ***Impact of OVA delivery in the context of GM-CSF on the expansion of APC (MHCII) [A] and activated T cells (CD3/CD69) [B] in the draining lymph nodes.***

Cells from lymph nodes were obtained at different time points of the sensitization (Ad/GM-CSF + OVA) or control protocols (Ad/GM-CSF) by mechanic disruption of lymph nodes (as described before) and stained with antibodies against either MHCII **[A]** or CD3 **[B].** T cells activation was evaluated based on the expression of CD69. A total of 50,000 events were acquired by FACScan (Becton Dickinson, USA) and analyzed using WinMDI software (The Scripps Research Institute, La Jolla, California, USA). Blocks indicate the % of each subset at selected time points.

■ Ad/GM-CSF+OVA ☐ Ad/GM-CSF


A.
MHC II
[cellsx10^6 /mouse]
5
4
3
2
1
0
16%
26%
44%
29%
49%
36%
1
3
5
7
9
11
TIME (Days)
Ad/GM-CSF, 10xOVA
Ad/GM-CSF
B.
CD3/CD69
[cellsx10^5 /mouse]
6
5
4
3
2
0
12%
12%
24%
14%
3
5
7
9
11
TIME (Days)

Continuation of Appendix I:

ANALYSIS OF THE EXPRESSION OF COSTIMULATORY MOLECULES B7.1 (CD80) AND B7.2 (CD86) BY MHC II$^+$ CELLS ISOLATED FROM THE LUNGS AND LYMPH NODES IN A MUCOSAL MODEL OF ANTIGEN INDUCED AIRWAY INFLAMMATION

Table1. Expression of B7 molecules on MHCII$^+$ cells at the peak of APC expansion (day 7)

	LUNG		LYMPH NODES	
	B7.1	B7.2	B7.1	B7.2
Ad/GM-CSF + OVA	**11.4** [3.9]	**15.4** [3.0]	**5.6** [5.1]	**25.0** [7.3]
Ad/GM-CSF	**12.5** [3.8]	**13.0** [2.6]	**10.2** [5.1]	**27.0** [4.8]

Cells were isolated from the lung or draining lymph nodes and stained for MHCII expression as the indication of antigen presenting cells (APC). For simplicity, B7.1 and B7.2 expression has been shown only at day 7 (bolded) or at day 1 [].

APPENDIX II

IMPORTANCE OF LYMPHOID ORGANS *vs.* THE LUNG IN RECALL RESPONSES TO ANTIGEN

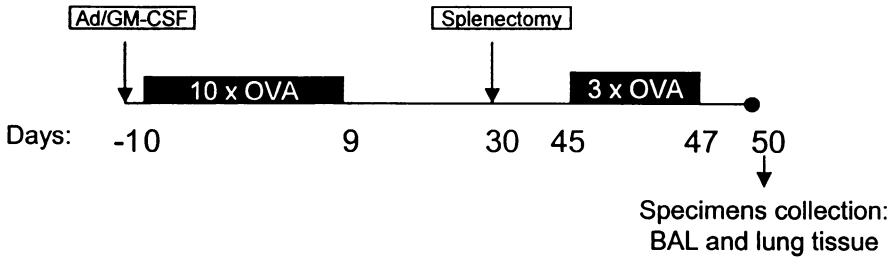
Figure A: Scheme of the protocol used for the study. One day prior to OVA aerosol exposure, LTαKO mice received Ad/GM-CSF intranasally at a dose $3x10^7$ pfu. After the resolution of airway inflammation (at day 30) spleens from 4 LTαKO mice were surgically removed. Following recovery from surgery (15 days), mice were re-exposed to OVA on three occasions. Specimens from either splenectomized (-SP) or non-splenectomized mice were collected 72 hours after the last OVA exposure (day 50).

Figure B: Airway eosinophilia in the BAL of splenectomized (+SP) and non-splenectomized (-SP) LTαKO. Data were obtained 72 hours after the last OVA exposure. (Mean ± SEM; n=4/group).

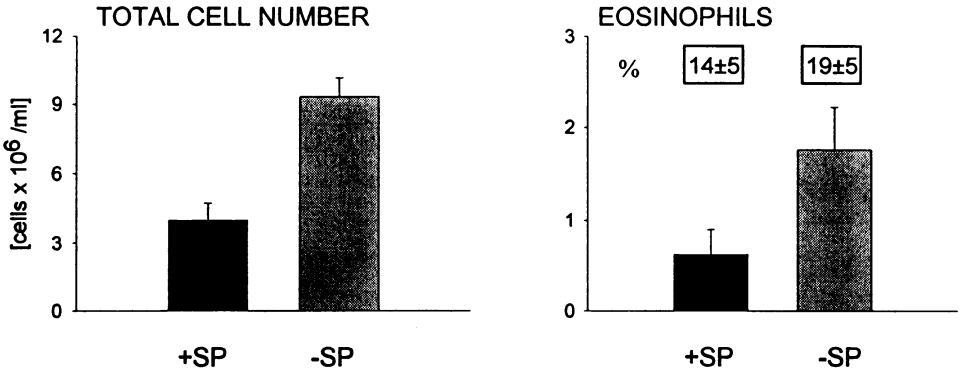
Figure C: *In vitro* production of IL-5 and IL-13 by lung cells cultured in medium alone or with OVA. Lung tissues were obtained 72 hours after the last OVA exposure, pooled from 4 mice/group, enzymatically digested and cultured for 5 days in RPMI (supplemented with penicillin-streptomycin, fetal calf serum, L-glutamine and β-mercaptoethanol). Cytokines were measured by commercially available ELISA kits [pg/ml](R&D Systems, MN, USA).

Figure D: Expression of the effector Th2-affiliated molecule ICOS. Lung tissues were obtained 72 hours after the last OVA exposure, pooled from 4 mice/group and enzymatically digested. The mononuclear fraction of cells was stained with CD3, CD4 (BD Pharmingen, ON, Canada) and ICOS (Millennium Pharmaceuticals, Boston, MA, USA). The histograms represent ICOS expression on $CD3^+/CD4^+$ gated T cells. The isotype controls have been presented as open histograms.

A



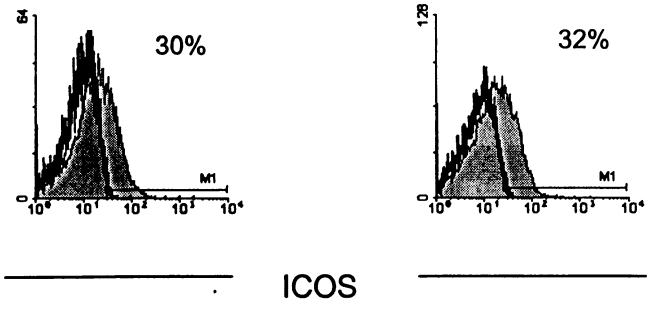
B



C

	+SP		-SP	
	MED	OVA	MED	OVA
IL-5	21.6	112.7	31.6	189.4
IL-13	47.6	122.9	744	3627.3

D



APPENDIX III

CD28 KNOCKOUT (CD28 KO) MICE FAIL TO DEVELOP ALLERGIC AIRWAY INFLAMMATION

Figure: CD28 knockout mice (CD28KO) and wild type mice (WT) were sensitized to ovalbumin according to a conventional protocol involving an intraperitoneal injection with aluminum hydroxide as an adjuvant, followed 7 days later by an aerosol challenge with ovalbumin (OVA). No airway inflammatory response to OVA was observed in CD28 knockout mice. **[A].** Eosinophils in the BAL of CD28 KO and WT mice 72 hours after challenge. **[B].** IL-4 and IL-5 in the BAL of CD28 KO and WT mice 24 hours after challenge. **[C].** The induction of ovalbumin-specific IgE in serum of CD28 KO and WT mice 72 hours after challenge.

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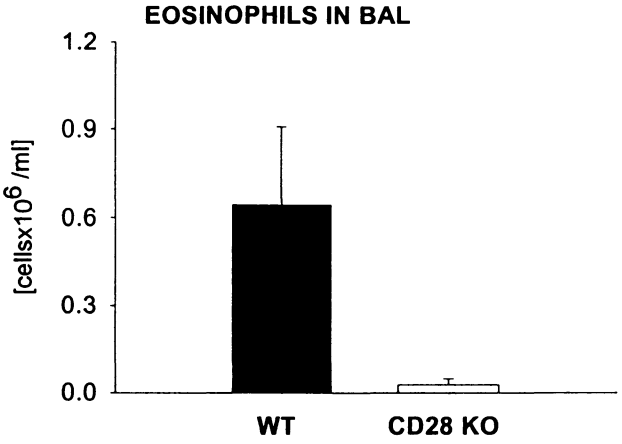
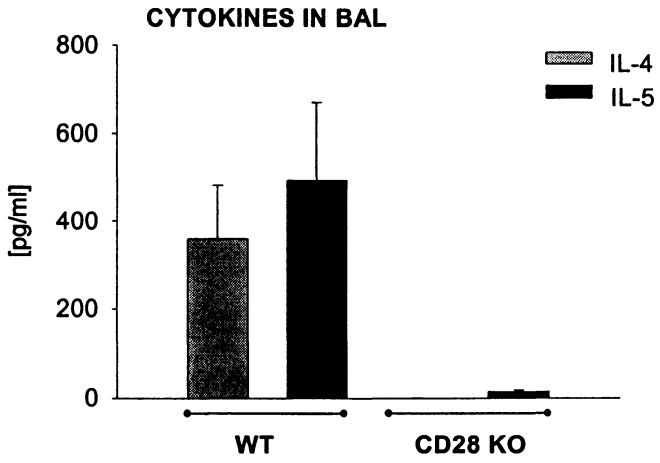
J Allergy Clin Immunol 2000, 106 (5): 206-212

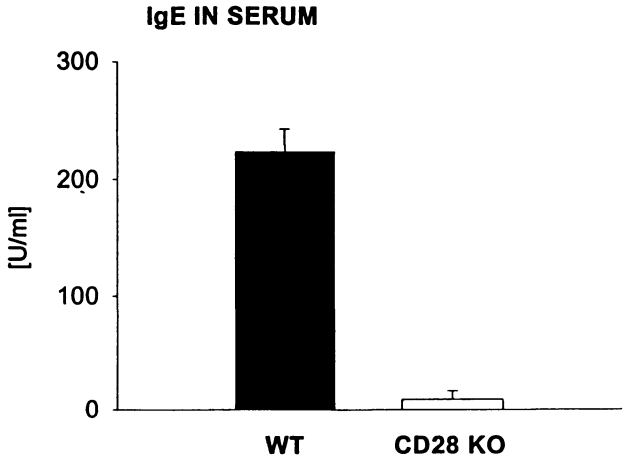
A.


EOSINOPHILS IN BAL
1.2
0.9
0.6
0.3
0.0
[cellsx10^6 /ml]
WT
CD28 KO

B.


CYTOKINES IN BAL
800
600
400
200
0
[pg/ml]
IL-4
IL-5
WT
CD28 KO

C.


IgE IN SERUM
300
200
100
0
[U/ml]
WT
CD28 KO

APPENDIX IV

GM-CSF AND DENDRITIC CELLS IN ALLERGIC AIRWAY INFLAMMATION: BASIC MECHANISMS AND PROSPECTS FOR THERAPEUTIC INTERVENTION

Gajewska BU, Wiley RE and Jordana M.

CURRENT DRUG TARGETS. IN PRESS

The attached review describes the involvement of dendritic cells in maintenance of homeostasis in the lung and in the generation of allergic airway inflammation.

GM-CSF AND DENDRITIC CELLS IN ALLERGIC AIRWAY INFLAMMATION: BASIC MECHANISMS AND PROSPECTS FOR THERAPEUTIC INTERVENTION

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ABSTRACT

The interaction between dendritic cells (DC) and naïve T cells is the first step in the evolution of an immune response, either tolerogenic or inflammatory. Therefore, the status of DC residing at mucosal sites, such as the airway, has a definitive impact on the character of the ensuing immune response. In the absence of pathogenic stimulation, DC serve to regulate immunological homeostasis in the lung; the generation of Th2-associated (allergic) inflammatory responses, which are directed at presumably innocuous antigens, represent a deviation from normal DC function. The dysregulation of DC phenotype leading to the development of allergy might be programmed by genetic pedigree, or might be induced by factors released in the airway. One potential candidate, GM-CSF, is abundant in the allergic airway and can condition DC to propagate Th2 responses. Moreover, that allergens, alone or in combination with other factors, can spontaneously induce GM-CSF production in the airway presents a compelling ætiological argument for the role of GM-CSF in allergic sensitization. The interplay between DC and mediators present in the allergic airway is likely critical to the establishment of allergic airway inflammation. Understanding these interactions may therefore afford insight into prospective therapeutic interventions to circumvent, and even reverse, the allergic diathesis.

INTRODUCTION

The incessant exposure of the respiratory tract to a plethora of both innocuous and harmful antigens imposes a monumental challenge on the immune system. Of paramount importance, the immune system must assess the prospective harm a given interloper represents and then execute the most efficacious programme—whether tolerogenic or inflammatory—to deal with it. Arguably, the effector T cell response that evolves, largely in the secondary lymphoid organs, is dictated by the set of instructions that naïve T cells receive from the lung. The lung, like all mucosal sites, must therefore comprise a system that can sample antigen, interpret the immunological context in which the antigen has arrived, and traffic to secondary lymphoid structures to convey this information to naïve T cells.

A dense network of dendritic cells (DC) in both the nasal and bronchial mucosae as well as in the lung interstitium is exquisitely equipped to serve this imperative [1]. DC possess an apparatus to capture and efficiently present antigen through different pathways; they have the capacity to assess immunological danger by interpreting signals transmitted during tissue damage, necrosis or infection; they have the flexibility to translate this immune-contextual information through the expression of distinct patterns of co-stimulatory molecules and cytokines; and, finally, they can migrate to the draining lymph nodes [2]. Functionally, then, DC can be understood as the nexus of mechanisms that maintain immunological homeostasis in the lung.

Th2-polarized T cells play a central role in allergic diseases such as asthma. The cardinal cytokines and chemokines produced by these cells critically mediate hallmark

features of the allergic phenotype, including airway eosinophilia, mucous hypersecretion and airway hyperreactivity [3]. The importance of the communication among antigen-presenting cells (APC), naïve T cells and allergen in the generation of allergen-specific Th2 responses is undisputable, although the underpinnings of this tripartite interaction have yet to be elucidated fully. Moreover, from the standpoint of protection—which is ostensibly the immune system's *raison d'être*—the biological advantage of an immune-inflammatory response against allergens remains perplexing.

Over millions of years the immune system has evolved sophisticated programmes to eliminate harmful replicating pathogens such as viruses and bacteria. An essential component of these programmes is a repertoire of innate recognition systems in DC, among which pattern recognition receptors (PRR) for molecules expressed ubiquitously by viruses and bacteria are pre-eminent [4]. That no such recognition systems have been identified for allergens has prompted consideration of alternative means by which allergen exposure can lead to DC activation and, ultimately, to the elaboration of an immune-inflammatory response. Since allergens, generally viewed as innocuous proteins, presumably cannot activate DC *directly*, the microenvironment in the airway at the time of initial allergen exposure is likely critical. Indeed, numerous antigens, including allergens, prompt epithelial cells to produce cytokines/chemokines [5, 6] that can decisively establish the context in which DC interact with allergens. One of these immunoregulatory signals is GM-CSF. GM-CSF is commonly used to propagate DC from bone-marrow progenitors and is noted for its ability to potentiate the antigen-presenting capacity of DC [7] . Uptake of allergen in the context of a GM-CSF-conditioned airway milieu could facilitate efficient

antigen presentation to T cells and influence the generation of an immune response. Indeed, growing evidence from both *in vitro* and *in vivo* studies supports the notion that GM-CSF is involved in DC activation in the lung and, concomitantly, in the development of allergic inflammation.

This review summarizes recent progress in our understanding of the role of DC in allergic inflammation, attempts to articulate a plausible connection between GM-CSF and DC in the generation of Th2 responses to aeroallergens, and considers potential therapeutic manipulations of DC for the treatment of allergy.

DENDRITIC CELLS IN ALLERGIC SENSITIZATION

The decision to elaborate an immune response, whether inflammatory or homeostatic (*i.e.* tolerance), against environmental antigens must logically be made at the point of entry, *i.e.* at either mucosal surfaces or the skin. In the respiratory mucosa, DC, the APC subset with the greatest antigen-presenting capacity, constitute a very dynamic population of lung cells with a decisive role in the development of lung/airway immune responses [2, 8]. DC constantly survey the mucosal environment but remain immature in the absence of pathogenic stimulation [9, 10]. The precise mechanisms responsible for keeping DC in an immature state are unknown, though it has been suggested that the presence of inhibitory/regulatory molecules (such as IL-10), or of cells equipped with inhibitory potential (*e.g.* macrophages), in the airway/lung environment may prevent DC from becoming activated and initiating unwarranted immune-inflammatory responses [11-

13]. Consequently, any alternation of this immunosuppressive environment may alter the DC compartment in a manner conducive to the development of Th2 deviated responses.

DC MIGRATION TO THE LYMPH NODES. The initial event in an immune response, either tolerogenic or inflammatory in nature, involves antigen capture by resident tissue DC [14]. Pulmonary DC are ideally situated to accomplish this task, especially airway intraepithelial DC whose distribution resembles that of the Langerhans cell network in the skin [15]. However, DC are also dispersed throughout the lung parenchyma, the connective tissue surrounding major airways and vessels, the pleura and the pulmonary vascular bed, thereby affording a comprehensive surveillance capacity to all lung subcompartments.

Secondary lymphoid structures furnish an essential environment for the elaboration of primary immune responses. They play a major role in the establishment of both inhalation tolerance [16] and allergic airway inflammation (AAI) [17]. By examining immune-inflammatory responses to ovalbumin (OVA) in lymphotoxin α (LTα)–deficient mice, which lack lymph nodes but retain an aberrant spleen, we have recently provided definitive experimental evidence for the irreplaceable role of secondary lymphoid structures in allergic sensitization [17]. Interestingly, while the spleen was found to be redundant for Th2 sensitization and airway eosinophilic inflammation in wild-type controls, it was essential in LTα-deficient mice, attesting to the plasticity of the immune system [17]. While it is possible that antigens penetrating the mucosa reach secondary lymphoid structures in the absence of a cellular vehicle, their arrival, immunologically, is probably inert. In contrast, antigen-loaded DC express immune-contextual signals that empower DC

to present antigen *informatively* to naïve T cells, thereby initiating an immune response. There is evidence that DC instilled into the trachea of naïve mice or rats migrate to T-cell dependent areas in the draining lymph nodes within 24 h [18, 19]; studies examining endogenous, rather than *ex vivo*-generated, DC in mice confirm this migratory behavior [20, 21]. The general consensus to date is that the key molecule in this migration is the chemokine receptor CCR7, which is upregulated in response to antigen. SLC/CCL21 and MIP-3β/CCL19, the major ligands for CCR7, are constitutively expressed in the lymph nodes, which are therefore able to attract and retain CCR7-expressing cells [22, 23]. Indeed, blockade of SLC prevented the recruitment of DC to the lymph nodes in a humanized SCID model of allergic inflammation, resulting in attenuated Th2 responses [24].

EVIDENCE IMPLICATING DC IN ALLERGIC SENSITIZATION. There are very few human studies examining the role of DC in allergic sensitization. However, it has been shown *in vitro* that DC from atopic individuals can efficiently present allergens to syngeneic naïve and memory T cells and elicit the production of Th2 cytokines [25-27]. In addition, monocyte-derived DC from allergic patients, pulsed with HDM and delivered intratracheally (i.t.) into SCID mice, were able to stimulate transferred, human-derived T cells and generate a productive Th2 response in the lung [24]. Notwithstanding the insight these human studies have afforded, studies in animal models are best suited to provide functional data about the importance of DC in the induction of allergic immune responses in the lung. In rodents, i.t. introduction of bone marrow-derived myeloid DC or splenic DC pulsed with OVA *in vitro*

resulted in the development of Th2 cell-dependent airway eosinophilia, goblet cell hyperplasia, IL-4 and IL-5 production, and bronchial hyperreactivity upon subsequent OVA challenge [28, 29]. Similarly, re-introduction of OVA-pulsed airway DC resulted in the development of Th2-associated IgG_1 and IgE antibody responses [10]. While the importance of these findings is undisputable, the experimental conditions that produced this evidence should be heeded. Indeed, *ex vivo* manipulation of DC introduces an intractable experimental artifact; that re-introduction of such cells elicits immune-inflammatory (Th2) responses to OVA says little about how DC might respond to OVA, or to allergens, *in vivo*. Furthermore, OVA is an archetypical innocuous antigen, and passive inhalation of OVA leads to the establishment of tolerance, not airway inflammation [30, 31]. Therefore, studies involving *ex vivo* manipulation of DC are intrinsically unable to elucidate how DC reconcile the maintenance of homeostasis with the initiation of an immune-inflammatory response to allergens.

THE INTERACTION OF DC WITH ALLERGENS AND ENVIRONMENTAL FACTORS DURING SENSITIZATION. The principle of *biological economy* would dictate that an inflammatory response should be mounted only when the benefit to the organism (survival) outweighs the risks (tissue damage). In this regard, the "danger theory" provides an elegant elaboration on the decision involved in engineering an inflammatory response. In brief, it proposes that cells injured by exposure to certain antigens, toxins or mechanical damage release signals that activate DC and alter their phenotype [32]. Once activated, not only are DC able to capture and process antigen efficiently, but they also mature and acquire

new (*i.e.* non-homeostatic) information from the immunologic milieu. In the context of allergic sensitization, the molecular identity of these "danger signals" has remained somewhat elusive; the innocuousness of OVA, however, has, somewhat paradoxically, provided a foundation to explore these issues experimentally.

While exposure to aerosolized OVA alone elicits tolerance, concurrent exposure to OVA and "environmental adjuvants" such as influenza A virus or tobacco smoke leads to the generation of a Th2-polarized response [33, 34]. More particularly, exposure to OVA in the context of exogenously delivered GM-CSF leads to *bona fide* allergic sensitization and airway eosinophilic inflammation, indicating that GM-CSF, distinguished among other inflammatory mediators, is capable of subverting inhalation tolerance [35]. Whether GM-CSF should be classified as a "danger signal", however, is perhaps moot; GM-CSF production is in all likelihood a downstream event, and therefore questions pertaining to the cellular source and the environmental signals that trigger its production are likely of greater significance.

How does this conceptualization inform our understanding of the role of DC in allergic sensitization? The justifiable convenience of using OVA as a surrogate allergen in experimental models has sadly obfuscated the fact that OVA does not possess the immunological or biochemical properties of "real-life" allergens. For example, the proteolytic activity characteristic of some aeroallergens could bypass mechanisms that would otherwise lead to the generation of "tolerogenic " DC; in this regard, exposure of rodents to *Aspergillus fumigatus* derivatives or house dust mite (HDM) extract readily induces allergic airway inflammation without the need for additional adjuvants [36, 37].

Exogenous proteases have been shown to degrade proteins forming tight junctions in airway epithelium [38], thereby facilitating antigen access to the subepithelial DC network. However, an intact epithelial layer does not prevent the induction of antigen-specific inhalation tolerance. Moreover, it has been shown that DC in the intestinal epithelium extend their dendrites through tight junctions into the lumen in order to sample local bacteria; during this process, DC express tight junction proteins that enable them to preserve continuity of the epithelial layer [39]. Thus, it is difficult to argue that subepithelial access to DC determines the immune-inflammatory outcome of antigen exposure, or that the proteolytic activity of some allergens is required to gain access to DC, an assertion further supported by the fact that some common allergens are not proteases. In our view, a more compelling scenario is that allergens—and the environmental stimuli with which they are delivered—interact with epithelial cells to induce the release of signals, such as GM-CSF and probably others, that favor the maturation and activation of DC. In this regard, there is clear evidence that a variety of inhaled stimuli, including typical allergens such as HDM, induce epithelial cells to release a panoply of cytokines including GM-CSF [40, 41].

In brief, the ability of an allergen to elicit allergic sensitization and, ultimately, airway inflammation depends on its presentation to naïve T cells in secondary lymphoid structures in the context of appropriate immunological signals. These geographical and contextual imperatives establish the critical importance of DC in this process. Clearly, the DC network must be activated; while the route and timing of DC migration to the lymph nodes are universal—*i.e.* independent of the type of antigen—the translation of immune-contextual information (induction of co-stimulatory molecules and cytokines) likely reflects

the antigen *per se*, the signals this antigen elicits from resident cells, as well as the untold environmental stimuli with which this antigen arrives in the lung. Some of these issues will be addressed in the sections that follow.

DC INVOLVEMENT IN TH2 POLARIZATION

The detailed processes that determine the preferential development of Th2 cells upon allergen exposure remain unclear. From a global perspective, T cell polarization is influenced, to different degrees, by the genetic predisposition of the host, environmental factors, the nature of the antigen, the cytokine milieu in which the antigen is presented to naïve T cells, and the status of DC [42-45]. To date, two models have attempted to conceptualize how DC may influence Th polarization. The first, referred to by Shortman *et al.* [46] as the *specialized lineage model*, presumes the existence of two distinct DC subsets—developed through separate pathways and expressing distinct receptors—predisposed to prime either Th1 or Th2 responses. The second, designated the *functional plasticity model*, argues that the ability of DC to induce Th1 or Th2 responses principally depends not on their lineage commitment but on functional instructions DC acquire in response to signals in the local microenvironment.

SPECIFIC DC SUBTYPES DETERMINE THE DEVELOPMENT OF TH2 ALLERGIC RESPONSES. With respect to the *specialized lineage model*, Rissoan and colleagues [47] were the first to identify two distinct human DC subsets, DC1 and DC2, capable of inducing different T cell responses. Whereas human monocyte-derived DC (myeloid-like cells or DC1) induced Th1

differentiation through the production of IL-12, DC derived from $CD4^+CD3^-CD11c^-$ plasmacytoid cells (lymphoid-like cells or DC2) promoted Th2 differentiation. Emerging from these findings is the hypothesis that a specific DC subset predisposed to elicit Th2 differentiation resides in the respiratory mucosa of allergic patients. Support for this hypothesis has been provided by Jahnsen *et al.* [48], who observed the dramatic accumulation of plasmacytoid DC (DC2) in the nasal mucosa of allergic rhinitis patients following topical allergen challenge. This observation is consistent with the preferential presence of DC2 in the blood of asthmatic patients compared to control subjects and could reflect either an evolutionary commitment or the preferential migration of DC1 to the lung [49, 50]. Indeed, the more recent observation that myeloid DC (DC1), but not DC2, rapidly accumulated in the bronchial mucosa of asthma patients within 4-5 hours of antigen challenge [51], and that this accumulation coincided with a reduction in circulating DC1, casts some doubts on the validity of the original hypothesis [52]. Although this apparent contradiction has not been reconciled, differences in the recruitment kinetics of each DC subtype may furnish one explanation.

A similar, functionally dichotomous lineage commitment has been observed in DC populations in the mouse. However, unlike human DC, mouse "lymphoid" $CD8^+$ DC induce Th1-polarized cytokine responses while "myeloid" $CD8^-$ DC tend to elicit Th2-affiliated responses [53, 54]. Studies illustrating the prevalence of myeloid DC over lymphoid DC in the lungs of experimental animals support this contention. In a murine model of allergy, for example, it has been shown that myeloid $CD8^-$ but not lymphoid $CD8^+$ DC phagocytose antigen (OVA) in the lungs and migrate to the draining lymph nodes [21]. In addition,

administration of GM-CSF to the lung, which leads to the preferential expansion of myeloid $CD11c^{+}CD11b^{+}$ DC, facilitates OVA-specific Th2 sensitization [55]. However, in the final analysis, both human and animal studies documenting changes in the blood/lung balance between DC1 and DC2 have not been able to distinguish the pre-established phenotypic commitment of DC from their adaptation to stimuli present in the inflamed allergic airway.

COOPERATION BETWEEN DC AND ENVIRONMENTAL FACTORS IN THE DETERMINATION OF TH2 POLARIZATION. The concept of *lineage commitment* has been challenged by evidence demonstrating the remarkable plasticity of DC subsets [56, 57]. In particular, data indicate that both the cytokine milieu and the nature of the antigen *per se* can differently regulate DC function and, by extension, T cell polarization. For example, it has been shown that cytokines such as IL-10 and TGF-β can subvert the alleged Th1-priming capacity of human DC1 and confer a Th2-polarizing phenotype [58]. Likewise, different antigens can modify the propensity of DC subsets to elicit Th-polarized responses. Indeed, LPS from *Escherichia coli* stimulates $CD8^{+}$ DC from mice to produce IL-12, whereas LPS from *Porphyromonas gingavalis* does not stimulate IL-12 production from lymphoid DC and preferentially induces Th2 responses [59]. Based on these intimations of DC plasticity, it appears that the character of the lung microenvironment determines the character of the ensuing immune response through the capacity of DC to interpret contextual signals and respond accordingly.

Intuitively, the nature of the inflammatory milieu in the airway reflects the interaction of antigens with resident cells. The significance of the latter has recently been

advanced by suggestions that the tendency of DC to trigger Th1- or Th2-polarized responses is influenced by the tissue in which the antigen is initially captured. Of direct relevance to allergic diseases, mucosal sites have been commonly designated *Th2-promoting environments* [60]; whereas DC isolated from the spleen preferentially induce Th1 differentiation *in vitro*, those harvested from Peyer's patches potentiate Th2 differentiation through a mechanism involving the production of IL-4 and IL-10 [61]. The Th2-educating propensity of mucosal sites has been validated *in vivo* by Constant *et al.* [62], who have shown that intranasal administration of Th1-biased *Leishmania major* to a Th1-biased mouse strain (C57BL/6) resulted in a Th2-polarized response. While the tendency of mucosal tissues to elicit Th2 responses requires further explanation, it is likely that the particular profile of resident cells in the tissue plays a significant role. For example, mucosal tissues are replete with mast cells, and recent studies have demonstrated that treatment of human DC with either histamine, PGE_2 or sphingosine 1-phosphate—all inflammatory mediators released by mast cells upon IgE cross-linking—promotes their Th2-stimulating capacity *in vitro* [63-66]. That these are mediators released upon IgE cross-linking precludes their contribution to the generation of Th2 polarization, but suggests an important role in the maintenance of the Th2 phenotype.

Synthesizing knowledge about DC in the lung mucosa, Stumbles *et al.* [10] have proposed that, under normal conditions, resident pulmonary DC will tend to induce Th2-biased responses while reserving the capacity to stimulate Th1 immunity upon receipt of additional signals. In fact, current evidence indicates that under steady-state conditions mucosal DC tend to induce tolerance, either through the induction of T cell anergy or

production of regulatory T cells, as the responses are mainly directed at either self-antigens (*e.g.* apoptotic epithelial cells) or innocuous environmental antigens (*e.g.* allergens) [67]. Therefore, building on Stumbles' hypothesis, any deviation from this tolerogenic programme would be expected to default to a Th2 response. Two possible explanations, not mutually exclusive, may account for the Th2-skewing predisposition of pulmonary DC: (i) DC in the lung promote Th2 differentiation directly by preferentially producing Th2-skewing cytokines or co-stimulatory molecules; and (ii) DC facilitate Th2 priming by inhibiting Th1 differentiation.

To date, a definitive Th1- or Th2-affiliated co-stimulatory molecule profile has not been reported for DC. The blockade of major co-stimulatory pathways such as CD28/B7, ICOS/ICOSL, and OX40/OX40L results in the inhibition not only of Th2 [68-71] but also, in the majority of cases, of Th1 responses [72-74]. Even the initial observation that B7.2 preferentially regulates Th2 responses has been discredited [75, 76]. This implies that additional DC-mediated signals are needed to educate effective Th1- or Th2-polarized responses. To this end, it is becoming increasingly evident that DC-derived IL-12 is instrumental in influencing Th polarization. The presence of IL-12 promotes Th1 differentiation, while its (relative) absence facilitates Th2 phenomena. That mouse $CD8^+$ DC (Th1-associated) isolated from IL-12-deficient mice fail to prime for Th1 responses and promote, instead, the development of Th2-type cells [77] indicates that IL-12 not only primes for Th1 but, under normal circumstances, also inhibits Th2 responses. Moreover, the capacity for DC and other resident cells at mucosal surfaces to secrete IL-12 in response to particular antigens underscores the relevance of antigen identity to the

outcome of Th polarization. In this regard, common bacterial components such as LPS, peptidoglycan and unmethylated CpG motifs all induce Th1 differentiation by a mechanism that involves the induction of IL-12 production by DC [78], whereas antigens from nematode worms, which do not elicit IL-12 production, induce Th2 responses [79]. While the nature of the antigen certainly plays a major role in the acquisition of IL-12 effector activity, the presence of other cytokines in the airway microenvironment also influences the DC phenotype. Notable in this regard are IL-10 and IL-6, both of which have been shown to inhibit IL-12 production [80]. Among its legion of immunosuppressive activities, IL-10 also inhibits MHC class II and costimulatory molecule expression on DC, presumably limiting the magnitude of the antigenic signal delivered to T cells [81, 82]. That freshly isolated lung DC produce IL-10 and IL-6 intimates that the primary physiological role of these cytokines in the lung may be to control the generation of Th1 responses [10, 83]. Conceivably, exposure to certain soluble antigens under these conditions may permit the development of Th2 responses.

We surmise that the selection of Th phenotype is dependent on the instructive programme acquired by DC in response to signals in the microenvironment. However, Th1 and Th2 are not the only possible outcomes of Th differentiation. There is at least a third possible outcome: the generation of T regulatory (T_r) cells [84]. Moreover, whether Th polarization actually translates into a *productive* effector response may also depend on the strength of the signals conveyed by DC to naïve T cells. If DC are to serve as cellular messengers, they must transmit reliable information about both the intruder and the outcome of the intruder's initial encounter with tissue resident cells. Speculatively, then, we

propose that the instructive programme acquired by DC in the respiratory mucosa encompasses a plastic response to three types of signals: (i) "pathogen identifiers", *i.e.* signals that convey the pathogenic signature of the intruder; (ii) signals with a primary role in DC maturation/activation; and (iii) polarizing signals. The intrinsic plasticity of DC—their capacity to interpret and respond to multiple instructions—is therefore the arbiter of immunological outcome.

Part of the difficulty in testing experimentally this conceptualization of the immune response elicited by allergens is that the majority of research has utilized ovalbumin. However, as mentioned before, the innocuousness of OVA has permitted research to evaluate the importance of microenvironment in the determination of immunological outcome. Indeed, our models of OVA aerosolization in the context of cytokine over-expression in the airway via gene transfer have invested us with a powerful experimental tool to investigate the context-dependent plasticity of immune responses. For instance, while passive exposure to aerosolized OVA alone leads to inhalation tolerance [30], concurrent airway expression of GM-CSF results in a robust Th2 response [35]. Importantly, the Th2-polarizing microenvironment established by GM-CSF is readily manipulable, as demonstrated by the finding that co-expression of IL-12 and GM-CSF privileges Th1-polarized sensitization to OVA [85]. Substitution of IL-12 with a recombinant adenovirus encoding the transgene for IL-10, on the other hand, conditions an immunologically inert response that mimics the features of active tolerance and intimates the differentiation of a memory regulatory T cell [86]. Finally, we have recently demonstrated that co-expression of GM-CSF and the Th1-associated chemokine IP-10

subverts the development of an allergic response to OVA and, through the preferential recruitment of IFNγ-producing Th1 cells, elicits sustained, Th1-polarized sensitization [87].

While each of these manipulations underscores the precedence of immunological microenvironment in the evolution of an adaptive response, whether the distinct immune responses we have documented are the consequence of cytokines providing discrete instructions to lung DC is likely but as yet unproven. However, because these cytokine and chemokine interventions elicit long-term modification of OVA-specific memory, it is probable that they influence the outcome of incipient contact between naïve T cells and APC by modulating the context in which antigen is initially presented and interpreted. In the section that follows, we shall consider in detail how over-production of one such signal, GM-CSF, may predispose this immunological context to the establishment of allergy.

THE ROLE OF GM-CSF IN ALLERGIC ASTHMA

That dendritic cells play a critical role in the evolution, and particularly in the initiation, of allergic phenomena is unequivocal. Less well understood, however, are the conditions under which DC become activated and equipped for Th2-polarized antigen presentation *during the incipient stages of an allergic response*. In the preceding discussion of DC in allergic sensitization and Th2 polarization, we alluded to the instrumental role of both danger signals in the licensing of DC function and the cytokine milieu in the outcome of Th polarization. In this section, we consider evidence implicating one candidate danger signal, GM-CSF, in the regulation of DC, the propagation of immune responses and, concomitantly, in the ætiology of allergic asthma.

ROLE OF GM-CSF IN THE MAINTENANCE OF PULMONARY HOMEOSTASIS. GM-CSF, initially described as an hæmatopoietic cytokine orchestrating myeloid differentiation in the bone marrow, is inexorably tied to the biology of the lung. Indeed, GM-CSF was first purified from the conditioned medium of cultured lung tissue harvested from mice exposed systemically to LPS [88]. Moreover, GM-CSF is, in general, more readily detected in the lung than in the blood, probably because the majority of hæmatopoietic and structural cells in the lung can produce this cytokine [89, 90]. While it is therefore reasonable to speculate on the immunoregulatory service GM-CSF might provide in the lung, it is also important to consider GM-CSF's inimitable role in pulmonary homeostasis. For instance, GM-CSF can stimulate the function of alveolar macrophages [91] and the growth of alveolar epithelium [92]. A critical role for GM-CSF in the lung has been demonstrated in transgenic mouse models in which the activity of GM-CSF was altered through the targeted ablation of genes for either GM-CSF ($GM^{-/-}$) [93] or its receptor ($GMR^{-/-}$) [94], or through overexpression of GM-CSF in various tissues [92]. Studies in $GM^{-/-}$ and $GMR^{-/-}$ mice indicate that GM-CSF signaling is required for pulmonary surfactant homeostasis, normal alveolar macrophage function and proper generation of innate immunity [89, 90, 93]. On the other hand, overexpression of GM-CSF in the lung results in progressive alveolar macrophage accumulation and alveolar type II cell hyperplasia [92].

EVIDENCE IMPLICATING GM-CSF INVOLVEMENT IN ALLERGIC AIRWAY INFLAMMATION (AAI). In addition to its contribution to normal lung function, GM-CSF has been credited with an important role in the elicitation and maintenance of inflammation [95]. Elevated levels of

GM-CSF have been documented in a number of pulmonary inflammatory conditions, including allergic airway inflammation [96]. Increased production of GM-CSF, both RNA and protein, has been observed in bronchial epithelial cells, BAL, sputum and antigen-stimulated PBMC from allergic patients (reviewed in [97]). Moreover, *in vitro* stimulation of either lung epithelial cells or PBMC from asthmatics resulted in enhanced production of GM-CSF compared to non-asthmatics [98, 99]; conceivably, this propensity to produce GM-CSF reflects documented polymorphisms in the gene encoding GM-CSF within the asthmatic population [100]. Experimentation in animal models has validated the permissive role of GM-CSF in allergic airway inflammation, offering mechanistic insight into the hyperproduction of GM-CSF in human asthmatics. We have already pointed out elsewhere in this review that the introduction of GM-CSF, either as a recombinant protein or through gene transfer, to the lungs of mice during concurrent exposure to a model innocuous antigen (OVA) elicits a cardinal, Th2-polarized, eosinophilic inflammatory response [35, 101]. In addition, local administration of GM-CSF during allergen challenge of previously-sensitized mice exacerbates and prolongs the inflammatory response, which may reflect the centrality of GM-CSF in the local activation of leukocytes recruited to the inflamed lung [102]. In the context of asthma, particular emphasis has been placed on the activation of eosinophils, as *in vitro* exposure of eosinophils to GM-CSF stimulates their differentiation, chemotaxis, survival and transendothelial migration [103-106]. However, GM-CSF is also indispensable for DC differentiation and maturation from bone marrow progenitors or peripheral blood monocytes *in vitro* [7, 107]. It is not surprising, then, that GM-CSF should play an important role in DC activation and expansion in the lung [35, 55].

GM-CSF AND DC IN THE CONTEXT OF AAI. As mentioned earlier, freshly isolated pulmonary DC from naïve mice have intrinsically low APC activity. While the adaptive advantage of a homeostatically inert APC population at a mucosal site in constant contact with foreign—and generally harmless—antigens is clear, maintenance of this immunological homeostasis may have more to do with the immunosuppressive microenvironment conditioned by, for example, alveolar macrophages (AM) than with the DC phenotype *per se*. Indeed, pulmonary DC exhibit rapid induction of APC potential following AM depletion *in vivo* [13]. AM are known to release nitric oxide (NO), which suppresses DC maturation and therefore optimizes DC for antigen capture and processing rather than antigen presentation [13]. Interestingly, however, maturational arrest of DC by AM is subverted by GM-CSF stimulation *in vitro*, which upregulates DC expression of MHC class II and B7 [10, 12, 108, 109]. It is therefore tempting to speculate that the establishment/perpetuation of allergic disease represents the sustained depression of AM activity through non-homeostatic expression of GM-CSF, which releases DC from their immunosuppressed state and facilitates maturation.

The contribution of GM-CSF to many facets of pulmonary DC biology has been demonstrated through *in vivo* administration of GM-CSF to the mouse lung, which induces not only the activation of DC, but also their massive accumulation [55]. This rapid recruitment of DC to the lung might represent mobilization of the rich pool of circulating DC precursors [110], whose exposure to GM-CSF *in vitro* induces their ability to activate T cells [111]. Alternatively, GM-CSF may both propagate the proliferation of macrophages in the lung [112] and condition their subsequent conversion to a DC phenotype, a plasticity

that is observed when human macrophages are cultured in the presence of GM-CSF [113]. However, irrespective of the material source of DC expansion upon delivery of GM-CSF, the activation of DC under these conditions seems to license this population for the generation of an immune response. While a GM-CSF-conditioned airway microenvironment in mice enhances the stimulatory capacity of lung DC, subverts the establishment of inhalation tolerance to OVA and orchestrates the development of a cardinal Th2 response [35], substitution of GM-CSF with different proinflammatory cytokines (TNFα, IL-2, IL-4 or IL-6) does not result in the generation of a Th2-polarized, or even an antigen-specific, inflammatory response, indicating that this effect is strictly related to the overexpression of GM-CSF in the lung at the time of initial allergen exposure (M. Stämpfli, personal communication).

Although the outstanding ability of GM-CSF to activate DC has been convincingly established, the associated tendency to induce a Th2 response to OVA awaits conclusive explanation. That GM-CSF can suppress IL-12 production by DC offers one explanation [114]. Moreover, it has been shown that ex *vivo* treatment of DC (splenic or bone marrow-derived) with GM-CSF typically yields a myeloid phenotype which, as mentioned earlier, preferentially polarizes Th2 responses [115, 116]; indeed, these cells elaborate a Th2 response *in vivo* when pulsed with OVA and administered intratracheally to rodents [28, 29]. However, if properly stimulated, rodent myeloid DC can also produce IL-12 and efficiently polarize Th1 cells [79]. This finding complements studies in which co-administration of GM-CSF and IL-12 to the lung supplants the Th2 bias and triggers the development of a Th1-polarized response to aerosolized OVA [85]. Collectively, these

findings intimate that exposure to an allergen in the context of GM-CSF—and the *absence* of concurrent Th1-polarizing signals—primes DC for the induction of an allergen-specific, Th2 response.

INDUCTION OF GM-CSF IN THE ALLERGIC AIRWAY: THE ROLE OF ALLERGENS. Informed by this simplified view, it is reasonable to postulate that allergens *per se* can elicit GM-CSF production by airway structural cells. Many allergens, of which HDM is the prototypic example, are known to have protease activity; indeed, Der p1 is a cysteine protease and Der p3 ,5 and 9 are serine proteases [117]. Although proteases have traditionally being viewed as degradative enzymes involved in extracellular remodeling, a number of these proteases are now recognized to be signaling molecules that regulate a variety of biological functions including mitogenesis, coagulation and inflammation. At least three type of protease receptors have been identified: the receptor for coagulation factor X_a, urokinase and a group of G protein-coupled receptors activated by proteolysis referred to as protease-activated receptors (PARs) [118]. Four different PARs, PAR-1 to –4, have been identified to date. It is known that PAR-2 is expressed by human epithelial, endothelial and bronchial smooth muscle cells as well as by fibroblasts [119]. In addition, King *et al.* [40] have shown that Der p 1 and Der p 9 induced the production of the cytokines IL-6, IL-8 and GM-CSF by human bronchial epithelial cells *in vitro*, and Sun *et al.* [120] have presented data showing that Der p 3 and Der p 9 induce bronchial epithelial cells to produce GM-CSF via interaction with PAR-2. That protease-mediated upregulation of GM-CSF might induce allergic sensitization in some, but not all, individuals may be

related to genetic variability or to the density of PAR-2 on the airway epithelium, with susceptible patients displaying a PAR-2 profile that renders them particularly sensitive to receptor engagement. Importantly, Knight *et al.* [121] have demonstrated that PAR-2, but not PAR-1, -3 or -4, staining on bronchial epithelial cells is increased in asthmatics compared to normal, control subjects.

However, that some allergens with weak or minimal protease activity can still elicit immune-inflammatory responses suggests the existence of protease-independent pathways to induce cytokine synthesis by epithelial cells. Indeed, exogenous proteolytic signaling is not the only environmental trigger for GM-CSF production in the airway [122]. Airborne pollutants such as diesel exhaust particles or ozone have been shown to induce GM-CSF production by epithelial cells, implicating these industrial emissions in the establishment of an airway milieu conducive to allergic sensitization [123-125]. It would seem, then, that allergens' own biological properties, the quality of the air in which they are conveyed, and the genetic pedigree of the individual who inhales this mélange determine the lung's propensity to overexpress GM-CSF and, presumably, to initiate allergic phenomena.

The upregulation of GM-CSF by aeroallergens through ligation of PARs is relevant to the interpretation of our own findings in *in vivo* models of aeroallergen exposure. We have recently investigated the impact of repeated intranasal exposure of mice to ragweed (RW), an allergen with modest proteolytic activity. Our data demonstrate that exposure to RW *alone*, in contrast to OVA *alone*, elicits Th2-polarized sensitization. However, the degree of Th2 sensitization and airway inflammation elicited by exposure to RW alone is

remarkably reduced by treatment with anti-GM-CSF antibodies. Moreover, when the airway microenvironment is enriched with exogenous GM-CSF, RW exposure results in considerable airway eosinophilic inflammation and RW-specific long-range Th2 memory, likely the result of a strengthened immune response as GM-CSF substantially expands and activates lung antigen-presenting cells, particularly dendritic cells [126].

RW is an airborne allergen and RW exposure is a seasonal event. In contrast, HDM is much less airborne and exposure to HDM is ubiquitous. Importantly, HDM has considerably more protease activity than RW. We have recently shown that repeated intranasal exposure to HDM *alone*, in contrast to RW *alone*, evokes a considerable

Table 1. Antigen Characteristics

		outcome of exposure to:	
antigen	biochemical properties	antigen alone	antigen + GM-CSF
ovalbumin (OVA)	no proteolytic activity	**tolerance**; no inflammation	robust inflammation; 30-40% eosinophilia
ragweed (RW)	minimal proteolytic activity	modest inflammation; 6-8% eosinophilia	robust inflammation; 30-40% eosinophilia
house dust mite (HDM)	known proteolytic activity of Der p 1, 3, & 9	robust inflammation; 20-30% eosinophilia	robust inflammation; 30-40% eosinophilia

expansion and activation of the antigen-presenting cell compartment, robust Th2 sensitization and considerable airway eosinophilic inflammation, which are prevented by intranasal administration of neutralizing anti-GM-CSF antibodies [101].

Recent evidence suggests that aeroallergens, in addition to expanding and activating the antigen presenting cell compartment via a GM-CSF-mediated mechanism, may further contribute to the generation of allergic airway inflammation by a mechanism that involves suppression of IL-12 production. Indeed, Ghaemmaghami *et al.* [127] have recently demonstrated that Der p 1 can proteolytically cleave CD40 from the surface of DC, rendering DC less responsive to stimulation by CD40L and therefore depleting a pathway essential for IL-12 production; as a result, T cells stimulated by DC in the presence of proteolytically active Der p 1 produce more IL-4 and less IFNγ. Whether this paradigm applies to other allergens awaits further investigation.

Since DC reside in close proximity to epithelial cells, local production of GM-CSF during allergen encounter can provide a necessary stimulus for allergen uptake and the maturation of DC. While cultured macrophages, interstitial cells and epithelial cells from human lung all stain positively for GM-CSF, only epithelial cells produce significant amounts of GM-CS*F in vitro* [128]. Moreover, epithelium-conditioned media has been shown to enhance substantially the ability of pulmonary DC to induce T cell proliferation, an immunostimulatory capacity that is ablated by GM-CSF neutralization [128]. Of course, GM-CSF is not the only signal liberated by airway epithelium that can activate DC with a Th2-priming disposition; human thymic stromal lymphoprotein (TSLP), for example, is upregulated in keratinocytes from patients with atopic dermatitis and can activate DC to

polarize Th2 cells *in vitro* [129]. In the context of chronic allergic inflammation, however, a broad spectrum of cells, including differentiated Th2 cells, eosinophils and macrophages/monocytes, has been documented to secrete GM-CSF [130, 131]. For instance, pulmonary macrophages from asthmatics produce 2-fold more GM-CSF when stimulated with LPS than macrophages from non-asthmatic controls [132]. It is also germane to point out that GM-CSF-induced activation of DC in the allergic lung may reflect enhanced DC responsiveness to GM-CSF as much as it does elevated levels of secreted cytokine in the airway microenvironment. One candidate signal documented to upregulate expression of GM-CSF receptors on DC is TNF-α, which is almost ubiquitous in inflammatory conditions such as asthma [133].

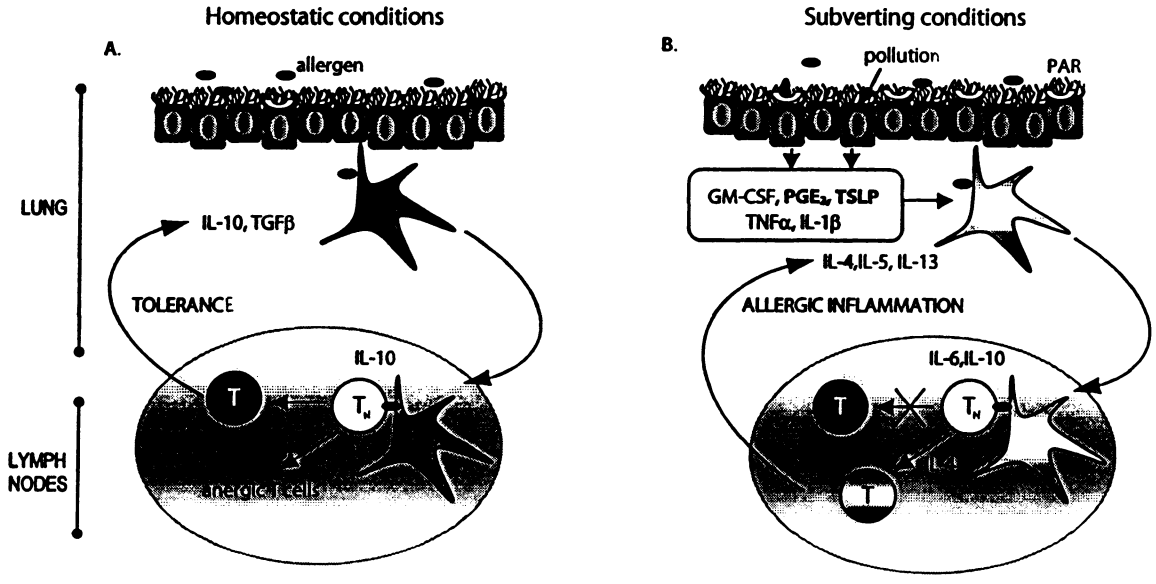

Figure 1. Role of DC in the development of Th2 allergic responses. A. Under homeostatic conditions, DC constantly endocytose apoptotic cells and inhaled proteins, including allergens. Key to the maintenance of inhalation tolerance is DC's intrinsic "homeostatic" status, which results in the generation of either anergic or regulatory T cells (T_R) that are able to control unwanted immune responses. The homeostatic status of DC in the lung is probably maintained by constitutive local production of immuno-inhibitory mediators (*e.g.* IL-10, TGFβ). B. In subjects susceptible to the development of allergy, allergens' own proteolytic activity, as well as concomitant exposure to other stimuli (*e.g.* pollution), induces epithelial cells to liberate mediators that activate DC. Activated DC migrate to the draining lymph nodes and present allergens in a manner that is permissive for Th2 generation. This Th2 bias may be dictated by the profile of cytokines (*e.g.* IL-6) or costimulatory molecules (*e.g.* OX40/OX40L, CD28/B7, ICOS/ICOSL) expressed by activated DC. T_N- naïve T cells

It seems, then, that DC in allergic inflammation are constantly exposed to elevated levels of GM-CSF. GM-CSF can therefore perpetuate allergic phenomena by (i) instructing DC to facilitate Th2-polarized allergic sensitization; and (ii) enhancing the survival and function of multiple inflammatory cells in the lung.

TREATMENT OF ASTHMA: AN IMMUNOLOGICAL PERSPECTIVE

CURRENT BENCHMARK TREATMENT FOR ASTHMA. Inhaled corticosteroids are currently the most effective therapy for allergic diseases [134]. There is no doubt, based upon their therapeutic index, that inhaled corticosteroids are excellent *symptom-controlling medicines*. The precise mechanisms by which corticosteroids are so effective in allergic airway inflammation remain to be elucidated. It is possible, however, that their efficacy resides precisely in the breadth of their documented effects; that is, their pervasive anti-inflammatory prowess overcomes immunological redundancy. With specific regard to DC, it has been shown that (inhaled) corticosteroids can suppress antigen uptake and presentation [135] as well as reduce the number of $CD1^{+}HLA\text{-}DR^{+}$ in the airways of asthmatics [136]. While the mechanism underlying this latter effect is not known, there is evidence that corticosteroids can both induce apoptosis of DC [137] and impair the differentiation of blood precursors to DC [137, 138]. It is also likely that the impact of corticosteroids on DC or DC function in part relates to the suppression of epithelial cell production of cytokines and/or chemokines that modulate DC [6, 139-141]. The long-term implications of the suppressive impact of corticosteroids on DC are, however, questionable, as it is also evident that corticosteroids are ineffective *curative medicines*,

most likely due to their inability to alter the underlying immunological abnormalities of the allergic diathesis.

NOVEL INTERVENTIONS DIRECTED AT TH2 RESPONSES. In contrast to the "shot gun" approach that corticosteroids typify, a number of proposed experimental interventions are directed at inhibiting specific, key effector molecules in allergic inflammation, including IL-4, IL-5, IL-13 and IgE (reviewed in [142, 143]). Some of these molecules are linked to DC activity, such as IgE. FcεRI-mediated uptake of antigen by DC can enhance the activation of antigen-specific T cells 100 to 1000-fold compared to regular antigen uptake [144, 145]. Thus, not surprisingly, anti-IgE antibodies for the treatment of allergy not only inhibit mast cell/basophil degranulation but also prevent T cell activation. Alternatively, chemokines/chemokine receptors involved in Th2 responses can be targeted (reviewed in [146]). DC are potent producers of chemokines under chronic inflammatory conditions. Matured DC have the capacity to produce, for example, MDC, TARC and MCP-4, which preferentially recruit Th2 cells expressing complementary receptors (CCR3, CCR4, CCR8) [147, 148]. The final verdict with respect to the efficacy of anti-cytokine/chemokine/IgE therapy has not yet been reached. However, whether strategies aimed at neutralizing a single downstream molecule—such as those mentioned above—will ever be superior from a clinical standpoint to inhaled corticosteroids is, at best, uncertain, due in part to the multitude of overlapping, often redundant, immune pathways at play.

Other strategies that are "cell-specific" as opposed to "molecule-specific" have been proposed. These strategies focus on targeting Th2 cells, the subset responsible for

the generation of Th2-affiliated cytokines, chemokines and, indirectly, immunoglobulins. One such proposal has been to "switch" the allergic immune response from Th2 to Th1 by using Th1-directing agents such as IL-12, IL-18, IFN-γ or CpG motifs [149-153]. There are several issues that should be considered when appraising this approach. The consensus is that Th phenotypes are, at the cellular level, irreversible; hence, rather than replacing a Th2 response, these types of interventions likely dilute the existing Th2 response by introducing a competing Th1 response. From this perspective, the concept of replacing one type of immune-inflammatory response with another, which itself is unlikely to be benign, is not particularly appealing [154-156]. In addition, it is possible that Th1 cells favour the development of autoimmune disease, as the application of CpG-ODN has demonstrated in animal models [157]. Finally, serious consideration should be given to reports documenting significant toxicity for some of the Th1-polarizing agents that have been examined, notably IL-12 [158], or that describe their paradoxical ability to exacerbate Th2 responses, as in the case of IL-18 [159, 160].

A more conceivable, therapeutically viable approach might hinge on the generation of T_r that have the ability to inhibit Th2 responses without causing inflammation, as evidenced in animal models of allergic airway inflammation [161, 162]. T_r cells can be produced *in vitro* in the presence of IL-10 [161], immunosuppressive drugs and most importantly allergens, leading to generation of allergen-specific suppressor cells [163]. Alternatively, T_r cells can be induced by targeting antigen (allergens) to immature DC , for instance by coupling antigens to anti-DEC205 monoclonal antibody [164]. Uptake of antigen delivered in this context does not lead to the maturation of DC and the concomitant

generation of antigen-specific effector cells, but instead promotes the establishment of T_r cells. Though promising in principle, the manipulation of T_r cells could deleteriously suppress T cell responses needed for efficient clearance of viral/bacterial infections.

TARGETING INITIATION OF THE IMMUNE RESPONSE. Since the outcome of an immune response critically depends on the instructions APC deliver to naïve T cells, targeting of APC, particularly DC, is likely to impact the generation of allergic responses. For example, there is evidence that manipulating the conditions in which DC are grown and pulsed with antigen *in vitro* can elaborate DC with tolerizing, rather that priming, ability *in vivo*. In addition, generation of DC in the presence of corticosteroids, vitamin D3 and suboptimal doses of GM-CSF leads to the emergence of "tolerogenic" DC [147, 165, 166], and DC genetically engineered to express constitutively viral IL-10, TGFβ, FAS-L or CTLA4-Ig can induce antigen-specific T cell hyporesponsiveness [167, 168]. While these studies illustrate that DC are highly plastic and readily manipulated *in vitro*, the clinical translation of this versatility appears remote. Indeed, the strict requirement for MHC haplotype identity implies that therapy would have to be implemented on an individual basis.

A more clinically plausible approach would involve modifying DC function *in vivo*. To this end, IL-10 represents a potentially interesting therapeutic agent due to its ability to downmodulate APC function. The fact that corticosteroids can upregulate IL-10 production, concomitantly resulting in DC dysfunction, could at least partially account for the improvement of clinical symptoms in asthmatics [169]. Moreover, the inhibition of Th2–polarized inflammation in mice sensitized to OVA in the context of IL-10 was associated

with reduced numbers of DC in the airway, possibly due to dysregulation of the chemokine environment [86].

A more selective approach to modify DC function might involve interfering with co-stimulatory pathways that deliver crucial second signals during the interaction between APC and T cells. A number of interventions designed to block a variety of co-stimulatory pathways have been considered for the treatment of many diseases including allergic inflammation. Not surprisingly, the inhibition of the major CD28/B7 pathway with CTLA4-Ig, which binds with high affinity to B7.1 and B7.2 on DC, inhibited airway inflammation in a murine model of allergic asthma [68, 170]. The use of antibodies to block either B7.2 or B7.1 selectively has likewise been shown to downregulate airway eosinophilia [171-173]. In addition, inhibition of two novel pathways, ICOS/B7RP-1 and OX40/OX-40L, leads to attenuation of Th2-driven mucosal inflammation in mice [71, 174]. Even interference with pathways not directly implicated in Th2 development, such CD40/CD40L, has resulted in diminished eosinophilic accumulation in the lung and attenuated IL-4-driven IgE production [175, 176]. Based on these promising results in murine models, it seems reasonable to explore such interventions in the clinical setting.

The maturation and activation of DC are the most upstream critical events in the generation of an allergic immune-inflammatory response and, in this regard, GM-CSF plays a pre-eminent role. Blockade of GM-CSF with antibodies prevented airway hyperresponsiveness in a model of diesel particle exposure [177, 178]. In addition, as we described earlier in this review, administration of anti-GM-CSF during sensitization to either RW or HDM prevents the development of Th2 responses (our unpublished observations).

However, GM-CSF is, as pointed out earlier, also essential for the maintenance of lung homeostasis; thus, in a broader immunological context, it is likely that impairing the maturation/activation of dendritic cells would have a detrimental impact on host defense against bacteria, viruses and parasites.

PRESENT CHALLENGES AND FUTURE SOLUTIONS. The approaches presented above, which aim to neutralize specific Th2 effector molecules, to introduce Th2-deviating signals, or to condition antigen presentation by dendritic cells during the generation of a primary immune response, are interventions that impact either an existing or an as-yet-to-exist Th2 response. Viewing these interventions from such a perspective provides a strategic sense of their strengths and limitations. Indeed, approaches designed to alter an existing Th2 response face a humbling comparison with inhaled steroids. On the other hand, approaches seeking to prevent the development of an as-yet-to-exist Th2 response must pass the test of clinical relevance; by definition, "patients" already have existing disease. The conundrum is that whereas efforts directed at producing newer, albeit not necessarily better, *symptom-controlling* therapies may not be warranted, efforts directed at fundamentally altering the immunological dysfunction underlying the allergic diathesis may ultimately prove futile.

The greatest therapeutic challenge in allergic disease is, in our view, not whether symptoms can be better controlled but whether the disease can be cured. However, we believe that several unanswered questions preclude sound assessment of the plausibility of this latter objective. Is allergen sensitization a remote process—a process that occurred

sometime in childhood, that is immunologically hermetic and, therefore, therapeutically intractable? Alternatively, is allergen sensitization an ongoing process whose persistence needs to be periodically maintained? The therapeutic implications, in our view, are evident. If allergic sensitization is an ongoing process, modulation of present and future, although not past, sensitization becomes a long-term but conceivable therapeutic goal. We suggest that intimations into these questions require insight into how the allergic phenotype might be maintained.

The generation of *immunological memory*, an essential component of adaptive immune responses, entails the development of specialized memory T and B cells, and long-lived effector B cells. With regard to the T cell compartment, memory is supported by both $CD4^+$ and $CD8^+$ T cells. At this time, CD8-memory responses, which establish an important protective mechanism against viruses and bacteria, are better understood than CD4-memory responses [179]. It is nevertheless clear that there are important differences between CD8 and CD4 memory. For example, in contrast to $CD8^+$ T cell memory, virus-specific $CD4^+$ T cell memory *decreases* over time [180]. Conceivably, $CD4^+$ Th2 memory cells play an important role in the inflammatory response allergic asthmatics develop when re-exposed to allergen, yet very little is known with respect to the persistence and maintenance of allergen-specific CD4-mediated memory responses [181]. Importantly, extrapolation of knowledge about memory responses against replicating entities, such as viruses, to responses against non-replicating, and often rather innocuous, entities such as allergens must be entertained with great prudence.

Our current understanding is that, in sensitized individuals, memory T cells become effector T cells upon antigen re-exposure; how, then, is the pool of memory T cells maintained? Sallusto and Lanzavecchia [182, 183] have proposed a model, based on evidence largely generated from *in vitro* studies with human peripheral blood mononuclear cells, that distinguishes between two distinct types of memory T cells: *effector memory T cells* (TEM) and *central memory T cells* (TCM). While TEM can rapidly execute effector activities upon encounter with antigen in the periphery, *e.g.* the airway, TCM might be understood as a central reservoir of future waves of TEM cells and, ultimately, of Th2 effector cells. While insightful and provocative, this theory ultimately equivocates on the initial question. That is, if TEM cells become Th2 effector cells, and the TEM pool is replenished by the TCM pool, *how is the pool of central memory T cells maintained?*

Speculatively, if allergen-specific CD4-memory decays over time as a consequence of the sequential exhaustion of memory pools upon repeated allergen re-exposure, then maintenance of memory requires the replenishment of these pools. This implies that allergen re-exposure of a sensitized individual not only triggers an inflammatory response, but also presumably initiates a neo-sensitization event. This hypothesis compels us to consider a combined therapeutic strategy capable, on the one hand, of suppressing inflammation and, on the other, of facilitating "homeostatic sensitization". Hypothetically, deliberate, recurrent *de novo* "homeostatic sensitization" would eventually overcome the alleged decay of established Th2 sensitization. A novel therapeutic approach would therefore be predicated on the delivery of a specific allergen in conjunction with a molecule that either prevents the generation of a pro-inflammatory

immune response or directly elicits an immunoregulatory response, likely through the generation of allergen-specific suppressor/tolerogenic T cells.

SUMMARY

The maintenance of immunologic homeostasis and the development of Th2-associated allergic responses in the lung are regulated by DC. The involvement of DC in allergic inflammatory responses is unequivocal and is supported by at least two pieces of evidence: (i) an increased number of DC in the lungs of asthmatic patients compared to healthy controls [25, 184, 185]; and (ii) diminished Th2 allergic responses in mice selectively depleted of DC [186]. The precise mechanisms by which DC induce tolerance *vs.* immunity are unclear, but the status of DC, characterized by the expression of a specific pattern of cytokines and costimulatory molecules, probably plays a decisive role. In the lungs, DC are regulated by manifold signals in the microenvironment, specifically antigens and factors released by resident tissue cells. Therefore, a propensity to develop allergy may stem from the subversion of mechanisms that otherwise prevent the activation of DC in the lung. In this review, we presented evidence for the permissive role of GM-CSF in allergic sensitization. GM-CSF, which is released by epithelial cells in response to allergens, establishes conditions conducive to the initiation and potentiation of allergic inflammation, in part through the modulation of DC phenotype. This intrinsic plasticity of DC in the lung may be marshaled for therapeutic strategies that aim to suppress, and even reverse, allergic responses.

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APPENDIX V

THE B7 FAMILY MEMBER B7-H3 PREFERENTIALLY DOWN-REGULATES T HELPER TYPE 1-MEDIATED IMMUNE RESPONSES

Suh W-K, **Gajewska BU**, Okada H, Bertram EM, Gronski MA, Dawicki W, Plyte S, Duncan G, Wakeham A, Itie A, Chung S, Da Costa α, Arya S, Horan T, Campbell P, Gaida K, Ohashi PS, Watts TH, Yoshinga SK, Bray A, Jordana M., Mak TW.

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The attached manuscript demonstrates the importance of a newly described costimulatory molecule B7-H3 (B7RP-2) in the regulation of immune responses. Part of the data presented in this manuscript was generated in our laboratory by me. Specifically, I used B7-H3 knockout mice in our Th2 and Th1 models in order to dissect the role of this molecule in two diverse inflammatory responses. Both models involve OVA exposure in the context of either Ad/GM-CSF (Th2 model) or a combination of Ad/GM-CSF and Ad/IL-12 (Th1 model). The data indicate: (1) redundancy of B7-H3 in the generation of Th2 responses, since none of the examined parameters were altered in B7-H3KO mice; (2) the involvement of B7-H3 in the regulation of lung Th1 responses based upon the observation of the increased accumulation of activated lymphocytes and elevated production of IFN-γ in B7-H3KO mice. The exact mechanism that accounts for the preferential regulation of Th1-mediated immune responses in these mice awaits further investigation.

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The B7 family member B7-H3 preferentially down-regulates T helper type 1–mediated immune responses

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We investigated the *in vivo* function of the B7 family member B7-H3 (also known as B7RP-2) by gene targeting. B7-H3 inhibited T cell proliferation mediated by antibody to T cell receptor or allogeneic antigen-presenting cells. B7-H3-deficient mice developed more severe airway inflammation than did wild-type mice in conditions in which T helper cells differentiated toward type 1 (T_H1) rather than type 2 (T_H2). B7-H3 expression was consistently enhanced by interferon-γ but suppressed by interleukin 4 in dendritic cells. B7-H3-deficient mice developed experimental autoimmune encephalomyelitis several days earlier than their wild-type littermates, and accumulated higher concentrations of autoantibodies to DNA. Thus, B7-H3 is a negative regulator that preferentially affects T_H1 responses.

An optimal T cell response is achieved by a combination of signals delivered through the antigen-specific T cell receptor (TCR) and accessory signals that are either stimulatory or inhibitory[1,2]. Members of the B7 family proteins are pivotal in the regulation of T cell responses[1,3–6]. The prototypical B7 family members B7-1 (CD80) and B7-2 (CD86) are induced or up-regulated on stimulated antigen-presenting cells (APCs) and bind to the T cell costimulatory receptor CD28. CD28 engagement augments TCR-mediated signaling and promotes T cell survival, leading to clonal expansion and differentiation of effector T cells[1,7]. Chronic antigenic stimulation results in the polarization of T helper cell subsets, namely T_H1 and T_H2 (ref. 8). T_H1 cells express interferon-γ (IFN-γ) as their effector cytokine, whereas T_H2 cells express interleukin 4 (IL-4), IL-5 and IL-10. However, B7-1 and B7-2 also bind to an inhibitory receptor called cytotoxic T lymphocyte antigen 4 (CTLA-4) that is induced on T cells after activation. Ligation of CTLA-4 leads to down-regulation of T cell proliferation medicated by TCR-CD28 (ref. 9). CTLA-4 thus provides a key inhibitory mechanism for B7-1- and B7-2-mediated T cell proliferation, as demonstrated by the fatal lymphoproliferative disorders noted in CTLA-4-deficient mice[10–12].

Recent studies have focused on the B7 family proteins PD-L1, PD-L2, ICOSL and B7-H3 (refs. 3–6). These proteins, which are structurally related to B7-1 and B7-2, are type I transmembrane proteins with two or four extracellular immunoglobulin (Ig) domains and 20–30% amino acid sequence identity. Unlike B7-1 and B7-2, these B7 molecules are expressed in multiple organs of nonimmune function as well as in APCs, indicating potential immunomodulatory functions at sites of inflammation. Initial *in vitro* experiments indicated that PD-L1 (ref 13; also known as B7-H1; ref. 14) and PD-L2 (ref. 15; also known as B7-DC[16]) have costimulatory functions in T cell proliferation and cytokine production[14,16]. However, more recent reports have contradicted those findings: PD-L1 and PD-L2 bind to a common inhibitory receptor, PD-1, and inhibit T cell proliferation and cytokine production[13,15,17,18]. The idea that PD-1 has an immune inhibitory function is supported by the phenotype of PD-1-deficient mice, which develop lupus-like diseases or autoimmune dilated cardiomyopathy[19,20]. Another B7 family member, the inducible costimulator ligand ICOSL (also known as B7RP-1 (ref. 21), B7h[22], B7-H2 (ref. 23), GL50 (ref. 24) and LICOS[25]), is constitutively expressed in APCs but can be induced in nonlymphoid tissues by tumor necrosis factor-α or lipopolysaccharide (LPS)[21,22,24]. ICOSL binds to the inducible costimulator (ICOS) that is expressed on activated T cells[21–25]. Interaction of ICOS with ICOSL promotes the delivery of T cell help to B cells and enhances cytokine production

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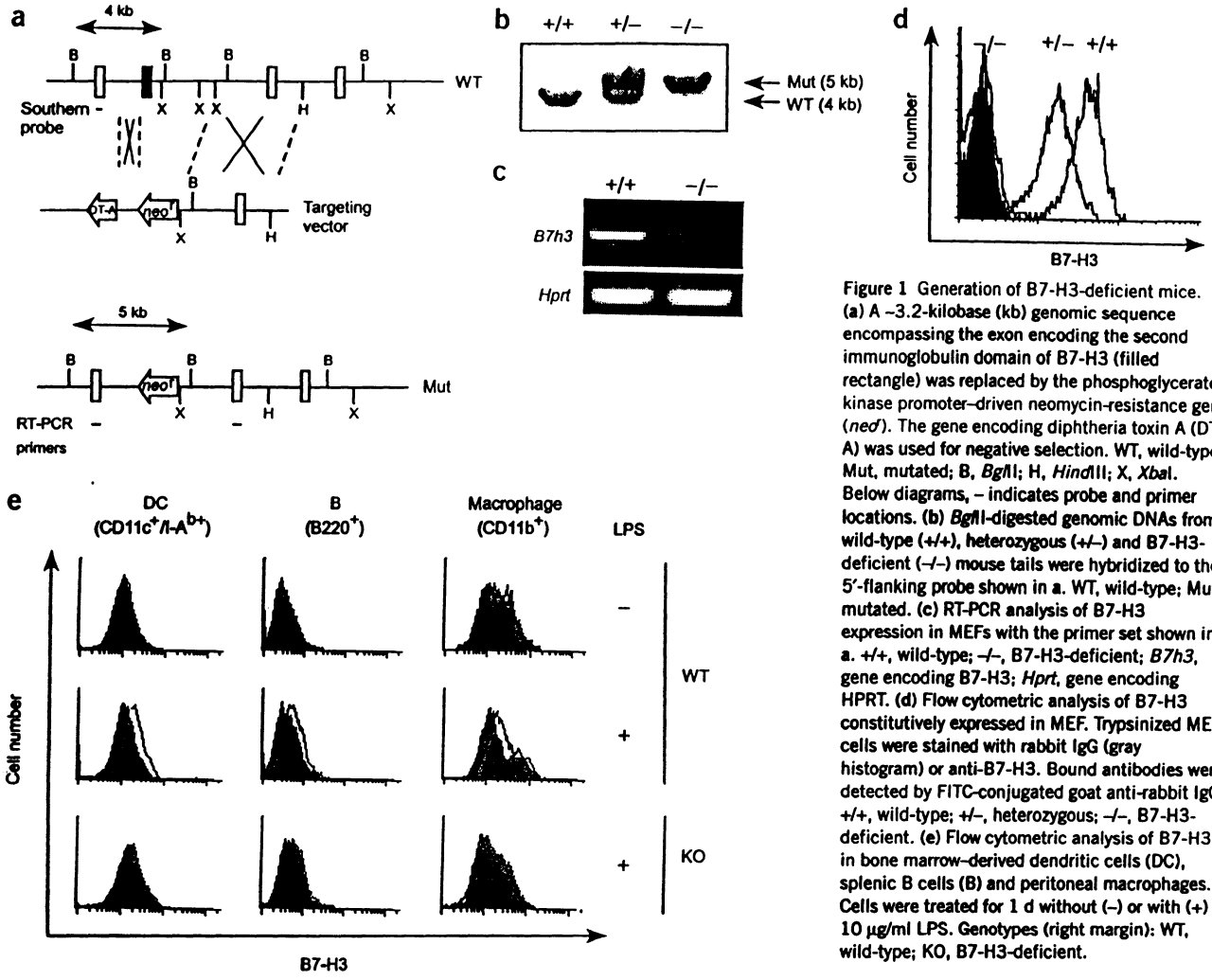

Figure 1 Generation of B7-H3-deficient mice. (a) A ~3.2-kilobase (kb) genomic sequence encompassing the exon encoding the second immunoglobulin domain of B7-H3 (filled rectangle) was replaced by the phosphoglycerate kinase promoter–driven neomycin-resistance gene (*neo*r). The gene encoding diphtheria toxin A (DT-A) was used for negative selection. WT, wild-type; Mut, mutated; B, *Bgl*II; H, *Hind*III; X, *Xba*I. Below diagrams, – indicates probe and primer locations. (b) *Bgl*II-digested genomic DNAs from wild-type (+/+), heterozygous (+/–) and B7-H3-deficient (–/–) mouse tails were hybridized to the 5′-flanking probe shown in a. WT, wild-type; Mut, mutated. (c) RT-PCR analysis of B7-H3 expression in MEFs with the primer set shown in a. +/+, wild-type; –/–, B7-H3-deficient; *B7h3*, gene encoding B7-H3; *Hprt*, gene encoding HPRT. (d) Flow cytometric analysis of B7-H3 constitutively expressed in MEF. Trypsinized MEF cells were stained with rabbit IgG (gray histogram) or anti-B7-H3. Bound antibodies were detected by FITC-conjugated goat anti-rabbit IgG. +/+, wild-type; +/–, heterozygous; –/–, B7-H3-deficient. (e) Flow cytometric analysis of B7-H3 in bone marrow–derived dendritic cells (DC), splenic B cells (B) and peritoneal macrophages. Cells were treated for 1 d without (–) or with (+) 10 μg/ml LPS. Genotypes (right margin): WT, wild-type; KO, B7-H3-deficient.

by T_H2 cells[26,27]. Consistent with these findings, ICOS-deficient mice show impaired antibody class switching and increased susceptibility to the development of experimental autoimmune encephalomyelitis (EAE)[28–30]. Another negative regulatory receptor, B and T lymphocyte attenuator (BTLA), is induced in activated T cells and down-regulates immune responses by engaging the B7 family member B7x[31], also known as B7-H4 (ref. 32) and B7S1 (ref. 33).

B7-H3 has been identified in both humans and mice[34,35]. Human and mouse B7-H3 share ~88% amino acid sequence identity[35]. B7-H3 is expressed in multiple organs both in human and mouse[34,35] and in several human tumor cell lines[34]. Human B7-H3 is induced in dendritic cells and monocytes by inflammatory cytokines[34]. B7-H3 binds to an unknown receptor expressed on activated CD4 and CD8 T cells[34,35]. This receptor is distinct from CD28, CTLA-4, PD-1 and ICOS[34], the receptors known to bind to other B7 family proteins. Human B7-H3 augments TCR-mediated T cell proliferation, IFN-γ production and generation of cytotoxic T lymphocytes (CTLs) *in vitro*[34], indicating that B7-H3 may have positive regulatory functions in CTL responses.

In this study, however, we show that mouse B7-H3 has a negative regulatory function in T cell–mediated immune responses both *in vitro* and *in vivo*. B7-H3 inhibited T cell proliferation and cytokine production mediated by antibody to CD3 (anti-CD3) *in vitro*. Furthermore, APCs derived from B7-H3-deficient mice had an increased T cell stimulatory capacity in a mixed leukocyte reaction (MLR) compared with that of APCs of wild-type mice. T cell responses developing in conditions favoring T_H1 differentiation were enhanced in B7-H3-deficient mice compared with that of wild-type mice, whereas T_H2 and CTL responses were normal. As the expression of B7-H3 in dendritic cells is promoted by the T_H1 cytokine IFN-γ but suppressed by the T_H2 cytokine IL-4, we propose that B7-H3 is involved in the selective feedback inhibition of T_H1-mediated immune responses.

RESULTS

Generation of B7-H3-deficient mice

To determine the *in vivo* function of B7-H3, we generated B7-H3-deficient mice. We targeted the exon encoding the second immunoglobulin domain of B7-H3 using a targeting vector containing a neomycin-resistance cassette in antisense orientation relative to B7-H3 transcription (Fig. 1a). We confirmed disruption of the gene encoding B7-H3 by Southern blot analysis of F2 progeny (Fig. 1b). RT-PCR (Fig. 1c) and flow cytometric analysis (Fig. 1d) of mouse embryonic fibroblasts (MEFs) derived from wild-type, heterozygous and B7-H3-deficient

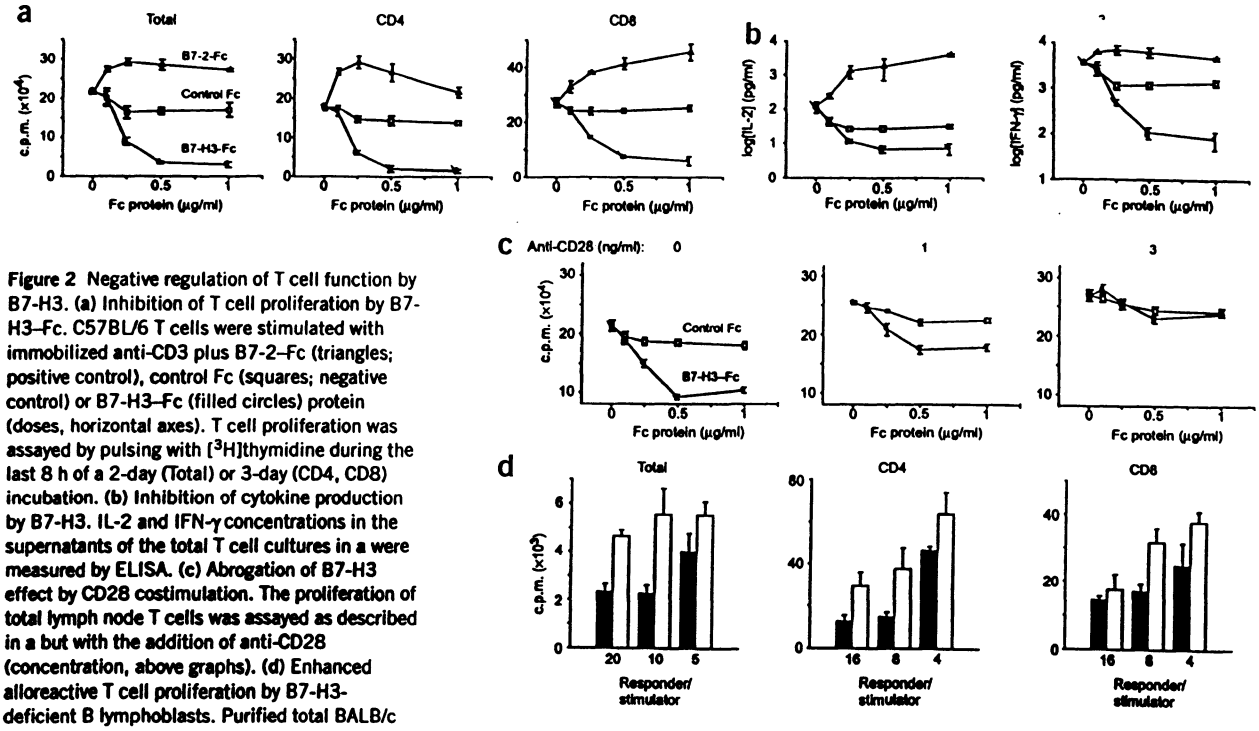

Figure 2 Negative regulation of T cell function by B7-H3. (a) Inhibition of T cell proliferation by B7-H3–Fc. C57BL/6 T cells were stimulated with immobilized anti-CD3 plus B7-2–Fc (triangles; positive control), control Fc (squares; negative control) or B7-H3–Fc (filled circles) protein (doses, horizontal axes). T cell proliferation was assayed by pulsing with [^{3}H]thymidine during the last 8 h of a 2-day (Total) or 3-day (CD4, CD8) incubation. (b) Inhibition of cytokine production by B7-H3. IL-2 and IFN-γ concentrations in the supernatants of the total T cell cultures in a were measured by ELISA. (c) Abrogation of B7-H3 effect by CD28 costimulation. The proliferation of total lymph node T cells was assayed as described in a but with the addition of anti-CD28 (concentration, above graphs). (d) Enhanced alloreactive T cell proliferation by B7-H3-deficient B lymphoblasts. Purified total BALB/c lymph node T cells were stimulated with irradiated B lymphoblasts prepared from wild-type (filled symbols) or B7-H3-deficient (open symbols) mice. T cell proliferation was assayed by pulsing cells with [^{3}H]thymidine during the last 8 h of a 3-day incubation (Total) or during the last 16 h of 4.5-day incubation (CD4, CD8). All data represent mean ± s.e.m. of triplicate samples and are representative of two to three independent experiments.

embryos confirmed that the targeted allele encoding B7-H3 had a null mutation. B7-H3-deficient mice were born at the expected mendelian frequency, and were of normal size, maturation and fertility. T, B and natural killer cell populations in the bone marrow, thymus, lymph node, spleen and peripheral blood were normal in B7-H3-deficient mice (data not shown). We found low expression of B7-H3 on the surfaces of wild-type but not B7-H3-deficient APCs after stimulating cells with LPS (Fig. 1e) and other stimuli (anti-IgM and anti-CD40 for B cells, and anti-CD40 for macrophages; data not shown). Although cell surface B7-H3 was present as early as 6 h after stimulation and was maintained until day 3 after stimulation, there were no appreciable changes in B7-H3 levels over time.

B7-H3 inhibits T cell proliferation

To investigate the function of B7-H3 in T cell proliferation, we examined the effect of immobilized B7-H3–Fc fusion protein on T cell proliferation induced by treatment with anti-CD3. Contrary to the results of a human T cell proliferation assay[34], we found that B7-H3 inhibited proliferation of both CD4 and CD8 T cells in a dose-dependent way (Fig. 2a). Production of IL-2 and IFN-γ was reduced accordingly (Fig. 2b). In contrast, activation with B7-2–Fc fusion protein (a positive control) increased anti-CD3-mediated T cell proliferation and cytokine production (Fig. 2a and Fig. 2b). The inhibitory effect of B7-H3–Fc on T cell proliferation was readily overcome by CD28-mediated costimulation (Fig. 2c). These results demonstrate that B7-H3 is a negative regulator of T cell proliferation and cytokine production, and indicate that B7-H3 may have a greater effect in conditions in which CD28-mediated costimulation is limited.

To assess the consequences of B7-H3 deficiency on the ability of APCs to stimulate T cell proliferation, we used MLR experiments in which B lymphoblasts derived from wild-type or B7-H3-deficient mice (H-$2^{b/b}$) were the stimulators and T cells from BALB/c mice (H-$2^{d/d}$) were the responders. Wild-type and B7-H3-deficient B lymphoblasts expressed similar amounts of major histocompatibility complex (MHC) class I and II, and B7-1 and B7-2 after LPS stimulation (data not shown). However, B7-H3-deficient B lymphoblasts showed a capacity to stimulate alloreactive T cells (both CD4 and CD8 populations) that was about twofold greater than that of wild-type B lymphoblasts (Fig. 2d). We found parallel, but smaller, differences when we used LPS-stimulated dendritic cells as stimulators (data not shown). Thus, the presence of B7-H3 on APCs negatively regulates T cell proliferation induced by T cell–APC interaction.

Enhanced T_H1-mediated hypersensitivity

To test whether the absence of B7-H3 *in vivo* increases T cell–mediated hypersensitivity reactions, we used airway inflammation models driven by either T_H1 or T_H2 cells. We achieved respiratory mucosal sensitization by establishing T_H1- or T_H2-polarizing airway microenvironments through the transient, adenovirus-mediated local expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) plus IL-12 (T_H1), or GM-CSF alone (T_H2)[36–38]. In these conditions, repeated exposure to aerosolized ovalbumin (OVA) leads to either T_H1- or T_H2-polarized airway inflammation. We analyzed the contents of the bronchoalveolar lavage (BAL) fluid of wild-type and B7-H3-deficient mice subjected to this treatment. In initial experiments with mice of mixed genetic background (C57BL/6 × 129/Ola), OVA sensitization in T_H1 conditions resulted in 2.7-fold more total immune-inflammatory cells in the BAL of B7-H3-deficient mice than in the BAL of wild-type mice (Fig. 3a). Numbers of infiltrating lymphocytes (Fig. 3a), macrophages (Fig. 3a) and neutrophils (data not shown) were increased to a similar degree. Histological examination of lung sections confirmed the increased

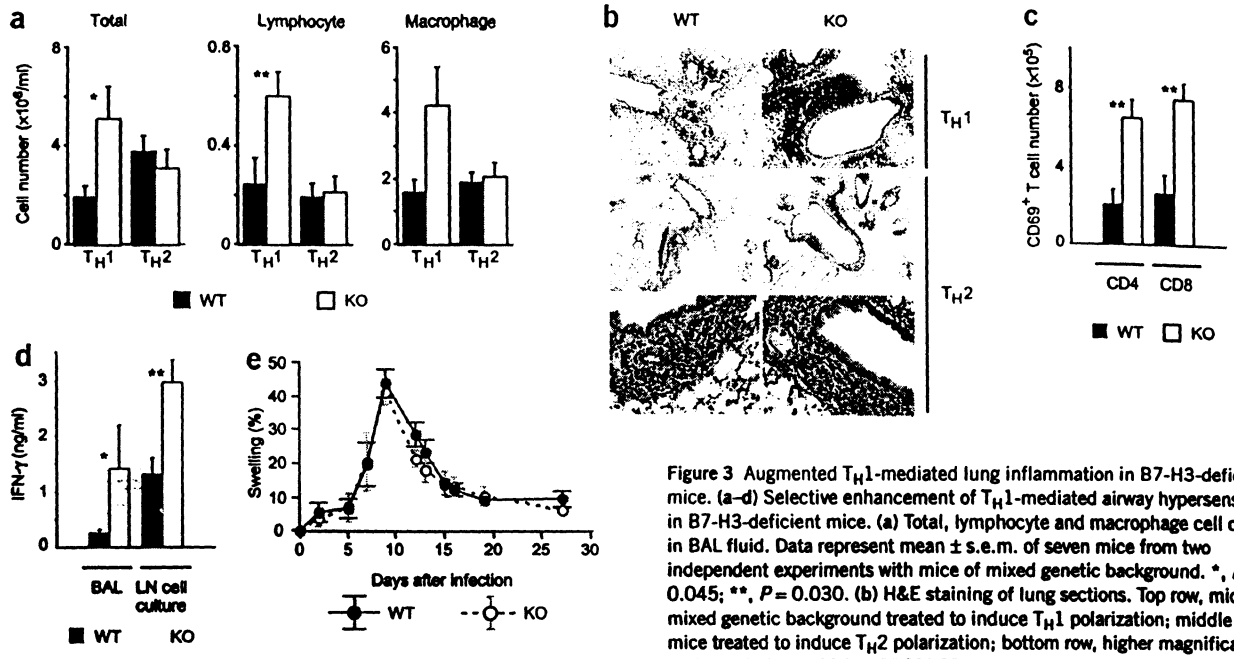

Figure 3 Augmented T_H1-mediated lung inflammation in B7-H3-deficient mice. (a–d) Selective enhancement of T_H1-mediated airway hypersensitivity in B7-H3-deficient mice. (a) Total, lymphocyte and macrophage cell counts in BAL fluid. Data represent mean ± s.e.m. of seven mice from two independent experiments with mice of mixed genetic background. *, $P = 0.045$; **, $P = 0.030$. (b) H&E staining of lung sections. Top row, mice of mixed genetic background treated to induce T_H1 polarization; middle row, mice treated to induce T_H2 polarization; bottom row, higher magnification of middle row. Original magnifications, ×50 (top and middle); ×200 (bottom). (c) Numbers of $CD69^+$ CD4 or $CD69^+$ CD8 T cells in mononuclear cell populations isolated from lungs of mice sensitized in T_H1 conditions. Data represent mean ± s.e.m of five wild-type and four B7-H3-deficient mice of the BALB/c background. **, $P < 0.01$. (d) IFN-γ concentration in the BAL or lymph node cell culture from mice sensitized in T_H1 conditions. Data represent mean ± s.e.m of five wild-type and four B7-H3-deficient mice of the BALB/c background. *, $P = 0.063$; **, $P = 0.016$. (e) Normal LCMV-mediated footpad swelling in B7-H3-deficient mice. LCMV (Armstrong strain; 3,000 PFU) was injected into the hind footpads of wild-type and B7-H3-deficient mice, and footpad thickness was measured with a caliper (time points, horizontal axis). Data represent mean percentage (%) swelling ± s.e.m. of four mice per group. WT, wild-type; KO, B7-H3-deficient.

severity of the airway inflammation in the absence of B7-H3 (Fig. 3b). We confirmed these data using mice that had been backcrossed six generations onto the BALB/c background. Flow cytometric analysis of isolated lung mononuclear cells showed B7-H3-deficient mice had about threefold more CD4 and CD8 T cells than did wild-type mice (wild-type versus B7-H3-deficient (mean ± s.e.m.): $4.2 \pm 1.7 \times 10^5$ cells versus $13.5 \pm 1.4 \times 10^5$ cells for CD4; $4.2 \pm 1.6 \times 10^5$ cells versus $15.7 \pm 2.8 \times 10^5$ cells for CD8). The number of activated ($CD69^+$) CD4 and CD8 T cells in the lung was also proportionally higher in B7-H3-deficient mice than in control mice (Fig. 3c). With respect to cytokine production, BAL samples from the B7-H3-deficient mice contained a concentration of IFN-γ approximately sixfold higher than that of control mice (Fig. 3d). Furthermore, draining pulmonary lymph node cells from B7-H3-deficient mice produced about three times more IFN-γ when restimulated *in vitro* with OVA, indicating the presence of enhanced T_H1 effector activity (Fig. 3d). In T_H2 conditions, however, we found similar numbers of inflammatory cells in the BAL and the lung sections of wild-type and B7-H3-deficient mice (Fig. 3a,b). Eosinophilia, a hallmark of T_H2-polarized airway inflammation, was also similar in these mice (Fig. 3b, bottom). OVA-stimulated wild-type and B7-H3-deficient splenocytes produced similar amounts of IL-4, IL-5 and IL-13 (data not shown). Thus, in T_H1- but not T_H2-polarizing conditions, B7-H3-deficient mice developed more severe airway inflammation than control mice and showed augmented T cell infiltration. To examine the function of B7-H3 in a

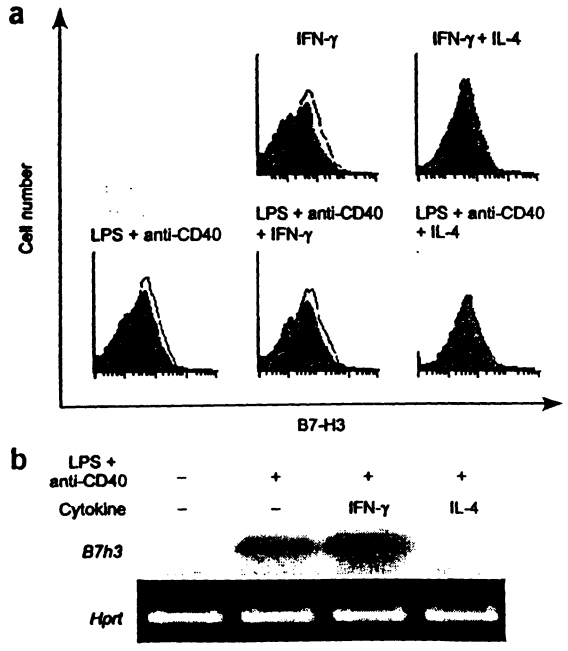

Figure 4 Regulation of B7-H3 expression by IFN-γ and IL-4 in dendritic cells. (a) Immature bone marrow–derived dendritic cells (C57BL/6) were incubated for 2 d in the presence of stimuli and/or cytokines (above histograms). Surface B7-H3 expression on dendritic cells ($CD11c^+I\text{-}A^{b+}$) was assessed by flow cytometry. Gray histograms represent control rabbit IgG staining. Data are representative of two independent experiments. (b) Dendritic cells (C57BL/6) were stimulated with LPS plus anti-CD40 for 1 d with stimuli and/or cytokines (above photos). B7-H3 mRNA (*B7h3*) was analyzed by RT-PCR using HPRT (*Hprt*) as a control. Data are representative of three independent experiments.

CTL-mediated hypersensitivity reaction, we injected lymphocytic choriomeningitis virus (LCMV) into the footpads of wild-type and B7-H3-deficient mice[39]. The extent and kinetics of footpad swelling were indistinguishable between these mice (Fig. 3e). These results indicate that B7-H3 is involved in the down-regulation of T_H1- but not T_H2- or CTL-mediated hypersensitivity reactions.

Regulation of B7-H3 expression in dendritic cells

To elucidate the basis of T_H1 selectivity of B7-H3 deficiency, we tested whether B7-H3 expression could be regulated by the key T_H1 or T_H2 cytokine (IFN-γ or IL-4, respectively). As shown for human dendritic cells[34], IFN-γ induced surface expression of B7-H3 on bone marrow–derived dendritic cells (Fig. 4a). IL-4 was able to abrogate IFN-γ-mediated B7-H3 induction (Fig. 4a). Cell surface B7-H3 was also induced on dendritic cells that were stimulated with LPS plus anti-CD40 (Fig 4a). Surface B7-H3 expression in these conditions was unaltered by IFN-γ but abrogated by IL-4. RT-PCR analysis showed that B7-H3 mRNA was enhanced by IFN-γ by about fourfold, but suppressed by IL-4 in dendritic cells stimulated with LPS plus anti-CD40 (Fig. 4b). The enhancement of mRNA by IFN-γ did not seem to be reflected by the amount of cell surface B7-H3. This may indicate additional mechanisms regulate cell surface B7-H3. These findings indicate that B7-H3 may provide a negative feedback mechanism during the amplification phase of T_H1 responses. The overall progression of T_H2 responses may not be affected by B7-H3 deficiency, as developing T_H2 cells produce IL-4, which suppresses expression of B7-H3.

EAE in B7-H3-deficient mice

We next investigated the function of B7-H3 in EAE, a T_H1-mediated autoimmune disease model[40]. We induced EAE with myelin oligodendrocyte glycoprotein peptide as described before[41]. The average day of disease onset (the first day when the clinical score was above 1; Methods) was ~2 d earlier in B7-H3-deficient mice (day 16.1; $n = 16$) than in wild-type mice (day 18.4; $n = 14$; Fig. 5a). This trend was more obvious when we compared mice from the same litter (Fig. 5b), presumably because of a minimal variation in genetic background. Despite the earlier onset, B7-H3-deficient mice had the same mean clinical scores as wild-type mice by later stages of the disease (beyond day 20; Fig. 5a). The rates of disease incidence were also similar: 14 of 16 wild-type mice versus 16 of 18 B7-H3-deficient mice. Nonetheless, the earlier onset of EAE in the absence of B7-H3 supports our view that B7-H3 negatively regulates T_H1-driven immune responses.

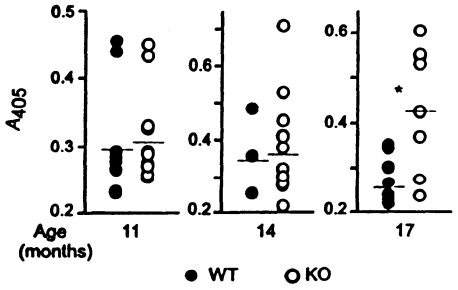

Figure 6 Accumulation of autoantibodies in B7-H3-deficient mice over time. The concentration of serum antibodies to single-stranded DNA was determined by ELISA in 3–11 mice at 11, 14 and 17 months of age. Genotypes (in key): WT, wild-type; KO, B7-H3-deficient. A significant difference was found at 17 months of age (*$P = 0.006$; $n = 8$ for each genotype).

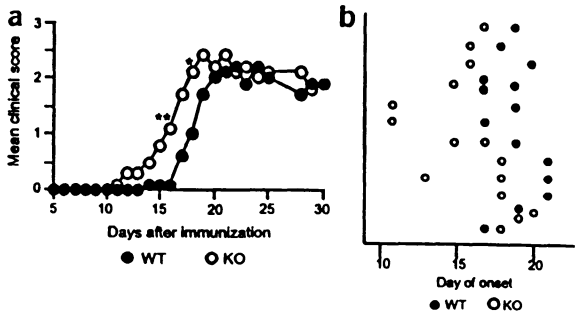

Figure 5 Earlier onset of EAE in B7-H3-deficient mice. After EAE was induced, clinical scores were assigned. (a) Disease course, as determined by the mean clinical score of all mice in each group (14 wild-type (WT) and 16 B7-H3-deficient (KO) mice). *, $P = 0.027$; **, $P = 0.009$. (b) Day of disease onset compared among littermates. Circles in the same row represent individual mice from the same litter. Data are a summary of four independent experiments.

Autoantibody accumulation in B7-H3-deficient mice

Consistent with their enhanced susceptibility to EAE, B7-H3-deficient mice accumulated substantially higher concentrations of serum autoantibodies to single-stranded DNA than did their wild-type littermates (Fig. 6). This spontaneous autoimmune phenotype became obvious only in later stages of life (~17 months) and did not result in immune complex deposition in the glomeruli of the B7-H3-deficient mice (data not shown). Furthermore, we did not find lymphocytic infiltration into multiple organs of B7-H3-deficient mice (data not shown). The marginal increase in the susceptibility of B7-H3-deficient mice to induced and spontaneous autoimmunity indicates that B7-H3 may be one of several molecules with overlapping negative immunoregulatory activities.

Antiviral CTL responses in B7-H3-deficient mice

B7-H3 enhances CTL generation *in vitro*[34]. To determine whether B7-H3 enhances CTL generation *in vivo*, we examined antiviral CTL responses to LCMV, influenza virus or vesicular stomatitis virus. Splenocytes collected from B7-H3-deficient mice on day 8 after infection with LCMV showed the same level of *ex vivo* CTL activity as those from wild-type mice (Fig. 7a, left). We also found similar levels of CTL activity when we collected splenocytes at day 30 after infection and restimulated them *in vitro* with antigenic peptides (Fig. 7a, right). These results indicate that normal primary and secondary CTL responses to LCMV can be mounted in the absence of B7-H3. To rule out the possibility that the strong antigenic stimulation associated with persistently replicating LCMV masked a need for costimulation[42,43], we examined CTL responses to influenza virus or vesicular stomatitis virus abortively replicating in mice. CTL responses to influenza virus or vesicular stomatitis virus are heavily dependent on costimulation[43,44]. We infected wild-type and B7-H3-deficient mice with influenza virus and monitored the expansion of the T cell subset specific for influenza nucleoprotein antigen NP366-374 using the NP366-374–H-2D^b tetramer as a probe[44]. The expansion of the CTL population specific for NP366-374–H-2D^b during primary and secondary influenza virus infections was indistinguishable in wild-type and B7-H3-deficient mice (Fig. 7b). When restimulated *in vitro*, wild-type and B7-H3-deficient splenocytes were equally cytotoxic (data not shown). Similarly, B7-H3-deficient mice mounted normal *ex vivo* CTL responses to vesicular stomatitis virus (data not shown). Thus, the generation of antiviral CTLs is not affected by a lack of B7-H3 *in vivo*.

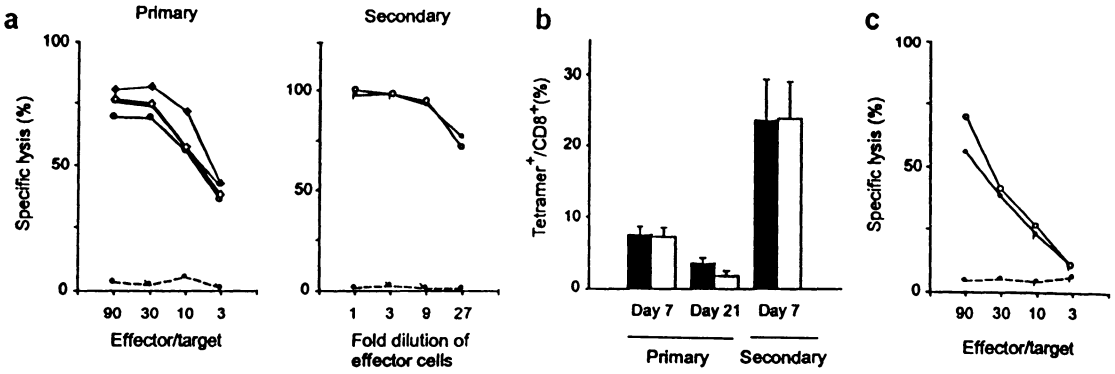

Figure 7 Normal antiviral CTL responses in the absence of B7-H3. (a) CTL responses to LCMV. Primary CTL response (left), splenocytes were collected on day 8 after infection and assayed for cytotoxicity using a ^{51}Cr-release assay (n = 2). Secondary CTL response (right), mice were killed on day 30 after infection and cytotoxicity was measured after restimulation for 5 d. Data represent mean of four mice per group. Filled symbols, wild-type; open symbols, B7-H3-deficient. Dashed line, negative control peptide; solid line, antigenic peptide p33. (b) CTL responses to influenza virus. The expansion of the nucleoprotein-specific CTL population was followed by tetramer staining during primary or secondary responses to influenza virus. Data represent mean ± s.e.m. of four mice per group. Filled bars, wild-type; open bars, B7-H3-deficient. (c) Effect of target cell B7-H3 deficiency on CTL killing. Immortalized wild-type (filled symbols) or B7-H3-deficient (open symbols) MEFs were used as target cells for recognition by anti-LCMV CTLs. Data are representative of two independent experiments using two MEF lines per genotype. Dashed line, negative control peptide; solid line, antigenic peptide p33.

Next, we tested whether the presence of B7-H3 on target cells could inhibit CTL activity. For target cells, we used immortalized MEFs that constitutively have high expression of cell surface B7-H3 (Fig. 1d). We treated target cells with IFN-γ to induce MHC class I molecules. We used splenocytes from LCMV-infected C57BL/6 mice as CTLs. A lack of B7-H3 on target cells did not result in a substantial increase in CTL killing (Fig. 7c). These results indicate that B7-H3 deficiency affects neither the generation nor the effector function of antiviral CTLs.

DISCUSSION

Our results show that B7-H3 has a different function in T cell responses than previously described. Although a positive regulatory function for human B7-H3 in the context of CTL responses has been proposed[34], we found that mouse B7-H3 is not involved in CTL responses. Instead, mouse B7-H3 negatively regulates T cell responses occurring in T_H1-polarizing conditions. Whether this discrepancy reflects a species difference between humans and mice has yet to be determined. The human locus encoding B7-H3 has four immunoglobulin domain–encoding exons, whereas the murine locus encoding B7-H3 has only two[35]. Through alternative splicing of mRNA, many human tissues can potentially express both B7-H3 (which has two immunoglobulin domains and shares ~88% amino acid sequence identity with mouse B7-H3) and a B7-H3b protein that has four immunoglobulin domains. The presence of B7-H3b in human cells may underlie some of these conflicting results. However, preliminary data indicate that mouse B7-H3–Fc inhibits human T cell proliferation in our experimental conditions (W.-K.S., J.B., T.H.W. and T.W.M., unpublished observations). Regardless, our data here show that, in mice, the absence of B7-H3 augmented T_H1 but not T_H2 or antiviral CTL responses: B7-H3-deficient mice experienced increased T_H1-mediated lung inflammation and earlier onset of EAE. As the expression of B7-H3 in dendritic cells is enhanced by the T_H1 cytokine IFN-γ, we propose that the interaction between B7-H3 on APCs and its unknown counter-receptor on T cells provides a negative feedback mechanism during the amplification of T_H1 responses. A lack of B7-H3 does not result in enhanced T cell responses in T_H2 conditions, most likely because IL-4 suppresses B7-H3 expression.

PD-L1 and PD-L2 down-regulate immune responses by binding to their common counter-receptor PD-1 (refs. 13,15,17,18). B7-H3, PD-L1 and PD-L2 are all expressed in multiple nonlymphoid organs[13,15,34,35] and can be induced in dendritic cells and/or monocytes by inflammatory cytokines[13,15,34]. This raises the possibility that B7-H3, PD-L1 and PD-L2 have redundant functions in down-regulating T cell responses. This redundancy may explain why B7-H3 deficiency does not affect antiviral $CD8^+$ T cell responses, the severity of EAE and oxazolone-induced contact hypersensitivity (data not shown). We were also unable to find any differences in the ratio of IgG2a versus IgG1 after immunizing mice with keyhole limpet hemocyanin–complete Freund's adjuvant or infecting them with influenza virus (data not shown). Whether B7-H3 expressed in multiple tumor cell lines[34] is involved in inhibiting antitumor immune responses, as is PD-L1 (ref. 45), remains to be determined.

So far, four B7 ligand–receptor pairs seem to down-regulate T cell activation: B7-1 and B7-2–CTLA-4; PD-L1 and PD-L2–PD-1; B7x-BTLA[31]; and B7-H3 with an unknown receptor. These multiple T cell inhibitory mechanisms may become involved in an orchestrated way at different phases of T cell activation (such as priming versus effector phase) as well as at distinct sites of immune responses (such as lymphoid organs versus inflammatory sites). Our findings indicate that B7-H3 and its unknown receptor provide a negative feedback mechanism for the developing T_H1 responses. A better understanding of these complex immune regulatory mechanisms should facilitate the generation of more powerful and selective tools to manipulate immunological disorders.

METHODS

Molecular cloning of B7-H3 cDNA. We cloned cDNA encoding mouse or rat B7-H3 from expressed sequence tag libraries. Mouse and rat B7-H3 share high amino acid sequence identity with each other (~97%) and with human B7-H3 (~88%).

Generation of B7-H3-deficient mice. A murine B7-H3 genomic clone was isolated from a 129/J phage library. E14 embryonic stem cells (129/Ola) were transfected with the targeting vector (Fig. 1a), and embryonic stem cell clones that had undergone homologous recombination were screened by PCR and verified by Southern blot analysis. C57BL/6 blastocysts were injected with the embryonic stem cells and implanted into pseudopregnant female mice. The

resulting chimeric mice were bred with C57BL/6 mice to obtain heterozygous F1 progeny, which were interbred to generate B7-H3-deficient mice. We used F2 or F3 progeny of a C57BL/6 × 129/Ola genetic background except where indicated otherwise. Mice that had been back-crossed six generations onto the BALB/c background were used for confirmatory airway inflammation experiments. B7-H3-deficient mice derived from two independent embryonic stem clones showed the same phenotypes. All live animal experiments were done with the approval of the University Heath Network Animal Care Committee (Toronto, Ontario, Canada).

Proteins and antibodies. Recombinant proteins B7-H3–Fc (the two extracellular immunoglobulin domains of murine B7-H3 fused in-frame to the Fc portion of human IgG1), B7-2–Fc (the two extracellular immunoglobulin domains of murine B7-2 fused in-frame to the Fc portion of human IgG1) and control Fc (human IgG1 Fc backbone protein) were synthesized as described[21]. Anti-B7-H3 antiserum was raised in rabbits with B7-H3–Fc as the antigen. The antiserum was purified through a protein A column and subsequently absorbed twice using human IgG columns to eliminate anti-human IgG activities. This antibody was specific for B7-H3 as shown by immunoblot (Supplementary Fig. 1 online) and was used for flow cytometric analyses of B7-H3.

MLR. Splenocytes from wild-type and B7-H3-deficient mice were stimulated with 10 μg/ml LPS (Sigma) for ~40 h, and the resulting $B220^+$ B lymphoblasts were purified by cell sorting (to ~98% purity). After γ-irradiation (20 Gy), these B lymphoblasts (stimulators) underwent twofold serial dilution (from 1×10^5 cell/well) in U-bottomed 96-well plates. Total BALB/c T cells (responders) were purified from lymph node cells by depletion of $B220^+$ and $CD11b^+$ cells with magnetic beads (Dynal). $CD4^+$ or $CD8^+$ responder T cells were purified from BALB/c splenocytes through positive selection using magnetic-activated cell separation microbeads (to >95% purity; Miltenyi Biotec). Purified responder T cells (1×10^5 cells per well) were added to the stimulator cells, and proliferation was assessed by measurement of [^{3}H]thymidine incorporation.

T cell proliferation assay. Flat-bottomed 96-well plates were pre-coated overnight at 4 °C with 10 μg/ml each of anti-hamster IgG (Jackson ImmunoResearch Laboratories) and anti-human IgG (Sigma). After the plates were washed, 0.1 μg/ml anti-CD3 (145-2C11; BD Pharmingen) plus increasing amounts (0, 0.1, 0.25, 0.5 and 1 μg/ml) of Fc proteins were added to the wells in triplicate. The plates were further incubated at 37 °C for 5–6 h. The wells were washed twice to eliminate unbound proteins before the addition of T cells (1×10^5 cells per well). In some cases anti-CD28 (37.51; BD Pharmingen) was added to the cells just before plating.

Airway inflammation. Airway inflammation was induced and analyzed as described[36–38]. For local cytokine microenvironments, GM-CSF (for T_H2 conditions) or GM-CSF plus IL-12 (for T_H1 conditions) were transiently expressed in the airway on day −1 by intranasal infection of the mice with a replication-defective adenovirus strain carrying the appropriate cytokine cDNA. Mice were exposed to OVA aerosol (1% weight/volume in 0.9% saline) for 20 min on 10 consecutive days (days 0–9). On day 11, the mice were killed and the immune cell populations in the BAL were differentially stained and counted. The remaining lung tissue was processed for histological examination with H&E staining. In some cases, mononuclear cells of the lung were isolated and T cells were analyzed by flow cytometry. Cells from draining pulmonary lymph nodes or spleens were cultured in the absence or presence of 400 μg/ml OVA for 5 d before cytokine concentrations were assessed by enzyme-linked immunosorbent assay (ELISA; R&D Systems).

Dendritic cells and macrophages. Dendritic cells were generated from bone marrow progenitor cells *in vitro* with GM-CSF (10 ng/ml; PeproTech) as described[46]. Immature dendritic cells were collected at day 6 of culture and then stimulated with 1 μg/ml LPS plus 5 μg/ml anti-CD40 (3/23; BD Pharmingen) for 1–2 d in the absence or presence of IFN-γ (30 U/ml; Biosource) or IL-4 (500 U/ml; PeproTech). Peritoneal macrophages were prepared from mice that had been injected intraperitoneally with 1 ml 4% thioglycollate 4 d before.

RT-PCR. Total RNA prepared using Trizol reagent (Life Technologies) was reverse-transcribed to generate first-strand cDNA using the cDNA Cycle Kit (Invitrogen). cDNA of B7-H3 and hypoxanthine guanine phosphoribosyl transferase (HPRT) was amplified by 30 cycles of PCR (94 °C for 1 min; 60 °C for 1 min; 72 °C for 1 min) using the following primer sets: for B7-H3, 5′-GCTACACCTGCTTTGTGAGC-3′ and 5′-CCAGTAGTACCACAAGACAG-3′; for HPRT, 5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGAAAAG GACCTCT-3′. B7-H3 RT-PCR products from dendritic cell samples were visualized by phosphorimaging after hybridization of ^{32}P-labeled B7-H3 probes.

Experimental autoimmune encephalomyelitis. Induction and assignment of clinical scores of EAE were done as described[41] with wild-type and B7-H3-deficient littermates 8–12 weeks of age.

ELISA for autoantibodies. Antibodies to single-stranded DNA were detected by ELISA with plate-coated sonicated salmon sperm DNA (10 μg/ml; Stratagene) as the antigen.

LCMV CTL assays. For primary CTL responses, wild-type and B7-H3-deficient mice were injected intravenously with 2,000 PFU LCMV (Armstrong strain). At day 8 after infection, splenocytes were prepared and *ex vivo* CTL activity was measured by a standard ^{51}Cr-release assay with ^{51}Cr-labeled mouse lymphoma EL4 cells pulsed with the LCMV glycoprotein peptide p33 (KAVYNFATM). For secondary CTL responses, wild-type and B7-H3-deficient mice were killed on day 30 after infection. Collected splenocytes were cultured for 5 d in the presence of 1 μM p33 peptide, and cytotoxicity was measured as described above. For assessment of the involvement of B7-H3 in the CTL killing of target cells, wild-type and B7-H3-deficient MEFs were immortalized with SV40 large T antigen through retroviral gene transfer[47]. The immortalized MEF cells were treated for 20 h with IFN-γ (1 U/ml) to induce cell surface MHC class I molecules. After trypsinization, these cells were loaded with ^{51}Cr and p33 peptides as described above and were used as target cells. Splenocytes prepared from C57BL/6 mice infected with LCMV were used as CTLs.

Influenza virus nucleoprotein-specific CTL responses. Primary and secondary CTL responses to influenza virus were monitored by tetramer staining and cytotoxicity assays as described[44].

Statistical analyses. Student's *t*-test was used to determine the statistical significance of differences between genotypes.

GenBank accession numbers. Mouse B7-H3, AY190318; rat B7-H3, AY190319.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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