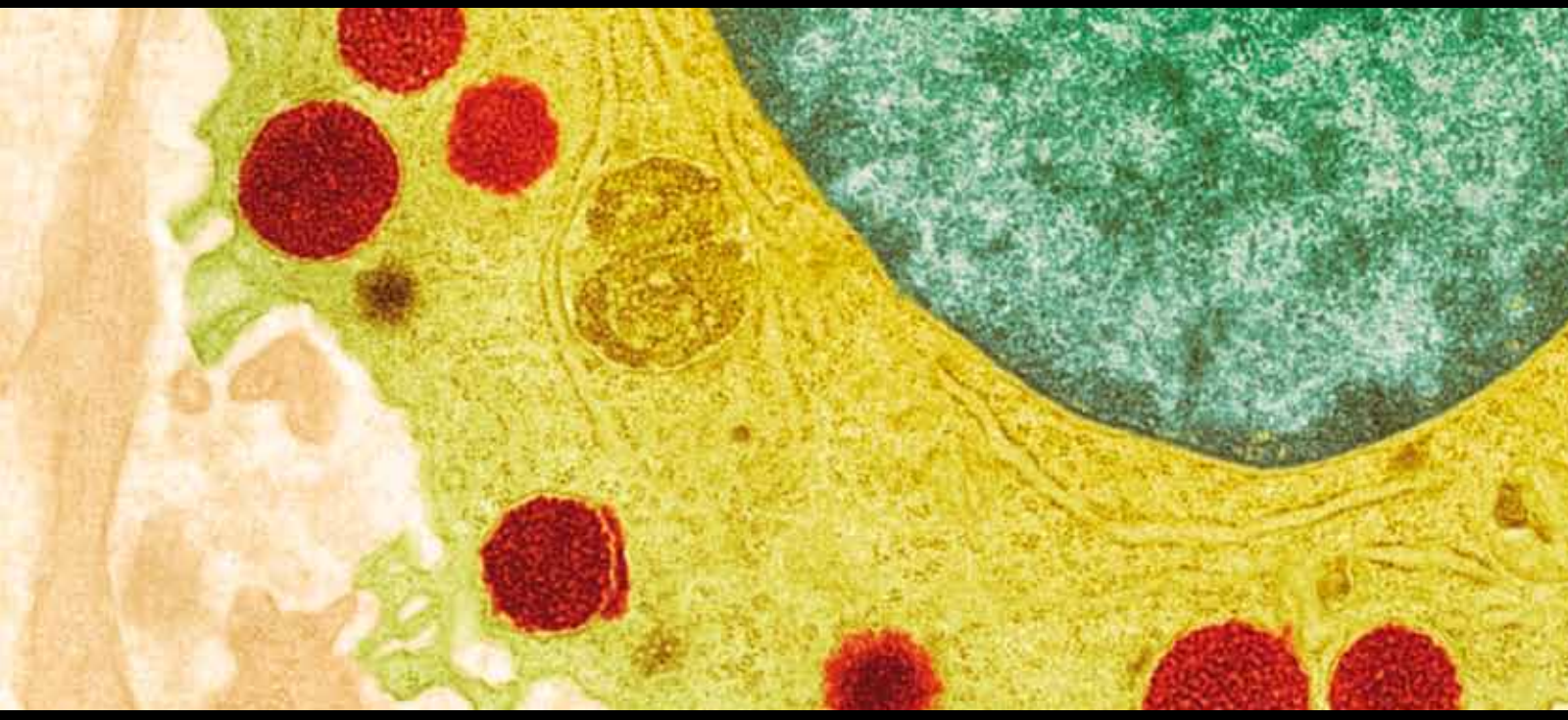
Journal of Allergy

Crosstalk between Innate and Adaptive Cells on Allergic Process

Guest Editors: Maria Leite-de-Moraes, Hamida Hammad, and Michel Dy



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Editorial

Crosstalk between Innate and Adaptive Cells on Allergic Process

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As guest editors of this special issue of the *Journal of Allergy*, we are pleased to introduce both original article, and reviews that address pertinent questions associated with a critical view of the current knowledge of *crosstalk between innate and adaptive cells on allergic process*.

Innate cells play a major role in the first line of defense against invading organisms and environmental challenges. However, in some cases they are deflected from their primary role to participate, in association with adaptive cells, on allergic process. Innate cells are constantly in contact with anodyne environmental molecules that for still not well understood reasons trigger allergic responses in a number of individuals. Both prevalence and severity of allergies are increasing worldwide. Allergic asthma is a disease characterized by a chronic airway inflammation with the infiltration of Th2 cells and eosinophils, airway obstruction, and remodeling. Notably, 250 000 people die of asthma every year. About 10% to 20% of the population are allergic to house dust mite (HDM), the causative agent of some types of atopic dermatitis or allergic asthma. Food allergy also represents a public health problem associated with diminished quality of life. The symptoms range from mild erythema and pruritus to anaphylaxis. Milk, eggs, fish, or peanuts allergies are very common in the general population, and the latter allergen is highly associated with anaphylaxis.

Innate cells are the first-line barrier in contact with allergens in skin, airways, and gut. Therefore, they will send messages by cell-to-cell contact or cytokines to positively or negatively influence adaptive cells. Epithelial cells can express many pattern recognition receptors (PPRs) to detect and respond to pathogen-associated molecular patters (PAMPs) currently found in microbes like mites or damaged tissues. This stimulation will induce the production of cytokines as TGF-β (transforming growth factor beta), TSLP (thymic stomal lymphopoietin), Mip2 (macrophage inflammatory protein 2), IL-1 (interleukin-1), IL-25, IL-33, or GM-CSF (granulocyte-macrophage colony-stimulating factor) that will act on other cells not only of the innate but also of the adaptive immunity [1]. In brief, TSLP, GM-CSF, IL-25, and IL-33 will activate dendritic cells to prime Th2 response by inhibiting the production of the Th1-polarizing cytokine IL-12, by inducing chemokines that attract Th2 cells or by favoring the development of Th2 cells through the upregulation of OX40L.

Dendritic cells (DCs) are the most important antigen presenting cells (APC) that will induce T-cell differentiation. However, this major capacity can vary depending on their distinct subsets. Several markers are used to distinguish these subsets, but the most simple discrimination is based on the level of expression of CD11c and CD11b. Conventional (c) DCs express high levels of CD11c compared with $CD11c^{dim}$ plasmacytoid (p) DCs [2]. Despite the fact that great advance on the understanding of the biology of DC subsets was already obtained, further studies are necessary to clarify how allergic sensitization is induced and the implication of the distinct DC subsets.

In addition to DC, innate cells as basophils can also influence adaptive immune responses. Basophils amplify allergic Th2 cells initiated by dendritic cells via a nonredundant role because of their capacity to generate IL-4 rapidly and efficiently on exposure to an increasing number of stimuli including IgE [3]. For instance, it has been established that basophils can be targeted in an IgE-independent manner by allergen proteases, such as papain, which promotes their recruitment and stimulation in draining lymph nodes, initiating a Th2 immune response. Moreover, basophils

directly respond to IL-33 by producing substantial amounts of the Th2 promoting factors IL-4, IL-6, and histamine [4]. It is notably that histamine will promote IL-4 production by invariant natural killer T (iNKT) cells [5].

iNKT cells are in the very frontier between innate and adaptive immune responses. They constitute a distinctive population of mature T lymphocytes that produce a broad range of cytokines few minutes after stimulation allowing them to modulate both innate and acquired immunity in a large spectrum of inflammatory diseases. They express a highly restricted T-cell receptor (TCR) repertoire composed of a single-invariant Vα14Jα18 chain in mice and a Vα24Jα18 chain in humans, preferentially paired with limited TCR Vβ chains. In contrast to conventional T cells that recognize peptides, iNKT cells recognize glycolipids presented by CD1d expressing Ag-presenting cells (APCs), such as dendritic cells (DCs). The major iNKT cell subset promptly and massively produces IL-4 and IFN-γ. iNKT cells enhanced the severity of asthmatic symptoms in experimental models, namely, airway eosinophilia, hyperreactivity, Th2 cytokine production, and mucus and IgE secretion and are implicated on food allergy [6–8]. Moreover, iNKT cells may have similar effects in humans since they are enhanced in bronchoalveolar lavage fluid of asthmatic patients [9, 10]. In addition to acting as effector cells, iNKT lymphocytes can also be considered as a novel biomarker for some pathologies. Indeed, it was recently provided the first demonstration in humans that early postallogeneic HSCT (hematopoietic stem cell transplantation) donor-derived iNKT cell recovery can be used as a new predictive marker of acute GVHD (graft-versus-host disease) with preserved GVL (graft versus leukemia) effect and improved overall survival [11].

Recently a new iNKT cell subset that preferentially produces IL-17 were discovered [12, 13]. This iNKT17 subset derives from a alternative thymic pathway of differentiation dependent on the transcription factor RORγt that, in contrast to the mainstream IL-4-producing RORγt^{neg} iNKT cell subset, maintains the expression of this transcription factor in the periphery [13]. iNKT17 cells are present in the lung and can exacerbate allergic airway inflammation. IL-17-producing iNKT cells were also observed in humans [14]. Further studies are in progress to better characterize the crosstalk of these cells with epithelial cells, DC, basophils and conventional T and B cells in allergic responses.

In this special issue, S. Schnyder-Candrian et al. reported that neutrophil inhibitory factor (NIF) can preferentially block the transmigration of eosinophils across endothelial cell monolayers. The inhibitory effect was confirmed *in vivo* in an experimental asthma model. These findings clearly suggest that NIF has an antiallergic effect and show a new facet of this molecule primarily implicated on neutrophil migration. In addition to endothelial cells, keratinocytes represent not only a mechanical barrier but also an important source of cytokines and chemokines as reviewed by the French group from Poitiers. In fact, F. Bernard et al. emphasized the critical role of cytokines, namely, IL-4, IL-13, IL-17, IL-22, and TNFα, in keratinocytes and the implication of this crosstalk in atopic dermatitis (AD) and psoriasis. They mounted an elegant model of reconstructed human epidermis (RHE) that mimics human skin. This group demonstrated that specific sets of cytokines-induced AD-like or psoriasis-like phenotype in human keratinocytes. The theme is, therefore, expertly reviewed and discussed in this paper based on their own experience and those from others groups.

As commented before, DCs are professional APC critically implicated on the sensitization phase of allergic responses acting as a bridge between innate and adaptive immunity. S. Awasthi et al. provide an overview of the lung DC developmental programming. It is noteworthy that lung DCs develop after birth. Considering the frequent exposure to allergens, pathogens and environmental chemicals during the critical window that encompass lung DC maturation, authors stress the fact that these factors potentially influence DC subsets with long-term respiratory and immunological consequences. The study of lung DC development in neonates is not possible in humans, but authors have an important experience with baboon models that could help to better identify the basis of childhood asthma, and here they discuss the more recent findings concerning this central issue of DC differentiation and allergen sensitization.

Another actor in this crosstalk between innate and adaptive responses in allergies are B cells and antibodies. The term atopy was first used by Coca and Cooke in 1923 [15]. At this time, atopy was associated with hypersensitiveness but not necessarily with immunoglobulin (Ig) production. In this issue, W. Williams et al. discuss the contribution of allergen-specific IgG to the development of Th2-mediated airway inflammation. This paper discusses the place of immunoglobulin in the asthmatic process highlighting the importance of IgG and FcγRs signaling. The authors propose a model whereby allergen-specific IgG promotes the expansion of secondary Th2 responses through ligation of FcyRs on innate cells. They also address the question concerning the implication of B cells and FcyRs on innate cells and their contribution to allergic immune responses.

We could not conclude this issue without discussing the implication of regulatory T cells on allergic process. The Portuguese group of Lisbon leaded by L. Graça addressed this point. Allergic process represents ultimately exacerbated responses to a given antigen that is not tolerated by the organism. The authors discuss why some antigens are tolerized by the immune system and why others are "seen" as allergens. Many questions discussed in this paper are still without answers but they stimulate the continuing efforts to understand the crosstalk between innate and adaptive cells on allergic process and potentially provide better care to patients.

Finally, this special issue provides an instructive overview of the different actors from both innate and adaptive immune cells and their crosstalk on allergic process. We would like to thank the authors' contributions, and we hope that this issue will bring new ideas to improve the scientific research against this health public scourge named allergy.

Maria Leite-de-Moraes
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Research Article

Neutrophil Inhibitory Factor Selectively Inhibits the Endothelium-Driven Transmigration of Eosinophils *In Vitro* and Airway Eosinophilia in OVA-Induced Allergic Lung Inflammation

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Leukocyte adhesion molecules are involved in cell recruitment in an allergic airway response and therefore provide a target for pharmaceutical intervention. Neutrophil inhibitory factor (NIF), derived from canine hookworm (*Ancylostoma caninum*), binds selectively and competes with the A-domain of CD11b for binding to ICAM-1. The effect of recombinant NIF was investigated. Intranasal administration of rNIF reduced pulmonary eosinophilic infiltration, goblet cell hyperplasia, and Th_2 cytokine production in OVA-sensitized mice. *In vitro*, transendothelial migration of human blood eosinophils across IL-4-activated umbilical vein endothelial cell (HUVEC) monolayers was inhibited by rNIF (IC_{50}: 4.6 ± 2.6 nM; mean ± SEM), but not across TNF or IL-1-activated HUVEC monolayers. Treatment of eosinophils with rNIF together with mAb 60.1 directed against CD11b or mAb 107 directed against the metal ion-dependent adhesion site (MIDAS) of the CD11b A-domain resulted in no further inhibition of transendothelial migration suggesting shared functional epitopes. In contrast, rNIF increased the inhibitory effect of blocking mAbs against CD18, CD11a, and VLA-4. Together, we show that rNIF, a selective antagonist of the A-domain of CD11b, has a prominent inhibitory effect on eosinophil transendothelial migration *in vitro*, which is congruent to the *in vivo* inhibition of OVA-induced allergic lung inflammation.

1. Introduction

Allergic asthma has increased dramatically in prevalence and severity over the last two decades. It is a manifold syndrome consisting of bronchospasm and airway hyperreactivity driven by chronic airway inflammation whose underlying immunological mechanisms are still matter of investigation [1, 2]. The accumulation of antigen presenting cells (APCS), Th_2 lymphocytes, and eosinophils is a prominent feature of chronic airway inflammation [2]. The immigration of leukocytes in the extravascular space is orchestrated by the endothelial barrier and follows an inducible process of stepwise interacting adhesion molecules and chemokines. Intensive research of the last two decades has delineated the molecular program of leukocyte emigration [3–6]. In allergic airway inflammation, the Th_2 cytokines IL-4 and IL-13 stimulate the endothelium to express VCAM-1 and, by virtue of its binding to very late antigen-4 (VLA-4) [7, 8], facilitate transendothelial migration of eosinophils [9, 10]. The fact that IL-4 and IL-13 do not provoke the endothelial lining to express the neutrophil recruiting E-selectin [11] further explains why eosinophils are preferentially accumulated

while neutrophils are often virtually absent in allergic inflammation [12]. As a major counterreceptor of ICAM-1, the β_2-integrin CD11b/CD18 has been associated to neutrophil and macrophage recruitment, although in severe asthma β_2-integrin-expressing lymphocytes and eosinophils are prominent participants [8]. Furthermore, β_2-integrins allow egress of newly generated plasma cells from lymph nodes to the bone marrow [13] while their *in vivo* role in eosinophil recruitment is not thoroughly investigated.

Neutrophil inhibitory factor (NIF) is a 41-kilodalton glycoprotein from the canine hookworm (*Ancylostoma caninum*) that binds to the A-(I)-domain of CD11b/CD18 ($\alpha M\beta_2$, Mac-1) [14, 15]. Although NIF, a heavily glycosylated polypeptide of 274 amino acids, neither contains the Arg Gly-Asp sequence nor the disintegrin motif, it binds with high specificity and affinity to the A-domain of CD11b/CD18 [15, 16]. The A-domain, is ≈200 amino acid peptide within the CD11b molecule binding divalent cations and representing a major recognition site for iC3b, fibrinogen, and factor X of the coagulation cascade [17]. By virtue of these multiple but distinct binding sites, the A-domain plays an essential role in phagocytosis, cytotoxicity, and leukocyte trafficking to inflammatory sites.

The function of NIF was so far investigated in *in vivo* models characterized by a dominant neutrophil response. In an acid-induced lung injury model in rabbits, NIF and anti-CD18 antibodies showed comparable attenuation with high number of accumulated neutrophils in the air space. The study indicates that rNIF rather blocks neutrophil cytotoxicity than interfering with their recruitment [18]. In the reverse passive Arthus reaction in rats, neutrophil recruitment depends on CD11a/CD18 and CD11b/CD18 and either of these integrins is sufficient for neutrophil trafficking [19], suggesting that CD11b/CD18 is dispensable for neutrophil recruitment. Given that CD11b/CD18 is expressed on different leukocytes including eosinophils, NIF may also block functions of these leukocytes. With regard to allergic inflammation, eosinophils were in the focus of our interest due to their destructive potential and their preferential accumulation in tissue.

This study shows that rNIF blocks transmigration of eosinophils but not neutrophils across endothelial cell monolayers in culture and inhibits eosinophil recruitment and infiltration, goblet cell hyperplasia, mucus secretion, and Th_2 cytokine production in the OVA-induced lung inflammation model in mice.

2. Material and Methods

2.1. Cytokines, Monoclonal Antibodies, and Reagents. Human recombinant IL-1 was kindly provided by Dr. P. T. Lomedica (Hoffmann-La Roche, Nutley, NJ), TNF by Dr. Z. Nagy, (Preclinical Research, Sandoz Ltd., Basel, Switzerland), IL-4 by Dr. J. Banchereau (Schering Plough, Dardilly, France), and recombinant NIF by Dr. Matthew Moyle (Corvas, San Diego, CA, USA). The ELISA kits for mouse IL-4, IL-5 were from R&D (Abingdon, UK). The mAbs IB-4 (IgG2a) recognizing CD18, mAb 60.1 (IgG1) against CD11b and H12 (IgG1) directed to CD11a were generous gifts of Dr. S. Wright (Rockefeller University, New York, USA). The mAb CLB54 against CD18 was kindly provided by Dr. J. Ghrayeb, Centocor Inc., Malvern, PA, USA. The mAbs HP2/1 (IgG1) against VLA-4 (CDw49d) and 25.3 (IgG1) against CD11a were from Immunotech S. A., Marseille, France. The mAb W6/32 (anti-HLA frames structure; Sera-Lab, Crawley Down, England) was used as negative control. The mAb 107 was a generous gift of Dr. M. A. Arnaout (Massachusetts General Hospital, Charlestown, MA 02129, USA). In functional inhibition experiments, all mAbs were used at proven saturating concentrations of 10 μg/mL as described [20].

O-phenylenediamine, 3-amino-1,2,4-triazole, horseradish peroxidase, and chicken egg albumin grade V were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Mice and Experimental Protocol. BALB/c mice were bred in our specific pathogen-free animal facility at CNRS, Orleans, France. Mice were maintained in a temperature-controlled (23°C) facility with a strict 12-hour light/dark cycle and were given free access to food and water. The experiments were performed with gender-matched mice aged 6 to 8 weeks. All protocols complied with the French Government's ethical and animal experiment regulations.

Mice were immunized subcutaneously twice at weekly interval with 0.2 mL saline containing 10 μg ovalbumin (OVA) with 1.6 mg aluminium hydroxide. One week after the second immunization, mice were challenged 3 times (at days 14, 15, and 16) as follows. Animals were hold under light i.v. ketamine, xylazine anesthesia and administered intranasally with 40 μL saline (0.9%) containing 10 μg OVA alone, or OVA with 25 μg rNIF. Control mice were challenged with saline alone, or 25 μg rNIF alone, given in 40 μL saline solution. One day after the last challenge, plethysmography analysis was performed. Two days after the last challenge mice were sacrificed and the lungs analyzed.

Mice were given a high dose of ketamine/xylazine i.p. and bled out. Via a tracheal cannula, the lungs were washed twice with 1 mL of ice-cold saline (see below Bronchoalveolar lavage fluid). After bronchoalveolar lavage, the lung was perfused via heart puncture with ISOTON II acid-free balanced electrolyte solution (Beckman Coulter, Krefeld, Germany). Lungs were fixed overnight in buffered 4% formaldehyde solution for histology analysis. BAL fluid was analyzed for cell composition and cytokine concentrations. Experiments were performed at least twice using groups of 4 animals.

2.3. Airway Resistance Using Whole-Body Plethysmography. Bronchial hyperreactivity (BHR) to aerosolized methacholine was investigated at 24 h after the last OVA challenge. Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic, Sharon, CO, USA). Mice were exposed for 50 seconds to 100 mM methacholine. The constriction was measured for 15 min after nebulization. Mean airway bronchoconstriction was estimated by the enhanced respiratory pause (Penh) index.

Penh can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance. Penh is calculated by the formula Penh = (Te/RT − 1) × PEF/PIF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. For the graphics, the Penh mean values are given for −3 min to −1 min (baseline) and 14 points or minutes after the methacholine nebulization.

2.4. Bronchoalveolar Lavage (BAL). Bronchoalveolar lavages (BAL) fluids were prepared by washing the lungs 4 times with 0.5 mL of ice-cold saline. The cells were sedimented by centrifugation at 400 ×g for 10 min at 4°C. The supernatants (cell-free BAL fluid) were stored at −70°C for cytokine analysis. An aliquot of the cell pellets was stained with Trypan Blue solution, counted, and 200,000 cells centrifuged on microscopic slides (cytospin at 1000 rpm for 10 min, at RT). Air-dried preparations were fixed and stained with Diff-Quik (Merz & Dade AG, Dudingen, Switzerland). Differential counts were made under oil immersion microscopy. Two times one hundred cells were counted for the determination of the relative percentage of each cell type present in the BAL.

2.5. Lung Histology. The organs were fixed in 4% buffered formaldehyde overnight and embedded in paraffin as described previously [21]. Lung sections of 3 μm were stained with hematoxylin and eosin or with periodic acid Schiff reagent (PAS) and examined with a Leica microscope (×40 and ×100 magnification). Peribronchial eosinophil infiltration and goblet cell hyperplasia with mucus hypersecretion were assessed by a semiquantitative score (0–5) by two observers independently.

2.6. Quantitation of IL-4 and IL-5 in BAL. Cytokine concentrations in BAL were determined by enzyme-linked immunosorbent assay (ELISA), using commercial kits from R&D (Abingdon, UK). The cytokine detection limit was 1 pg/mL.

2.7. Recombinant NIF. Recombinant NIF was expressed in Chinese hamster ovary (CHO) cells and purified from conditioned cell supernatant by immunoaffinity chromatography as described [15]. Stock solutions of purified recombinant NIF (herein called as rNIF) were in PBS, pH 7.3 at an approximate concentration of 10 mg/mL.

2.8. Endothelial Cell Cultures. HUVECs were harvested as previously described [22]. The cells were seeded on purified human fibronectin (Winiger AG, Wohlen, Switzerland) and grown in medium 199 enriched with sodium heparin (90 μg/mL; Novo Industries, Copenhagen, Denmark) and endothelial cell growth supplement (15 μg/mL; Collaborative Research, Inc., St. Waltham, MA) in the presence of 20% pooled human serum. Final monolayers were used in their second to fourth passage exhibiting the cytoplasmic factor VIII von Willebrand as tested by indirect immunofluorescence with rabbit anti-human factor VIII Ab [23].

2.9. Preparation of Bilayer Vascular Constructs. Bilayer vascular constructs consisting of confluent HUVEC layers on the top basal layers of extracellular matrix from human fibroblasts were prepared as described [9]. Briefly, human lung fibroblasts were cultured in MEM alpha containing 10% of FCS for 10 d in 24-well plates. The resulting multilayers were washed once with PBS and lysed with 1 mL/well of a 0.5% aqueous solution of ammonium. The remaining extracellular matrix layers were rinsed 5 times with PBS, before the HUVECs were seeded in a 1 : 2 ratio and grown in complete culture medium until they reached confluence.

2.10. Purification of Neutrophils and Eosinophils. Granulocytes were separated from 400 mL of heparinized (20 U/mL) blood; (Novo Industries, Copenhagen, Denmark) whole blood from normal individuals by methocel-metrizoate sedimentation and subsequent buoyant density centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described [22]. The resulting sediment was, essentially free from monocytes and lymphocytes and contained mostly neutrophils (96–99%) and eosinophils (1–4%). Eosinophils were further enriched by negative selection using CD 16 immunomagnetic beads according to the method of Hansel et al. [24] and were cultured overnight in Iscove's medium containing 10% FCS, 10 pM recombinant human GM-CSF, and 10 pM recombinant IL-3 (Sandoz Ltd., Basel, Switzerland) as described [20]. The activated eosinophils accumulated between distinct layers of a discontinuous Percoll gradient at densities between 1.070 g/mL and 1.075 g/mL. This behavior characterizes the metabolically activated hypodense phenotype found in allergic diseases [20].

2.11. Determination of Eosinophil and Neutrophil Transendothelial Migration. Bilayer vascular constructs were preincubated with cytokines at indicated concentrations for 4 h or 18 h at 37°C, washed twice with HBSS, and coincubated for 2 h at 37°C together with 2×10^5 neutrophils or eosinophils resuspended in 500 μL of MEM-α medium containing 10% FCS with or without NIF and/or mAbs at indicated concentrations. Transendothelial migration was determined after 2 hours as described [9, 10]. In brief, the bilayers were fixed with 1% paraformaldehyde in PBS-A and transmigrated eosinophils were counted in a blind fashion in 4 arbitrarily chosen high power fields focused on a subendothelial plane. The number of eosinophils/high power field (0.25 mm^2) was calculated as the percentage of migrated eosinophils.

2.12. Statistical Analysis. Statistical validation was performed using Student's two-tailed t-test for unpaired observations. P values of less than 0.05 are considered statistically different.

3. Results

3.1. Recombinant NIF Decreases Eosinophil Recruitment and Th_2 Cytokines in OVA-Challenged BALB/c Mice. The heterodimeric β_2-integrin CD11b/CD18 is expressed on

most leukocytes including eosinophils participating in the airway hyperresponsiveness of allergic asthma. Disruption of the β_2-integrin-ICAM-1 interaction by rNIF may attenuate the allergic airway inflammation. Therefore, the potential inhibitory effect of rNIF together with the antigen challenge in immunized mice was investigated and the allergic response analyzed.

In the absence of rNIF total BAL (bronchoalveolar lavage) cell counts increased up to 24-fold in OVA-challenged BALB/c mice as compared to saline treatment (15×10^4 to 354×10^4 cells/BAL). The composition of the OVA-induced leukocytes in the BAL (Figure 1) was dominated by invading eosinophils ($P < 0.01$) and lymphocytes ($P < 0.01$). In contrast, macrophages were not significantly increased compared to the saline control, and the number of recruited neutrophils in OVA-challenged BALB/c mice was negligible (Figure 1). Recombinant NIF when given by the intranasal route at 25 μg per mouse with the OVA challenge reduced the total BAL cell counts by 40% ($P < 0.01$), with a 50% reduction of eosinophils ($P < 0.01$) and lymphocytes. Macrophages numbers in the BAL were not reduced by rNIF (Figure 1).

In addition, production of IL-4 and IL-5 was determined in the BAL samples. Ova challenge induced a significant increase of IL-4 and IL-5 in BAL fluid, while IL-13 could not be detected. Intranasal administration of rNIF reduced OVA-induced IL-5 and IL-4 production in the BAL fluid by 80% and 75%, respectively (Figures 2(a) and 2(b)).

To investigate the effect on airways hyperresponsiveness rNIF was given by the intranasal route at a dose of 25 μg together with each antigen challenge in OVA-sensitized BALB/c mice. OVA-challenge of OVA immunized BALB/c mice, but not NaCl or rNIF alone, developed a robust response to aerosolized methacholine expressed as enhanced respiratory pause (Penh) values as assessed by whole-body plethysmography (Figure 2(c)). Penh values provide an estimate for airway obstruction and may indicate airway hyperreactivity. The CD11b antagonist NIF when given together with the OVA challenge revealed no significant inhibition of the methacholine response (Figure 2(c)) as calculated by AUC assessment of Penh values over time.

3.2. Recombinant NIF Decreases Mucus Hypersecretion and Eosinophil Recruitment in the Lung Tissue. The morphological hallmarks of allergic asthma are peribronchial eosinophilic inflammation, mucus overproduction, and bronchial smooth muscle cell hyperplasia. OVA challenge in immunized mice caused distinct peribronchial cell recruitment with eosinophils together with hyperplasia of bronchial smooth muscle and increased mucus production. Recombinant NIF alone had no effect (data not shown), but drastically reduced OVA-induced mucus hypersecretion and hyperplasia of goblet cells and peribronchial eosinophil infiltration (Figures 3(a) and 3(b)). The effect of rNIF on OVA-induced eosinophil infiltration and mucus overproduction (goblet cell hyperplasia) was assessed semiquantitatively. Upon OVA challenge rNIF significantly reduced both eosinophil recruitment (Figure 3(c)) and mucus hypersecretion ($P < 0.05$), while rNIF on its own had no effect (Figure 3(d)). Together, the data suggest that the CD11b antagonist NIF inhibits OVA-induced allergic inflammation *in vivo*.

3.3. Recombinant NIF Inhibits Transendothelial Migration of Eosinophils. As rNIF significantly reduces eosinophil infiltration in the peribronchial space and the cytokine-activated endothelium is known to govern leukocyte trafficking in inflammation, it was of interest whether rNIF would inhibit the passage of eosinophils across endothelial layers *in vitro*.

In these experiments, human blood eosinophils were conditioned with GM-CSF and IL-3 for 24 h to induce changes mimicking the active phenotype found in allergic inflammation [20].

Bilayer vascular constructs were used to determine transendothelial migration (TEM) *in vitro*. They consisted of HUVEC monolayers grown on extracellular matrix from human fibroblast multilayers [9]. Pretreatment of the bilayers with IL-1 and TNF preferentially provoked TEM of neutrophils [9]. Conversely, IL-4-pretreated bilayers selectively induced TEM of eosinophils [9]. Considering these cell type specific conditions for transmigration, we investigated the impact of rNIF on TEM of eosinophils and neutrophils in a series of separate experiments (Figure 4). TEM of eosinophils and neutrophils across unstimulated bilayer vascular constructs was $3.1 \pm 0.6\%$ and $3.2 \pm 0.5\%$, respectively (means of triplicate determinations $\pm$SD of a representative experiment). With IL-4-pretreated bilayers a more than 10-fold increase of the TEM of eosinophils was observed which was inhibited in a dose-dependent manner by rNIF (Figure 4(a)). Half maximal inhibition of TEM (IC_{50}) was obtained at 4.6 ± 2.6 nM rNIF (mean $\pm$ SEM of three experiments). In contrast, TEM of neutrophils provoked by TNF stimulation of the bilayers was not significantly impaired by rNIF even at concentrations up to 1 μM (Figure 4(b)).

Although the process of eosinophil emigration depends on CD11b/CD18, our data indicate that the interaction is more complex involving different adhesion molecules. Therefore, the inhibiting property of rNIF was further investigated together with blocking mAbs against CD18 (CLB54), VLA-4 (HP2/1), and CD11a (25.3; Figure 5). In the presence of saturating concentrations of these mAbs TEM of eosinophils was partially inhibited. The inhibitory effect conferred by the mAbs CLB54, HP2/1, and 25.3 was considerably enhanced by rNIF indicating independent blocking mechanisms. In contrast, the finding that rNIF showed no additional inhibition in the presence of mAb 60.1 suggests shared functional epitopes (Table 1). Similar data to using mAb 60.1 were obtained, when mAb 107 against the metal ion-dependent adhesion site (MIDAS) of the CD11b A-domain was applied.

In summary, the experiments allow the conclusion that rNIF inhibits TEM of eosinophils by binding to the MIDAS region in the A-domain of CD11b. These data are congruent to our *in vivo* observation that rNIF significantly reduces accumulation of eosinophils in the peribronchial space.

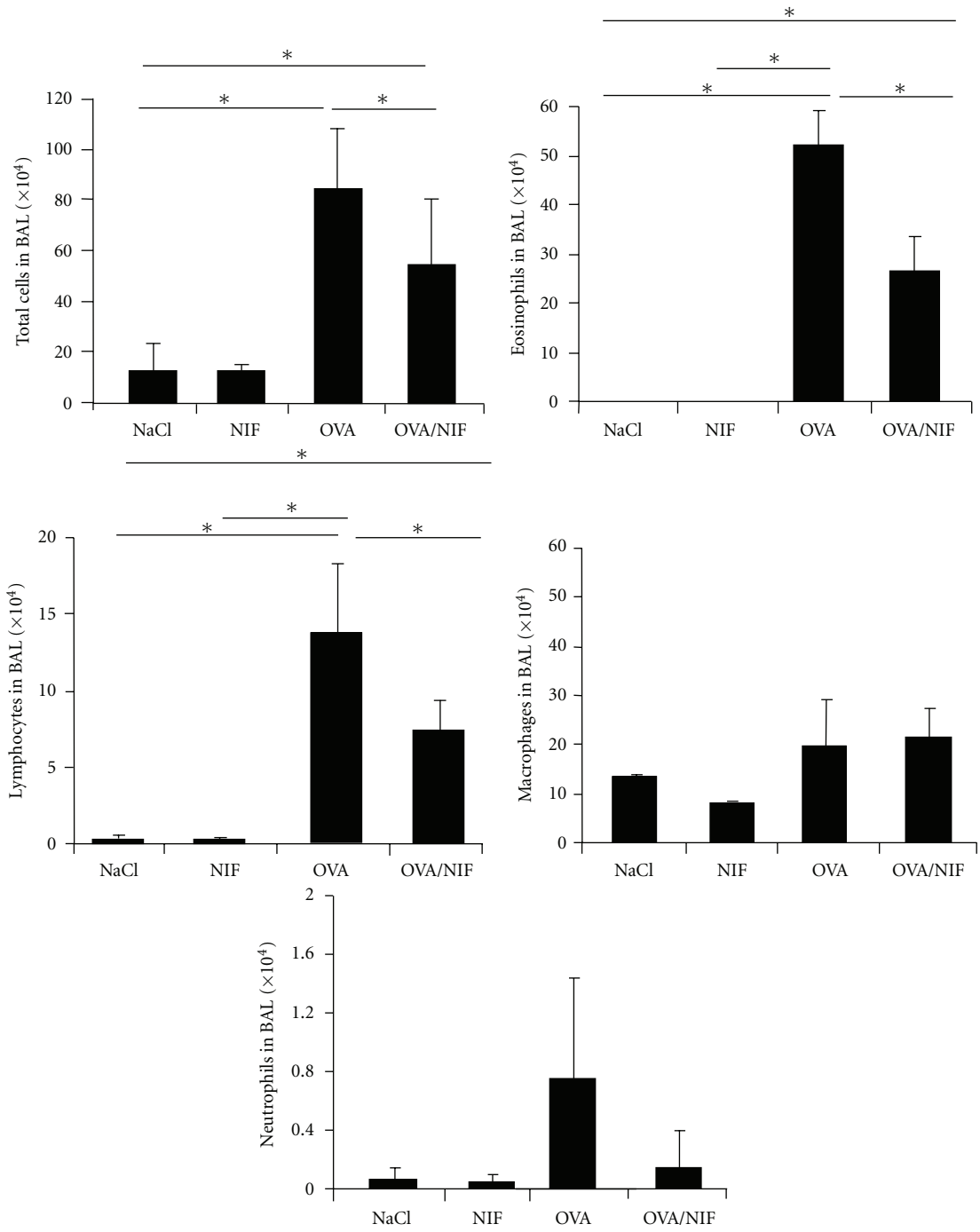

Figure 1: Recombinant NIF inhibits lung eosinophil infiltration, and Th_2 cytokine release. Immunized BALB/c mice were challenged intranasaly with either saline (NaCl), rNIF alone (NIF), OVA alone (OVA), or the combination OVA with 25 μg rNIF (OVA/NIF). Forty-eight hours after the third challenge, bronchoalveolar lavage (BAL) was performed and the cell composition determined. The total numbers of eosinophils (dotted bars), lymphocytes (black bars), macrophages (hatched bars), and neutrophils (white bars) are presented. Figure represents means ± SD of 6 animals per group (* indicates $P < 0.05$).

4. Discussion

Blood eosinophilia is closely associated to parasitosis representing the first line of defense. Once attached to targets, such as larvae of schistosomula mansoni, eosinophils kill by producing large amounts of cationic proteins and oxidative metabolites [25]. Blood eosinophilia is also a hallmark of allergic inflammation, and similar mechanisms may drive eosinophil recruitment and accumulation in the tissue [26]. The discovery and characterization of a potent neutrophil

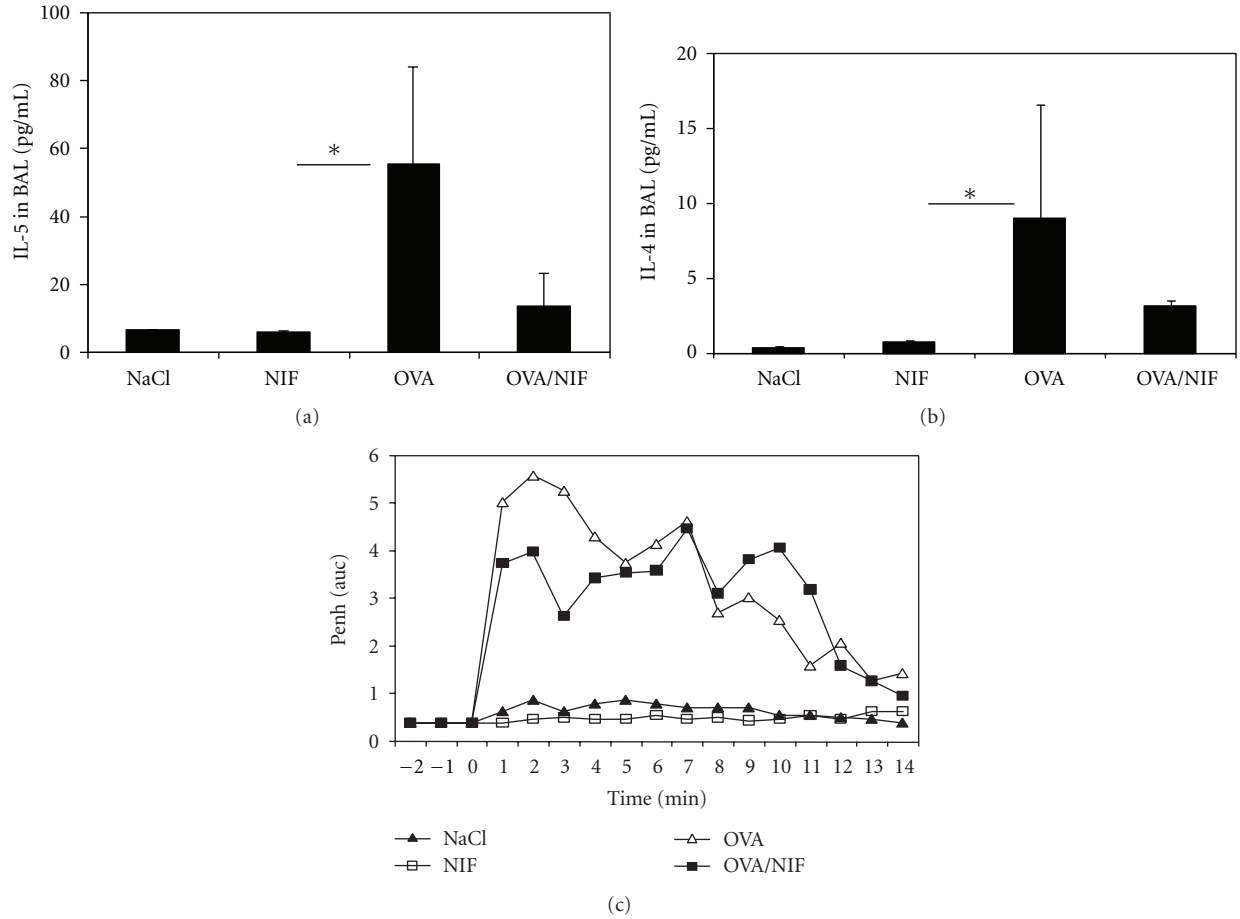

Figure 2: The presence of rNIF reduced IL-5 and IL-4 secretion into the BAL but not acute bronchial hyperreactivity. Forty-eight hours after the third challenge, bronchoalveolar lavage (BAL) was performed. The concentrations of IL-5 ($P < 0.05$; (a)) and IL-4 (ns; (b)) were measured by ELISA. Bronchial hyperreactivity (BHR) to methacholine nebulization was determined 24 h before sacrificing using whole-body plethysmography (c). BHR intensity was measured in Penh arbitrary units after indicated challenges and expressed as AUC values. The results represent means ± SD ($n = 8$ animals per group; * indicates $P < 0.05$).

Table 1: Cumulative effects of blocking mAbs in the presence of rNIF on transendothelial migration of eosinophils across IL-4-activated HUVEC monolayers.

Antigen	mAb	Inhibition of eosinophil transendothelial migration (%)			
		Buffer		rNIF 10 nM	
		Mean	SD	Mean	SD
HLA I α chain	W6/32	9.1	0.7	75.2	0.9
CD18	CLB54	71.3	1.9	100.4	0.4
VLA-4	HP2/1	41.7	2.2	85.7	0.9
CD11a/CD18	25.3	48.7	1.0	76.5	1.2
CD11b/CD18	60.1	71.3	0.9	67.8	1.2
CD11b A-domain MIDAS	107	65.2	1.0	66.1	1.0

Eosinophils were either incubated with the indicated mAbs alone or together with 10 nM rNIF. Data are given as mean of triplicate determinations ±SD.

inhibiting factor, a 41 kD glycoprotein from the canine hookworm (*Ancylostoma caninum*) [14], sheds light to a parasitic survival strategy and provides a concept to attenuate allergic inflammatory response of the host. Whereas many studies have characterized the potential of rNIF to antagonize neutrophil activation and thereby treat postischemic inflammation [27–30], there are no studies published that are focusing on allergic inflammation.

Here we show that rNIF has a dominant antiallergic potential. In OVA-immunized BALB/c mice, rNIF given by the intranasal route with the OVA challenge strongly reduced the number of eosinophils and lymphocytes in the BAL.

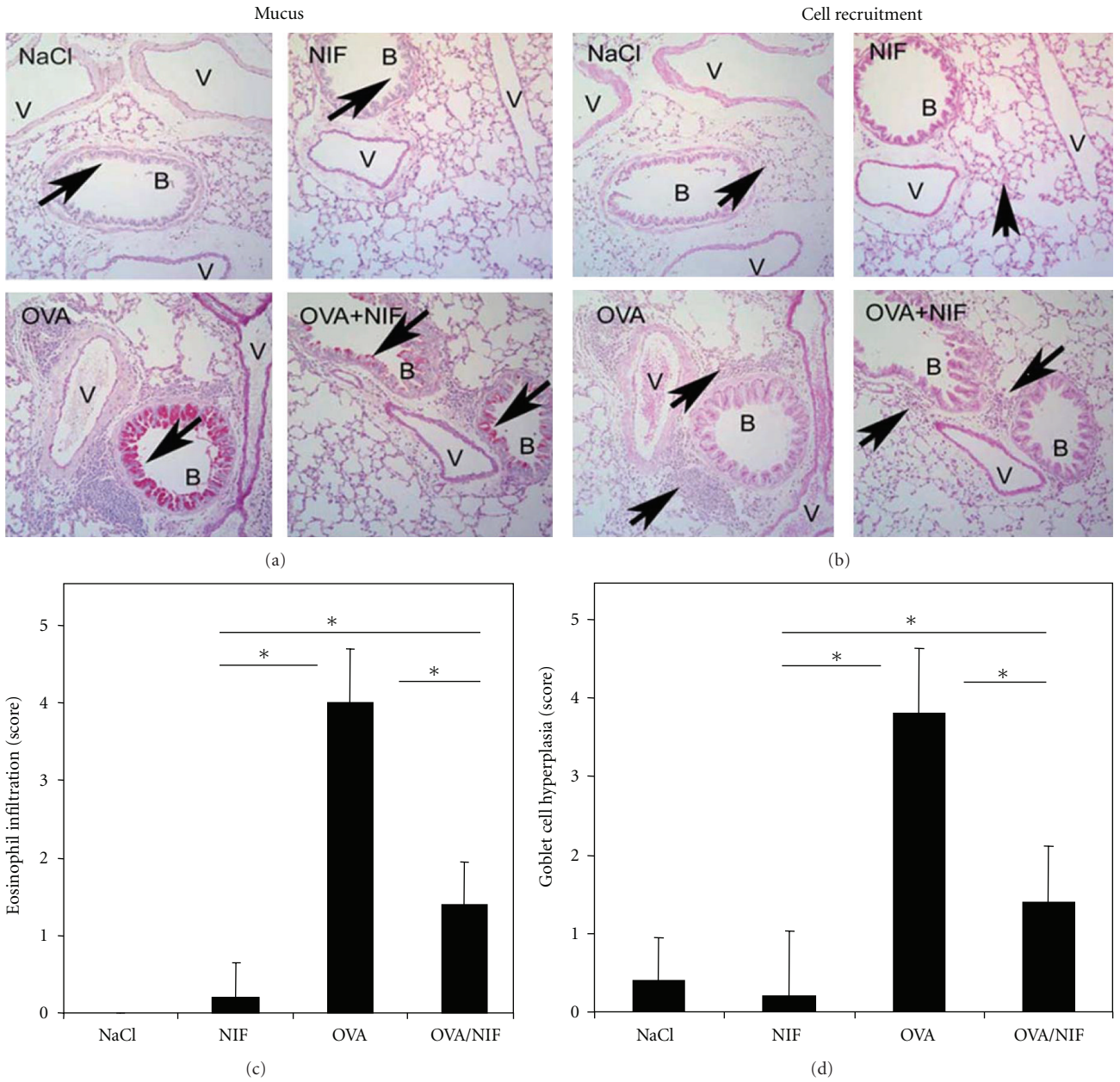

Figure 3: Role of rNIF in cell recruitment to the lungs and mucus hypersecretion. Lung sections of BALB/c mice sacrificed two days after the third challenge with either saline (NaCl), rNIF alone (NIF), OVA alone (OVA), and OVA with rNIF (OVA/NIF) are shown. The formalin-fixed tissue sections were stained with periodic acid Schiff reagent (PAS) to visualize mucus (a) and with hematoxylin/eosin to visualize cell recruitment (b), as shown by the arrows. Representative lung sections are shown at magnification ×20. V: blood vessel; B: bronchioles. Eosinophil recruitment (c) and goblet cell hyperplasia with mucus hypersecretion (d) were assessed semiquantitatively. Results represent means ± SD ($n = 5$ animals per group; * indicates $P < 0.05$).

Furthermore, in the presence of rNIF, IL-4 and IL-5 secretion in the airways was reduced, which may be linked to the diminished recruitment of eosinophils and lymphocytes. Recombinant NIF also reduced mucus secretion and hyperplasia of goblet cells in OVA-induced lung inflammation. In contrast, antigen-specific IgE production was not impaired (data not shown) suggesting that T and B cell interactions established during OVA immunization are not disturbed by rNIF. Similarly, rNIF had no influence on the airway hyperresponsiveness when given together with the OVA challenge.

At the endothelial barrier the process of transmigration orchestrates composition and localization of the leukocyte infiltrate. We and others have shown that IL-4 and IL-13 activate endothelial cells to express VCAM-1 and provoke transmigration of eosinophils and other VLA-4-expressing

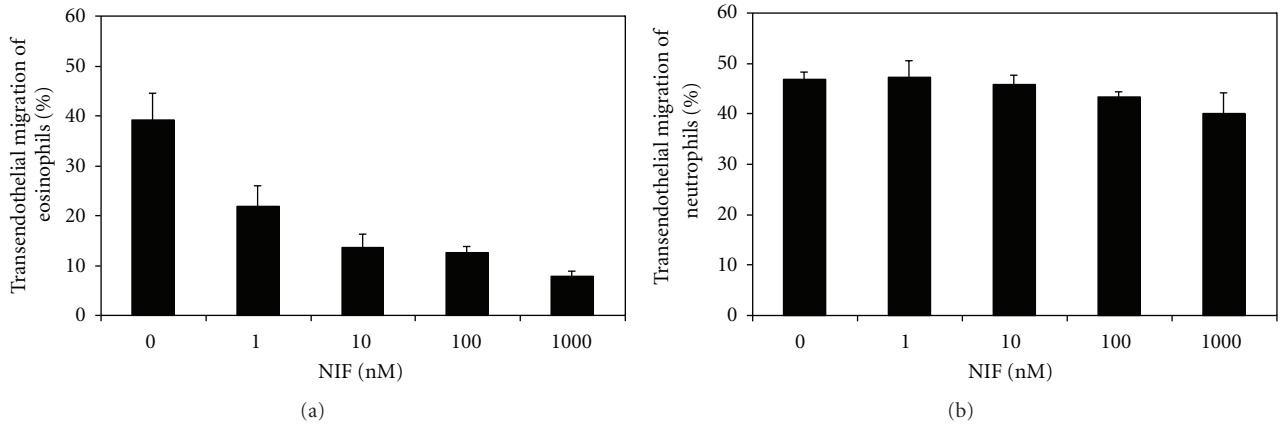

Figure 4: Recombinant NIF inhibits transendothelial migration of eosinophils but not neutrophils. Bilayer vascular constructs were activated with 1 ng/mL IL-4 for 16 h before transendothelial migration of eosinophils was determined in the presence of indicated concentrations of rNIF (a). In separate experiments bilayer vascular constructs were preincubated with 10 ng/mL TNF for 4 h. Thereafter, transendothelial migration of neutrophils was determined in the presence of indicated concentrations of rNIF (b). Graphs depict means of triplicate determinations ± SD representing typical results of a series of 3 independent experiments.

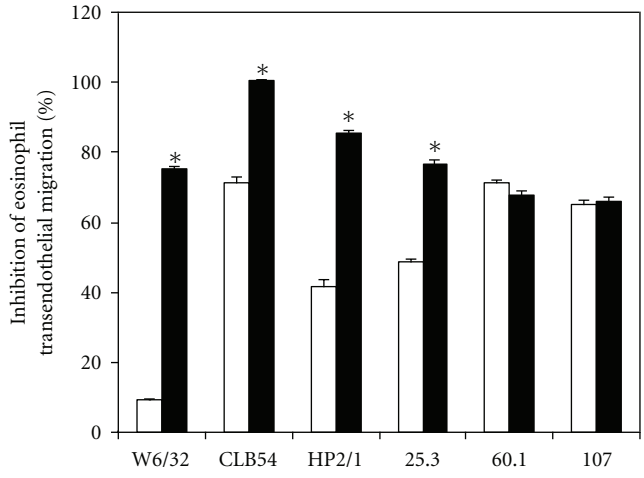

Figure 5: Effects of blocking mAbs in the presence of rNIF on transendothelial migration of eosinophils across IL-4activated HUVEC monolayers. Eosinophils were either incubated with the control mAb W6/32 (anti HLA α-chain) or CLB54 (anti-CD18), HP2/1 (anti-VLA-4), 25.1 (anti-CD11a), 60.1 (anti-CD11b), or 107 (anti-CD11b MIDAS) alone (white bars) or with 10 nM rNIF (black bars). All mAbs were used at saturating concentrations of 10 μg/mL. Results are means of triplicate determinations ± SD of a representative example out of a series of three experiments (* indicates $P < 0.05$).

leukocytes [9, 10, 31, 32]. Recombinant NIF inhibited the IL-4-provoked transendothelial migration of eosinophils in a dose-dependent fashion.

CD18, VLA-4, and CD11a are crucially involved in the transmigration process [4]. Blocking mAbs against these ligands partially blocked eosinophil transmigration, and rNIF enhanced the inhibitory effects providing independent blocking mechanisms. The fact that rNIF binds specifically and with high affinity to recombinant constructs containing to the A-domain of CD11b [15, 16] explains these observation. In contrast, rNIF together with mAb 60.1 against CD11b showed no extra inhibition. Similar data were obtained when rNIF was incubated together with mAb 107 against the metal ion-dependent adhesion site (MIDAS) of the CD11b A-domain. The mAb 60.1 is known to specifically inhibit the adherence of activated neutrophils to nonstimulated endothelium while not impairing adhesion of nonactivated neutrophils to cytokine activated endothelium [33, 34]. The mAb 107 has been shown to preferentially bind to the inactive low-affinity form of the CD11b A-domain, and it was suggested that its antagonistic effect is exerted in part by stabilizing the receptor in the low-affinity state [35]. Together, these data indicate that distinct epitopes in the A-domain are functional in the interaction leading to transmigration of eosinophils. Surprisingly, even at concentrations up to 1 μM rNIF did not inhibit TEM of neutrophils across TNF or IL-1-activated endothelial cells. These experiments cast doubt on the potency of rNIF to block neutrophil invasion in acute inflammation.

Still, the A-domain of CD11b is crucial for neutrophil spreading, chemotaxis, adhesion-dependent degranulation, and superoxide generation and rNIF substantially inhibits these functions. In addition, adherence of activated neutrophils and eosinophils to unstimulated endothelial cells in culture is CD11b/CD18 dependent [36–39]. All these interactions mediated by activated granulocytes are strongly inhibited by rNIF [14]. On the other hand, non-activated neutrophils interact with cytokine-activated endothelial cells in a sequential interaction leading to transendothelial migration *in vitro* and to the rapid localization of neutrophils in microbial infections and acute inflammation [5]. Although the A-domain of CD11b is indispensable (for review see [40]), its function is complex involving both inside-out and outside-in signals that convert conformational changes in

integrins to control ligand binding affinity [41]. Given that rNIF binds specifically and with high affinity to the A-domain of CD11b our data support the evidence that the blocking function of NIF is restricted to distinct functional epitopes and does not imply a total blockade of the A-domain [15, 16].

Together, the study shows congruent *in vivo* and *in vitro* data suggesting that rNIF operates at the crossroad of antiparasitic defense and allergic inflammation by interfering with the recruitment of eosinophils at the endothelial barrier and thereby modulates allergic inflammation.

Abbreviations

BAL: Bronchoalveolar lavage
EPO: Eosinophil peroxidase
rNIF: Recombinant Neutrophil Inhibitory Factor
TEM: Transendothelial migration.

Authors' Contribution

R. Moser and B. Schnyder have shared senior authorship.

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Review Article

Lung Dendritic Cell Developmental Programming, Environmental Stimuli, and Asthma in Early Periods of Life

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Dendritic cells (DCs) are important cells of our innate immune system. Their role is critical in inducing adaptive immunity, tolerance, or allergic response in peripheral organs—lung and skin. The lung DCs are not developed prenatally before birth. The DCs develop after birth presumably during the first year of life; exposures to any foreign antigen or infectious organisms during this period can significantly affect DC developmental programming and generation of distinct DC phenotypes and functions. These changes can have both short-term and long-term health effects which may be very relevant in childhood asthma and predisposition for a persistent response in adulthood. An understanding of DC development at molecular and cellular levels can help in protecting neonates and infants against problematic environmental exposures and developmental immunotoxicity. This knowledge can eventually help in designing novel pharmacological modulators to skew the DC characteristics and immune responses to benefit the host across a lifetime.

1. Introduction

Asthma is a serious pulmonary disease that affects about 300 million people worldwide [1], and 8.2% (about 25 million) of the population within the USA [2]. A significant number of patients develop asthma during early childhood. A number of cross-sectional and longitudinal cohort studies in adult asthmatic patients suggest that the childhood asthma poses a risk for more severe asthma or relapse during adulthood [3–6]. One among ten children has asthma, and this trend has increased over the recent years [7]. Characteristics (airway obstruction, airway hyperresponsiveness, atopy, and recent wheeze) observed in children have been reported as predictors of asthma symptoms in adulthood. This is supported by evidence that the sensitization to allergens at young age increases the likelihood of asthma in adulthood [8–11].

In allergic asthma, an immune reaction is caused by inhaled allergens with an overwhelming inflammatory response and obstruction in the airways. As a first step in sensitization, the antigen presenting cells (dendritic cells—DCs, macrophages, and lung epithelial cells) take up and process the inhalant allergens. The DCs are recognized as the key immune sentinel cell in the peripheral organs, including lung [12], and are at the cross-roads of inducing tolerance or inflammation [13]. The DCs are activated directly or via cell-cell interaction [14]. The activated DCs in turn stimulate T and B cells and other immune cells, which release a variety of cytokines, chemokines, and chemical mediators. These mediators are responsible for affecting the local microenvironment and generating inflammation and obstruction in airways. Traditionally, asthma has been known as the Th2-mediated disease (Figure 1). Both Th2- and non-Th2-dependent immune elements and mechanisms are now recognized for a number of phenotypes and endotypes of asthma [15–17].

While asthma phenotypes and endotypes are not fully characterized, resident lung DC types could be important [12, 18–22]. Lung DCs exhibit unique phenotypes than

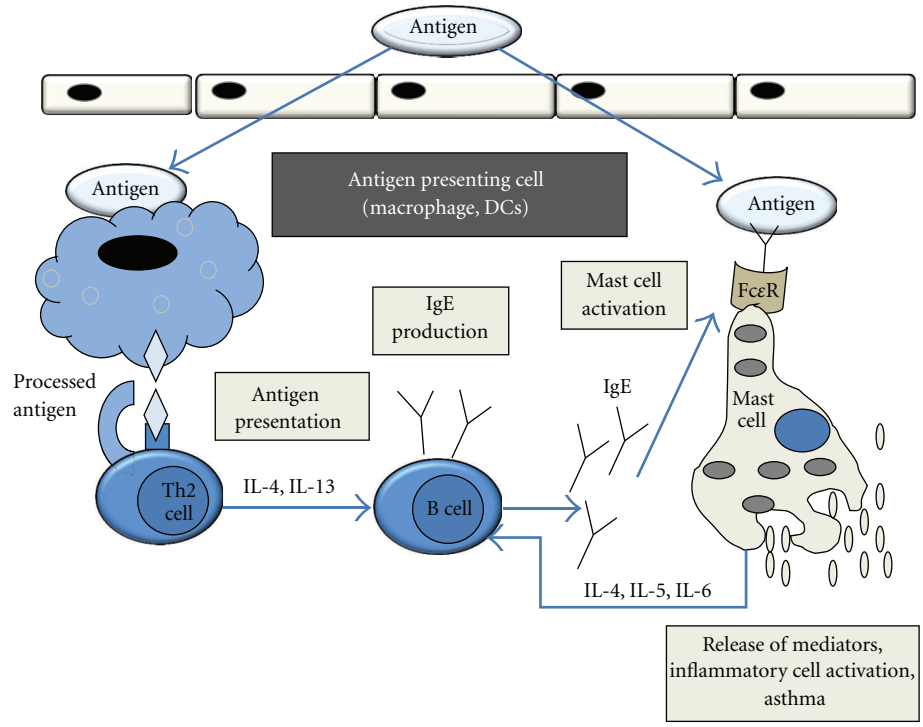

Figure 1: An illustration depicting the types of immune cells involved in a Th2-mediated allergic response. Antigen presenting cells (macrophages or DCs) take up the antigen, process it, and present it on the MHC molecule on the cell surface. The antigen presenting cells induce naïve T cells towards Th1 or Th2. Th2 response is mainly responsible for downstream events that include activation of B cells, production of IgE, and binding of IgE to Fcε receptor on the cell surface of mast cells, resulting into mast cell degranulation and inflammation.

those present in other organs or in circulation [23], and are distributed throughout the alveolar epithelium, alveolar parenchyma, and nasal mucosa [24, 25]. A variety of investigations in rodents and human patients have reported the importance of different lung DC types in asthma [26–29]. This corresponds with the reported results on alterations in selective DC populations in the bronchoalveolar lavage fluids (BALFs) and in peripheral blood of patients with asthma (Table 1). A number of distinctive reports are available in the literature on the characteristics and functions of lung DC types [23, 30, 31], and are not reviewed here. In this paper, we provide an overview of lung DC development, exposure to pathogens, allergens, and environmental chemicals during early childhood, and their long-term impact on asthma development.

2. Critical Window of Immune Vulnerability

Over the last several years, prominent research studies have demonstrated that the late fetal and early postnatal periods are phases of reduced immune competence [12, 32, 33]. This reported reduced immune competence corresponds with the immaturity of immune system. In particular, the lung DCs are underdeveloped prenatally and close to term at birth [18, 34–38]. The development of lung DCs during infanthood has not been studied. Concurrent exposures to allergens and other environmental factors during this "critical window of immune vulnerability" have the potential to program DC types, DC functions, and DC-mediated tolerance or sensitization, which can have long-term respiratory and immunological consequences (Figure 2) [32, 39, 40].

3. Developing Immune System and Exposure to Asthma-Triggering Agents in Early Childhood

Particular events during early childhood can set the stage for specific developmental programming of DCs. The flexibility of a developing immune system and simultaneous exposure to allergens and other environmental stimuli can be important compounding factors for both the establishment and a long-term persistence of asthma.

In addition to allergens, other risk factors for DC developmental programming towards particular DC phenotype and function during this "critical window period" may include environmental chemicals, drugs, certain dietary factors, infectious agents, and physical and psychological stressors. Not surprisingly, research studies suggest that the maturation and function of DCs are shifted by some of these predisposing risk factors. These include heavy metals, such as lead [41], and air pollutants particularly those from traffic [42] and environmental tobacco smoke [43]. Among relevant shifts that have been reported are: (1) reduced expression of Toll-like receptors (TLR)2 and TLR4 [44], (2) shift to

Table 1: DC subsets in patients with asthma.

Clinical Condition	Altered DC phenotypes	References
Asthma	pDC (HLA-DR+, CD123+) increased in BALF mDC (HLA-DR+, CD11c+) increased in BALF	[99]
Allergic asthma	mDC (BDCA-3+, mannose receptor+) increased in BALF	[100]
Allergic asthma	pDC (BDCA4+) with increased FcεRI in blood mDC (CD1c+) with increased FcεRI in blood	[101, 102]
Asthma	Increased DC proportions in peripheral blood	[103]
Allergic asthma patients challenged with allergen	Increased pDC and mDC in sputum	[104]
Asthma	Increase in pDC1 and pDC2 expressing FcεRI	[105]
Repeated exposure to allergen	Depletion of mDCs	[23]
Asthmatic children	DC2 (CD11c−, CD123high+) decreased in blood	[106]
Asthmatic patients	Increased CD1a+ cells in bronchial mucosa	[107]
Experimentally elicited allergic rhinitis	pDC increased in nasal mucosa	[108]
Asthma	pDC increased; decreased mDC : pDC ratio in blood	[109]
Children with asthma	Deficiency of circulating pDC	[110]
Atopic patients with chronic rhinosinusitis	Increased FcεRI on DC (CD1+)	[111]

Abbreviations: BALF: bronchoalveolar lavage fluid, mDC: myeloid DC, pDC: plasmacytoid DC, BDCA: blood dendritic cell antigen.

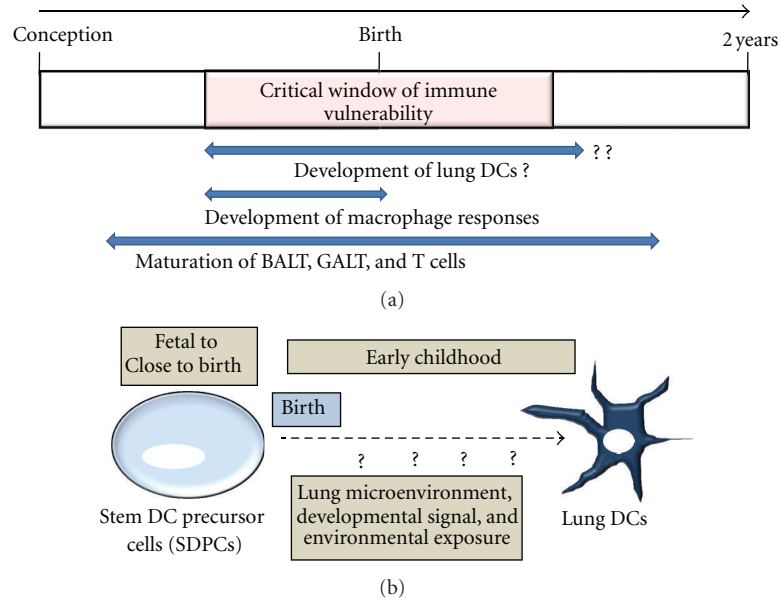

Figure 2: Immune development during critical window of vulnerability. (a) Timeline of maturation of bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT), T cells, and macrophages. (b) Lung-resident SDPCs could be the plausible source of lung DCs in early childhood. The timing of lung DC development, environmental factors triggering this transition, and signaling mechanisms involved in DC development remain unknown.

Th2-biased adaptive immune response [45], and (3) promotion of misregulated (unresolved) inflammation [46, 47].

A number of recent studies have demonstrated that early exposures to *Chlamydia muridarum* (an intracellular pathogen) [48–50], Bacillus Calmette-Guérin (BCG) [51], and influenza A virus [52] alter the immune responses against allergens in adulthood via affecting the DC types and functions. Bacterial infections or stimulation with TLR4 ligand (Gram-negative bacteria-derived lipopolysaccharide) have been shown to skew the T-cell response to Th1 type during childhood [53]. In this regard, "Probiotics" and "Hygiene hypothesis" have been discussed elsewhere in the literature for controlling asthma-related immune response [54, 55].

4. Respiratory Syncytial Virus (RSV) Infection and Asthma

Respiratory syncytial virus infection is the most common cause of bronchiolitis and pneumonia in children under 1 year of age (Centers for Disease Control and Prevention, Atlanta, GA, USA). Severe forms of the RSV lower respiratory

tract infections (LRTI) are characterized by airway obstruction and prominent wheezing. Furthermore, RSV infection in infancy has been linked to the development of asthma in childhood. Thus, there has been great interest in determining whether the pathogenesis of RSV bronchiolitis in infancy induces a persistent Th2 bias, leading to the development of Th2-dependent asthma in later childhood.

Some studies have focused on the expression of the prototype Th1 cytokine, interferon gamma (IFN-γ), and the Th2 cytokine, interleukin-4 (IL-4). Bendelja and colleagues studied the expression of IFN-γ and IL-4 in peripheral blood lymphocytes (PBL) of infants with various forms of RSV infection [56]. Among RSV-infected infants, the percentage of PBL positive for IL-4 was slightly greater than the percentage positive for IFN-γ, thus suggesting a Th2 bias. However, the expression of IL-4 was greater in subjects with mild upper respiratory tract infection (URTI) than in subjects with bronchiolitis or pneumonia. IFN-γ expression remained unaffected. Therefore, patterns of IFN-γ and IL-4 expression by PBL could not be associated with the severity of RSV infection.

Others have determined the quantities of IFN-γ and IL-4 cytokines in respiratory tract secretions of infants with RSV infections. In one study, IFN-γ was found to be the predominant cytokine in subjects with all forms of respiratory tract illness related to RSV infection, with slightly higher ratios of IFN-γ to IL-4 in those with LRTI in comparison to those with URTI alone [57]. A second, larger study similarly demonstrated that the Th2 cytokines, IL-4, IL-5, and IL-13, were usually undetectable in secretions from infants with all forms of RSV infection. IFN-γ appeared to be protective against severe illness, in that IFN-γ concentrations were greater in subjects with milder, nonhypoxic forms of RSV-induced LRTI than in those with more severe LRTI accompanied by hypoxia [58]. In all of these studies, the differences in ratios of IFN-γ to Th2 cytokines were determined only by variations in IFN-γ concentrations between the groups. The findings of subsequent studies have also suggested a protective role for IFN-γ in RSV infection of infants [59, 60].

How the DC phenotypes and DC-induced T-cell responses are skewed following RSV infection is an important question, and could provide clues to the severity of the disease and predisposition to asthma. Infection of human infants with RSV and other viruses is followed by the appearance of DCs in nasopharyngeal and tracheal secretions [61, 62]. RSV infection of monocyte-derived DCs causes maturation of the cells, with expression of costimulatory molecules that participate in the instruction of T cells. However, it results in impaired CD4-positive T cells [63]. Although DC types were not addressed, the lung tissues from infants with fatal RSV demonstrated a lack of CD8−positive T cells [64]. Studies in mice have suggested unique roles for myeloid (mDC) and plasmacytoid DCs (pDC) in RSV infection. The pDCs in RSV-infected mice reduce the viral replication, while depletion of pDCs results in enhanced inflammatory responses and greater airway hyperreactivity [65]. A balance between mDC and pDC seems to determine the immune responses to RSV and airway reactivity following RSV infection [66]. The recruitment and activity of DC subsets occurring after RSV infection could skew immune responses toward either Th1 or Th2 cytokine pathways, thereby determining the eventual development of atopic disease or long-term airway hyperreactivity following RSV infection in infancy.

5. Lung DC Development

Despite growing understanding about the DC characteristics and functions in adult patients and animal models, the natural processes of lung DC development in prenatal or neonatal phase, as well as differentiation, maturation, and functional specialization of DCs, have not yet been studied. An understanding of perinatal and infant DC maturation in environmentally exposed tissues (including lung) is critical to better manage immune maturation for a healthier life course.

Histological details reveal that the MHC class II-positive cells start to appear in lung tissues of rat and human fetuses at 30–58% of term. Since the MHC class II is expressed ubiquitously by a variety of immune and nonimmune cells, the interpretation may not be DC specific. The appearance of MHC class II-positive DCs increases only after birth [37, 67–70].

Importantly, the airway structures and epithelial cell system are also not fully developed at the time of birth or during the neonatal period. It is reasonable to believe that direct cell-cell interaction or chemical mediators and growth factors released by another cell type can have a significant impact on the establishment of normal lung DC infrastructure as the lung microenvironment and the normal lung physiology evolve [13, 71, 72]. Repeated exposure to potentially harmful allergens or other environmental stimuli during this period can significantly affect the DC programming, phenotypes, and functions. Although it remains to be studied, these events may prompt a long-term memory for the generation of asthma-promoting DCs.

6. Animal Models for Studying Lung DC Development

Technical and ethical issues related to the availability of neonatal and infant lung tissues limit the enthusiasm to conduct studies that address the issues related to lung DC development. Studies are limited to first challenging the neonatal animal with infectious or allergenic stimuli and then investigating the DC types and functions later in life in the same animal. This approach may not adequately reflect the dynamic process of DC development or programming during early childhood in humans. Since DCs make <1% of total lung cells [34], it is not possible to harvest sufficient DC populations from rodent pups because of their small-sized lung. Hundreds of small-sized, age-matched mouse pups (most commonly used model) would be needed to harvest an adequate number of cells; it is almost impossible to have simultaneous births and enough age-matched mouse progeny available for this purpose. Also, the cells cannot be

pooled from pups born close together, because murine pups (between birth and one-month) age at 150 times faster rate than humans [73]. There are significant differences in the lung anatomical [74], developmental, and immunological aspects of humans and mice (e.g., the lymphocyte and neutrophil distribution in blood, DC phenotypes) which make it difficult to interpret and translate the results to human infants [75–77]. As such, murine DC precursors/DC phenotypes are different from those reported in humans [23, 78]. Cellular intermediates within the hematopoietic stem cell hierarchy tree have been identified in tissues of mice and humans [79, 80]; significant differences have been noted in regards to their subsets and frequency [78]. Lack of reagents and paucity of information on DC-precursors or DCs limit such studies in other rodents. These studies are also not possible in human neonates or infants due to ethical reasons. Large animal models are expensive and require diligent work; these models can mimic the conditions of human infants very closely. To this effect, an asthma model is available in *Rhesus* monkeys. Similarities have been reported in asthmatic response among *Rhesus* monkeys and humans; the house dust mite antigen induces asthma conditions with clinical profiles; the biochemical and immunological markers are similar to those in human patients of asthma [74, 81, 82].

The baboon model seems ideal for studying the early human immune maturation and the developmental programming [83] because of similarities in ontogeny, immunology, reproductive physiology, placentation, and maternal-fetal transfer [84–88]. They are very close to humans in the evolutionary tree [89], and the lung development pattern in preterm baboons is similar to that found in preterm human babies [90]. Some of the immunological aspects [91–93], the bronchoconstriction, and the airway response against platelet-activating factor [94] in baboons are also analogous to those of humans and asthmatic patients, respectively. The advantages of the baboon over other commonly used primates, such as *Rhesus* monkey, include the ease of timed pregnancies due to the estrogen-sensitive sex skin in cycling females, the availability (baboons breed year-round), and the relative ease of handling (reviewed in [91]). Moreover, it allows harvesting of sufficient number of cells of interest from relatively large tissues of neonate and infant baboons.

We have studied the development of pulmonary innate immunity, including DCs, in a non human primate baboon (*Papio* species) model [34, 35, 95–97]. We have investigated the DC phenotypes and functions in prematurely delivered and close-to-term baboons (67–95% of gestation). Our results demonstrate that lung DC population having low density (similar to those of adult baboon lung DC population) remains underdeveloped until close to birth [34]. Since we do not know the stage of differentiation and the cell subsets, it is probably more appropriate to call them stem DC precursor cells (SDPCs) [36]. We have recently observed that the SDPCs can differentiate into DCs *in vitro* under DC-promoting conditions (Figure 3). A significant increase in expression of DC markers and tentacles is observed over time. It has been proposed by others that the tissue DCs can be generated from hitherto unknown resident stem cell populations. Isolation of stem cells has been reported from adult human lung [98]; their differentiation into lung DC-precursors or DCs has not yet been studied.

7. Molecular and Immunologic Basis for DC Programming and Therapeutic Opportunities

In summary, our results indicate that the lung DCs are not developed at least until birth. We do not know when the DCs develop after birth during childhood, what triggers the lung DC development, and how the pathogenic stimuli affect the DC development leading to DC phenotypes with different functions. We speculate that pathogenic stimuli, such as allergens, may alter the lung DC developmental programming or SDPC→DC transition during early childhood in a way that an immunological memory is created for generation of asthma-promoting DC phenotypes or immune responses. Persistent reexposure to allergens may bolster the generation of these DC phenotypes so that an allergic response is maintained for a long time.

An understanding of the basic immunobiology of DC development and the early programming of asthma-promoting DC phenotypes and functions against allergenic stimuli can pave the way to identify the basis of childhood asthma. Although it can be challenging to study the molecular mechanisms for lung DC development *in vivo*, studies with baboon lung SDPCs and DCs can provide useful tools to unravel the molecular and immunologic basis of the lung DC development and develop novel pharmacological modulators. A focused effort in this direction can provide a unique opportunity to effectively manage the pediatric immune system for optimized maturation and reduced health risks. This would include opportunities to intervene at an early age and skew the DC programming towards normal DC phenotype and function using pharmacological modulators. Additionally, this information may be useful in better protecting neonates and infants from environmental insults that increase the later-life health risks. It is the magnitude and persistence of downstream immunoinflammatory effects of tissue DC function that position this topic as a central health issue for allergic and other chronic diseases.

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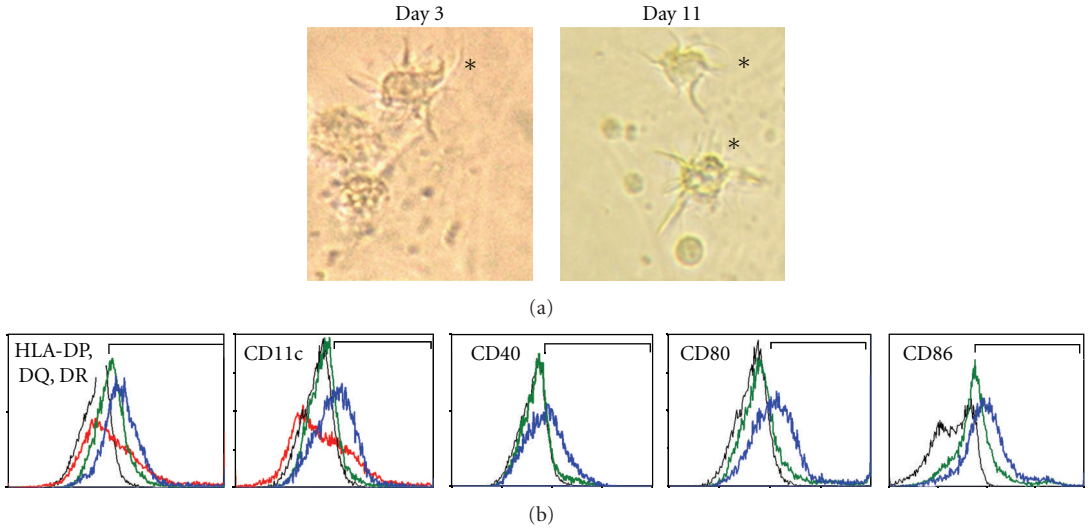
Authors' Contribution

All authors have contributed and reviewed the paper. Specific contributions by the authors are as follows: B. Singh compiled Table 1 in the paper. R. C. Welliver and R. R. Dietert contributed to the text related to RSV, critical window of immune vulnerability, and environmental exposures, respectively. S. Awasthi conducted studies related to the baboon DCs in her lab and coordinated with co-authors for compilation of this paper.



Markers → / Days in culture ↓	HLA-DP, DQ, DR	CD11c	CD40	CD80	CD86
Isotype control	4.4% (160)	9% (148)	4.4% (160)	9% (148)	5.3% (79)
6 days	30% (366)	19% (355)	10% (280)	28% (1601)	39% (328)
11 days	37% (629)	44% (322)	26% (382)	54% (1049)	61% (295)
17 days	41% (617)	34% (355)	Not done	Not done	Not done

(c)

Figure 3: The SDPCs harvested from a close-to-term fetal baboon differentiate into DCs when cultured in presence of GM-CSF, IL-4, and TNF-α. The lung SDPCs were harvested on OptiPrep density gradient as per the method published earlier [34]. (a) Photomicrograph showing cells with dendrites (*). (b) Flow cytometry data showing increase in DC-marker expression. Black line: isotype control antibody-stained cells, green line: 6 days, blue line: 11 days, red line: 17 days—cells stained with antibodies to particular marker. (c) Data in the table shows % cells (fluorescent intensity) gated in the marked region of histogram charts in (b) staining positive for the specific marker.

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Review Article

Keratinocytes under Fire of Proinflammatory Cytokines: Bona Fide Innate Immune Cells Involved in the Physiopathology of Chronic Atopic Dermatitis and Psoriasis

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Cutaneous homeostasis and defenses are maintained by permanent cross-talk among particular epidermal keratinocytes and immune cells residing or recruited in the skin, through the production of cytokines. If required, a coordinated inflammatory response is triggered, relayed by specific cytokines. Due to numerous reasons, troubles in the resolution of this phenomenon could generate a cytokine-mediated vicious circle, promoting skin chronic inflammation, the most common being atopic dermatitis and psoriasis. In this paper, we discuss the biological effects of cytokine on keratinocytes, more particularly on specific or shared cytokines involved in atopic dermatitis or psoriasis. We report and discuss monolayer or 3D in vitro models of keratinocytes stimulated by specific sets of cytokines to mimic atopic dermatitis or psoriasis. IL-22, TNFa, IL-4, and IL-13 combination is able to mimic an "atopic dermatitis like" state. In psoriasis lesions, over expression of IL-17 is observed whereas IL-4 and IL-13 were not detected; the replacement of IL-4 and IL-13 by IL-17 from this mix is able to mimic in vitro a "psoriasis like" status on keratinocytes. We conclude that specific cytokine environment deregulation plays a central role on skin morphology and innate immunity, moving towards specific pathologies and opening the way to new therapeutic strategies.

1. Introduction

Skin constitutes the largest physical and chemical barrier against various stresses including pathogens, constituting the first line of defense of the body. Cutaneous homeostasis and defenses are maintained by permanent cross-talk among dermal fibroblasts, epidermal keratinocytes, and cells of the immune system residing or recruited in the skin, through the production of cytokines. Recent years showed that skin appears as a multitasking immune organ, and highlighted the key role of keratinocytes, which are the epithelial cells of epidermis (the outer skin layer in contact with environment), in this protection [1]. Keratinocytes are no longer considered as a passive protection barrier but as true innate immune cells. Indeed, skin represents a very attractive tissue that is a paradigm for studying the cross-talk between innate and adaptive immunity system and an organ.

To be effective for protection against a large panel of injuries, skin requires sensitivity and selectivity to detect a signal of danger, a strong reactivity to develop a rapid response, and efficiency. These requirements need sophisticated interactions between keratinocytes and "sentinel" immune cells infiltrating normal epidermis, that is, T lymphocytes and Langerhans cells [2]. These interactions are conducted by cytokines, maintaining the homeostasis of skin; if required, a coordinated inflammatory response is triggered, relayed by specific cytokines. Due to a number of known or unknown reasons (genetic, environmental, etc.), troubles in the resolution of this phenomenon could generate a cytokine-mediated vicious circle, promoting

chronic inflammation. This state is characterized by resident/infiltrating immune cells in the epidermis or dermis, altered differentiation of keratinocytes and the increase of epidermal thickness in some cases. They are the result in particular of an altered dialogue between keratinocytes and activated immune cells, due on a balance of Th subsets, especially Th1, Th2, Th17, and Th22 producing specific sets of cytokines. Various inflammatory responses can be observed, leading to different clinical entities, the most common being atopic dermatitis (AD) and psoriasis. AD affect up to 3% of adults and 25% of children, and psoriasis 2.5% of the world's population [3]. Both are classically considered as two opposing models due to the polarization of the Th response.

Atopic dermatitis has a complex pathogenesis associating inflammatory reactions to epidermal barrier dysfunctions, allowing allergen sensitization. Genetic studies showed the importance of filaggrin in AD [4]. Filaggrin is a protein of the stratum corneum involved in the maintenance of keratin cytoskeleton, the assembly of the cornified envelope, and the water-binding capacities of skin. Inflammatory cytokines are implicated in skin barrier disruption by downregulating the protein expression of the cornified envelop, including filaggrin.

Chronic lesions of AD are characterized by lichenification with skin thickening and hyperplasia of the epidermis.

Plaques of psoriasis are characterized by parakeratosis, but also skin thickening and acanthotic epidermis with abundant dermal mononuclear cell infiltrate. These two inflammatory chronic skin disorders were opposed by the long standing Th1/Th2 paradigm, despite the fact that they share some histological similarities. The discovery of a role for IL-17-producing T helper cells (Th17) in psoriasis and for IL-22 producing (Th22) cells in AD emphasize the complexity of cytokine network involved in the induction and/or maintenance of these disorders.

Our paper is focused on the cytokines associated to AD compared to those in psoriasis and their direct biological effects on keratinocytes. For a general recent review comparing clinical features, immune cells, and therapeutic strategies in AD and psoriasis, refer to Guttman-Yassky et al. [2, 3]. Likewise, review and the experimental studies reported herein are focused on the chronic phases, occulting the description of initiating events and earlier phases of these skin diseases.

We study *in vitro* the respective effects of AD or psoriasis-associated cytokines on parameters of innate immunity and histology using the (normal human epidermal keratinocytes) NHEKs and (reconstituted human epidermis) RHE, and discussed the results in the context of Th polarization. We shall see that the inflammatory skin phenotype is largely the consequence of the effect of cytokines on keratinocytes. We will comment on the view that, if AD and psoriasis were opposed by long standing paradigms, they also have common features.

2. Cytokines and Keratinocytes

During the past few years, an increasing number of reports demonstrated that keratinocytes are direct targets for a specific set of cytokines, conducting dramatic changes in their biological properties such as inducing the secretion of chemokines and antimicrobial peptides or modulating the differentiation states or migration capacities. In the 1990s, functional receptors for the classical inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF) or the Th1-derived interferon (IFN)-γ have been described on keratinocytes (review in [5]). More recently, IL-17, IL-21, IL-22, and oncostatin M (OSM) have also been described as important regulators of epidermis functions, and their receptors have been detected on keratinocytes [6–9]. Other cytokines are able to induce skin inflammation in animal models, as do IL-12 or IL-23. However, keratinocytes do not display functional receptors for IL-12 and IL-23 [10], suggesting that other cell types are the targets, and that the effect on keratinocytes is indirect.

As an example, amongst the cytokines of the IL-10 family, IL-22 is the strongest activator of keratinocytes, and to a lesser extent IL-24, followed by IL-20 and IL-19 [11]. Analysis of the gene expression profile induced on NHEK showed that these cytokines upregulate the expression of genes associated with inflammation and innate immunity, such as S100A7-psoriasin and β-defensin2 (BD-2); they also downregulate differentiation-associated genes, including CK10, and induce hyperplasia and hypogranulosis of RHE [11, 12] and upregulated CK16, which is associated with suprabasal keratinocyte proliferation.

The simultaneous analysis of a large panel of cytokines demonstrate that a number of them directly target keratinocytes, that is, cytokines of the IL-1 (IL-1α and β), IL-2 (IL-4, IL-13, IL-21), IL-4 (IL-4, IL-13), IL-6 (IL-6, OSM, IL-31), IL-10 (IL-19, IL-20, IL-22, IL-24), IL-17 (IL-17A and IL-17F), IFN (IFNα, IFNγ), or TNF (TNFα, TNFβ) family [5]. Note that the biological effect of IL-4 or IL-13 on the inhibition of defensin production is observed only after preactivation of keratinocytes by the IL-4, IL-13, IL-22, and TNFα cytokine combination, which enhance IL-13RA2 chain expression as defined below. Regarding IL-21 receptor, IFNα and γ enhance the IL-2Rγ chain expression; since IL-21R is also expressed by keratinocytes (unpublished data), it suggest the sensitivity of keratinocytes to IL-21. It has been confirmed by Caruso et al, demonstrating that IL-21 induces *in vitro* the proliferation of isolated keratinocytes [13] and induces Erk phosphorylation (unpublished data). In contrast and in our hands, there is no evidence for a direct effect IL-2, IL-7, IL-9, and IL-15 on keratinocytes [14]. Transcriptomic analysis of the keratinocyte gene expression profile demonstrates that most cytokines active on keratinocytes have overlapping activities, sometimes displaying completely redundant biological properties. The use of shared molecules in intracellular signaling pathways, such as STAT3 in OSM or IL-22 signaling or NFκB in IL-1 or TNF signaling could explain redundancy [6, 7]. Recently, the IL-36 members of the IL-1 family have been reported to induce

by an autocrine effect their own secretion and antibacterial peptides production by keratinocytes [15].

Usually upstream of cytokine action during an inflammatory process, TLR engagement activates NFκB pathways, as IL-1 or TNF receptors engagement do, and may have partial redundant activities with these cytokines on keratinocytes. Keratinocytes express TLR 1-6 and 9 [16, 17]. TLR engagement have been described to induce cytokines, such as TSLP [18] and IFNα and β. Synergistic and/or overlapping biological activities of TLR ligands and cytokines on keratinocytes are very likely, as discussed below for cytokine combinations. Poorly documented up to now, the study of these costimulations could give a more dynamic view of the course of skin inflammation.

In another hand, powerful synergistic effects on keratinocytes have been also evidenced when cytokines of different families are associated [14]. These cytokines modified the expression of genes associated with inflammation, innate immunity, and differentiation. However, comparative and quantitative analysis demonstrates huge differences of expression induced by specific cytokines. For example, IL-22, as OSM, both via the STAT3 activation pathway induced a strong inhibition of keratinocyte differentiation, as objectived by the decreased expression of filaggrin, loricrin and involucrin and the increase in the overall thickness of RHE [6, 7]. In contrast, the induction of chemokines, S100A7, or BD-2 by IL-22 and OSM is moderated. On the other hand, whereas IL-17, IL-1, and TNFα strongly induced chemokines, S100A7, or BD-2 expression, they have weaker effect on keratinocyte differentiation [14, 19]. Nevertheless, it appears that single cytokine stimulation generates a rather limited effect on keratinocytes, namely, a limited number and/or a limited modulated expression of targeted genes.

Since in physiological or physiopathological conditions, tissues are surrounded not by one cytokine but a complex milieu, study of the biological activities of cytokine combinations is of great interest. For example, combination of IL-17A and IFN-γ or IL-17A and TNF-α results in a synergistic effect on CXCL8 production by keratinocytes [20, 21]. IL-17A and IL-22 synergize in the upregulation of BD-2 and S100A9 production [22, 23]. The association of IL-1α, IL-17, IL-22, OSM, and TNFα demonstrated a very strong synergy in increasing the expression of inflammatory molecules such as psoriasin/S100A7 or BD-2, or IL-8 *in vitro* by NHEK [14]. When IL-22 is removed of the cytokine mixture, CXCL8 and BD-2 expression is reduced by 30%, whereas the decrease is about 70% after IL-17 removing. In addition, *ex vivo* studies on human skin explants demonstrated upregulation of BD-2, S100A7, and CXCL8 expression in response to the same combination of cytokines. *In vivo* intradermal injection of these five cytokines in mouse increased CXCL1, CXCL2, CXCL3, S100A9, and BD-3 expression, associated with neutrophil infiltration and an early epidermal acanthosis [14] (and submitted).

3. Specific and Shared Cytokines in Atopic Dermatitis and Psoriasis

The presence of $CD4^+CCR4^+$ Th2-cells in lesional skin of acute atopic dermatitis patients was described [24]. Th2 secreted predominantly IL-4 and IL-13, which induce both pro- and anti-inflammatory effects depending on the target cell type and on the nature of the receptors expressed. IL-4 and IL-13 inhibit TNFα and IFNγ-mediated induction of antimicrobial peptides BD-2 and BD-3 by keratinocytes [25–27]. It has been suggested that the increased expression of IL-4 and IL-13 in atopic dermatitis skin may explain the susceptibility to bacterial and viral skin infections by reducing antimicrobial peptide expression [26, 27]. On the other hand, as TLR3 agonists [18], IL-4 or IL-13 synergizes with TNFα or IL-1β [28] to induce expression of thymic stromal lymphopoietin (TSLP) in keratinocytes and subsequently induces the maturation of $CD11c^+$ dendritic cells involved in allergic inflammation. Besides regulation of their production, IL-4 and IL-13 activities can be modified by differential expression of their receptors. Indeed, IL-4 and IL-13 upregulate IL-13R2 expression in keratinocytes [29] whereas IFNγ or IL-13, but not IL-4, upregulates expression of IL-13R1 on keratinocytes *in vitro* [30].

IFNγ, mainly produced by the Th1 lymphocyte subset and by natural killer cells, is implicated in the regulation of different cellular processes such as antiviral responses, cell growth and differentiation, and immunoregulatory functions [31]. IFNγ levels are increased in psoriatic skin, but not in acute AD lesions [32] reflecting an altered Th1/Th2 balance [33–35]. Interestingly, these IFN levels are reduced after antipsoriatic therapy [36, 37]. It is classically admitted that psoriasis is a Th1 disease. However, the coexistence of both IL-4-producing Th2 and IFN-γ-producing Th1 cells or Th1 dominance is observed in chronic AD lesion [38].

Several models strengthen the role of the members of the IL-1 family in inflammatory skin diseases. Transgenic mice constitutively expressing IL-1α in basal keratinocytes (under the control of the CK14 promoter) develop a spontaneous skin disease characterized by hair loss and inflammatory skin lesions displaying hyperkeratosis, acanthosis, parakeratosis, and a mononuclear cell infiltrate [39]. In addition, IL-1RA deficient BALB/c mice develop arterial inflammation, arthritis, and a localized skin inflammation resembling human psoriasis [40]. Recently, IL-36, α, β, and γ, 3 other members of the IL-1 family, have been found to be expressed in a psoriasis-like animal skin, as well as in the lesions of psoriatic patients [15].

TNFα is a multifunctional cytokine also produced by Th1 cells that mediates inflammation, immune response, and apoptosis. Biological activities are mediated by two distinct cell surface receptors: TNFR1 and TNFR2. Analysis of mice lacking either TNFR1 or TNFR2 has demonstrated that TNFR1 is critical for induction of skin inflammation by TNFα [41]. Intradermal injection of TNFα leads to skin inflammation with elevated IL-6 production and induction of ICAM-1 expression by keratinocytes [41]. TNFα is also involved in the production of antimicrobial peptide such as BD-2 and BD-3 [26, 42]. Extensive analysis

of the transcriptional profile of keratinocytes treated with TNFα shows activation of innate and adaptive immune responses by inducing a large panel of chemokines that attracts neutrophils, macrophages, and T cells. The fundamental role played by TNFα in skin inflammation has been confirmed in mice lacking either IKK2 or IκBα, two molecules of the TNFα/NFκB signalling pathway. Finally, expression of TNFα and TNFR1 is increased in lesional psoriatic compared to normal skin [43]. These *in vitro* and *in vivo* observations are confirmed by numerous clinical studies of successful anti-TNF therapy in psoriasis since the first report in 2000 [44–46]. In contrast, a pilot study on the effect of infliximab on 9 patients with moderate to severe AD was disappointed [47]. Nevertheless, these cytokines do not seem to be involved in hyperplasia of epidermal keratinocytes observed in chronic skin lesions of AD and psoriasis. The hyperplasia and the alteration of barrier-related functions are linked to Th17 and Th22 cell subsets. Regarding the presence of Th17 lymphocytes in lesional psoriatic skin and the critical role of IL-17 in the pathogenesis of psoriasis [2, 8], presence and involvement of Th17 in AD has been evaluated. The percentage of Th17 cells was increased in peripheral blood of AD patients, in correlation with the severity of AD, and dermis IL-17-immunostaining was strongest in acute lesions when compared to the chronic one [48, 49]. Nograles et al. showed that the Th2 microenvironment has a suppressive effect on Th17 pathway by decreasing IL-17 receptor expression [50]. Whatever the case, IL-17A expression in lesional AD is almost unchanged when compared to normal skin, whereas it is increased in psoriasis [8] (unpublished data). In addition, expression of IL-23p19, IL-12p40, but not IL-12p35, is increased in psoriatic skin [51–53] and IL-23p19 expression is associated with dendritic cells infiltrated in the skin [8].

As soon IL-22 has been described to target keratinocytes, it has been observed that the cytokine induces a "psoriasis-like" phenotype on RHE, that is, hyperplasia with a thickening of the spinous layer and a disappearance of the granular layer [6, 11], and that the expression and secretory patterns of IL-22-treated keratinocytes resembled most of the features of psoriatic lesions [54]. Indeed, IL-22 is overexpressed in psoriatic lesions, whereas IL-22R1 and IL-10R2 are expressed at a similar level in psoriatic and healthy skin [37, 55]. T cells infiltrating psoriatic lesions are an important source of IL-22, higher than peripheral T cells from psoriatic patients or controls [55]. Further analysis of T cell infiltrating psoriatic skin showed IL-17 and IL-22 coproduction by Th17 lymphocytes [56, 57]. Innate sources of IL-17 have been also considered in skin. Recently, a dermal RORγt $\gamma\delta$ T cells producing IL-17 following exposure to IL-1β plus IL-23 have been described [58, 59]. In psoriatic patients, these $\gamma\delta$ T cells were greatly increased in affected skin and produced large amounts of IL-17 [59]. Attention could also be paid to RORγt+, CCR6+, CD4neg, NK1.1neg iNKT cells [60] as a potential source of IL-17 in psoriasis, since these cells have been also described in skin and draining lymph nodes and respond to inflammatory signals in mice [61].

Beside the Th17 cells, a so-called Th22 subpopulation producing IL-22 but nor IL-17 or IFNγ has been recently described in circulation and in normal human dermis, expressing CCR6 and the skin-homing receptors CCR4 and CCR10 [62–64]. Th22 clones derived from psoriatic lesional skin have been further described, mostly in the epidermis compartment of the skin [65]. Not specific of psoriasis, Th22 are also increased in lesional skin of atopic dermatitis and a correlation is observed between the number of Th22 cells and disease severity [66]. In the chronic phase of AD, Th22 subset is induced by Langerhans cells [67] along with a Th1 cell response believed to be induced by IDCs. Otherwise, both in murine and human, it has been suspected that non-T cells in skin produce IL-22, such as dendritic cells, NK cells, macrophages, and so forth. [68, 69]. In our hands, IL-22 expression in lesional AD and psoriasis was enhanced when compared to normal skin [55] (submitted results). Interestingly, rare patients with simultaneous occurrence of psoriasis and AD have been recently described [70]. Predominant antigen (Ag)-specific Th1 and Th17 lymphocytes infiltrates psoriatic lesions whereas Th2 infiltrates AD lesions, and IL-22 are detected in both lesions. Taken together, it appears that psoriasis is overall Th1 and Th17 mediated and AD is overall mediated by Th2 and Th22 cells, and that Th1 cells also contribute to the chronic phase of AD. In most cases, psoriasis and AD are mutually exclusive for individuals, but in rare cases can be concomitants, in which case Ag-specific T cells determine the specific pathogenesis [70].

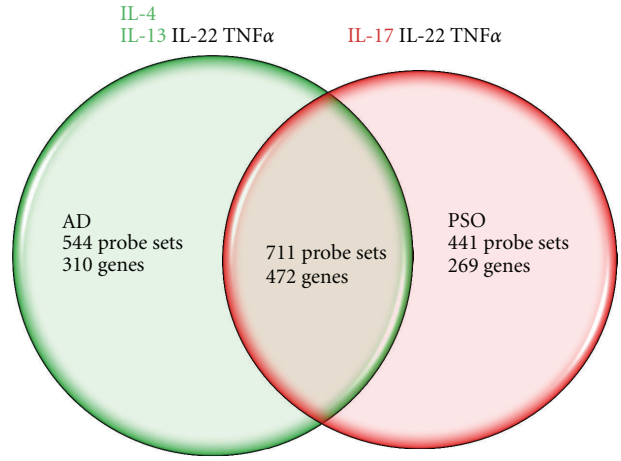

Figure 1: Overall effects of IL-22, TNFα, IL-4, and IL-13 combination (AD) or IL-22, TNFα, and IL-17 combination (PSO) on the transcriptome of keratinocytes in monolayer culture (NHEK). NHEKs were cultured in duplicate cultures with 10 ng/mL of each cytokines for 24 h and the gene expression profiles were analysed using Affymetrix hU219 chips. The number of significantly modulated ($\pm$2-fold the control) genes is indicated for each treatment (average of duplicate analysis) as well as the number of genes modulated by the two treatments.

4. Modelization of AD versus Psoriasis by Specific Sets of Cytokines

Taking into account (a) the preferential cytokine environment in AD vs psoriatic lesions and (b) those of these

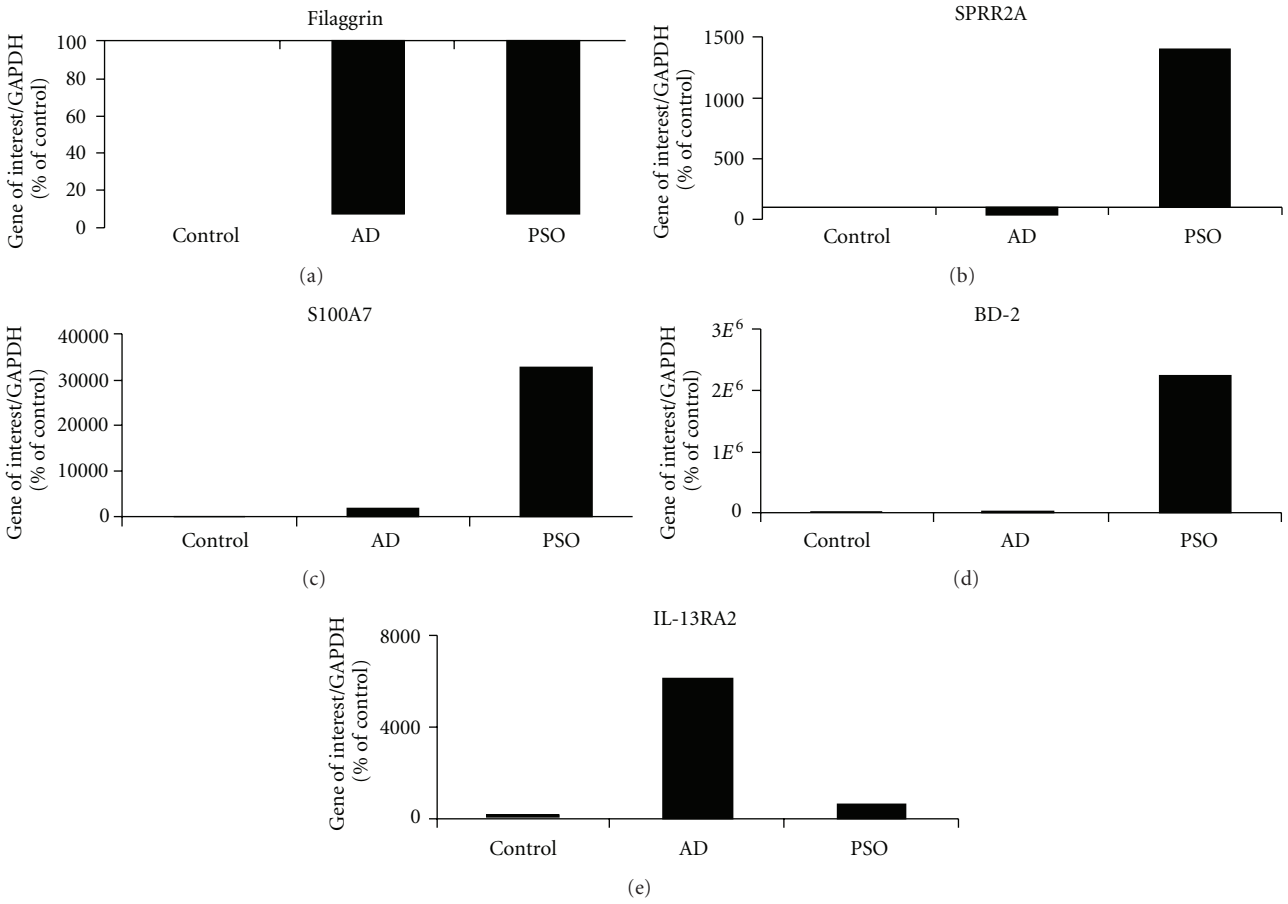

Figure 2: RT-qPCR analysis of the transcript expression of the keratinocyte differentiation markers filaggrin and SPRR2A, of the antimicrobial peptides S100A7 and BD-2, and the IL-13RA2 receptor chain after 24 h NHEK treatment by AD or PSO mixes. Results are expressed as percentage of the relative expression of stimulated cells over control cells.

cytokines able to target keratinocytes [14], we design *in vitro* experiments aiming to culture NHEK and RHE with an IL-22, TNFα, IL-4, and IL-13 mix or an IL-22, TNFα, and IL-17 mix, with the objective to, respectively, mimic AD and psoriasis epidermis. In these models, we finally analyzed the effects of the change from IL-17 (Th17, psoriasis) to IL-4/IL-13 (Th2, AD), in the same cytokine background (TNFα and IL-22). We hypothesize that these cytokines are not only specific biomarkers of the diseases, but are responsible for the phenotypes and the molecular signatures of these diseases.

The overall response of human keratinocyte monolayers (NHEK) to defined cytokines aiming to mimic AD or psoriasis environment was analyzed using Affymetrix technology, covering almost all the human transcriptome (Figure 1). In the representative experiment reported, among more than 20 000 individual genes analyzed, about 750–800 genes were down- or upregulated by more than 2-fold by each treatment. These results are indicative; it should be noted that this model is a "picture" of the transcriptome of these keratinocytes under defined culture conditions, at a given time (24 h); many additional genes should have been detected under additional experimental conditions. Taking into account these remarks, we expected from these models a representative approach of a global physiopathological situation.

Interestingly, there is a large overlap of more than 60% between the two treatments. Among these modulated genes, are many epidermis differentiation-related genes, such as filaggrin (Figure 2), which is known to be strongly downregulated by IL-22 (present in the two cytokine mixtures), alone or in combinations [14]. Most of the so-called epidermis differentiation markers are downregulated in NHEK by both AD and PSO NHEK treatments, as observed in pathologic skin. However, some of these markers such as genes from the small prolin rich proteins (SPRRs) family are differentially expressed in AD and PSO conditions. For example, SPRR2A is strongly overexpressed in the IL-17-containing mix (PSO) and downregulated in the IL-4/IL-13-containing mix (AD) (Figure 2).

In addition to differentiation markers, many other genes are commonly modulated by both cytokine mixes and TNFα by itself also strongly contribute to the "common" stimulation through. On the other hand, great differences between the two stimuli are observed for the antimicrobial peptides S100A7/psoriasin and BD-2; (Figure 2). S100A7 is dramatically overexpressed by the IL-17-containing mix

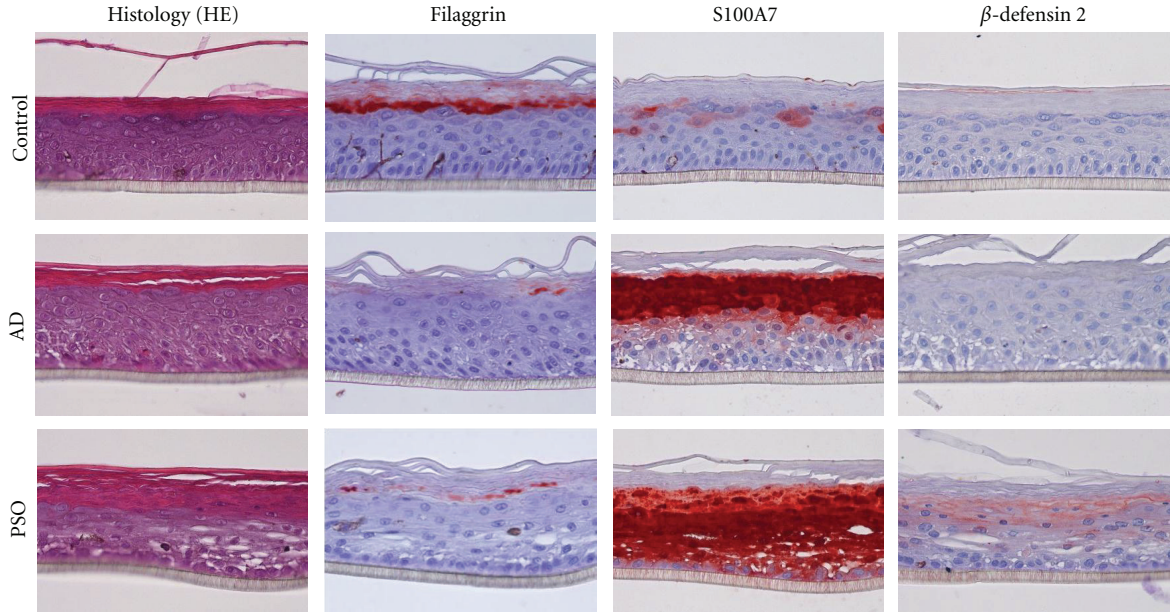

Figure 3: Histological and immunohistochemical analysis of RHE either unstimulated or stimulated with the IL-22, TNFα, IL-4, and IL-13 mixture (AD) or IL-17, IL-22, and TNFα mixture (PSO) for 48 h. Individual cytokine concentration was 3 ng/mL. RHE were cultured for 10 days at air-liquid interface before treatment. Tissues were fixed and embedded in paraffin and 4-μm vertical sections were stained with hematoxylin-eosin or immunolabeled using antifilaggrin, anti-S100A7, or anti-hBD2 mAbs.

(PSO) when compared to the weak enhancement induced by the IL-4/IL-13-containing mix (AD). According to its background expression in nonstimulated NHEK, BD-2 is dramatically enhanced in PSO model, and almost unmodified following AD stimulation. In contrast, the IL-13RA2 is overexpressed in the IL-4/IL-13-containing mix (AD) (Figure 2). These results parallel the *in vivo* situation, in which psoriatic skin is characterized by high expression levels of these antimicrobial peptides, functionally associated to a large degree of resistance to infections in psoriasis, and exacerbated infection sensitivity in AD skin [3].

A large number of chemokines are also produced, as indicated in Table 1. Whereas a chemokine such as CCL20 expression is more induced by the PSO mix as expected, CXCL10 is overexpressed only by the AD mix.

Since NHEK are undifferentiated primary keratinocytes cultured as monolayers, we further transpose the study to a more relevant epidermis model, reconstructed human epidermis (RHE), which closely resembles human skin epidermis (Figure 3). RHE are cornified 3D epithelia emerging from NHEK cultured at the air-medium interface [71]. The treatment of RHE with DA mix for 2 days led to significant modification of the histology of the epidermis, with an apparent loss of cohesion of the tissue, and orthokeratosis. The replacement of the Th2 cytokines by IL-17 (PSO model) leads to more dramatic tissue damage with destabilization of the epidermis, small pycnotic cells associated to parakeratosis, and a tendency to the disappearance of the granular layer. These *in vitro* phenotypes, respectively, parallel histology of AD and psoriasis lesions [3]. The decrease of expression of adhesion molecules could account for this phenotype. Clearly expressed in the epidermal layer of control RHE (Figure 3) as in normal skin, the treatment for only 48 h of these tissues by the two cytokine mixes resulted in a clear decrease in filaggrin accumulation. We previously showed that IL-22, present in the 2 mixes, decrease filaggrin expression [6]. As this effect was stronger in the AD mix, we suggested that it is due to an additive effect of IL-4/IL-13.

Regarding the antimicrobial peptides, we observed a local expression of S100A7 in untreated RHE, a strong overexpression following AD treatment and, as expected, a dramatic expression with the PSO mix. The BD-2 protein is neither detected in "healthy" RHE nor in the AD model, but was strongly induced by the PSO mix. This huge enhancement results from the synergistic effect of IL-17 added to the IL-22/TNFα combination. We demonstrated that

Table 1: RT-qPCR analysis of the expression of the transcripts for chemokines. Results are expressed as the fold increase expression of stimulated cells over control cells.

Gene	Fold increase in NHEK-PSO mix	Fold increase in NHEK-AD mix
CCL5	24	43
CCL20	30	7
CCL27	11	16
CXCL1	31	16
CXCL2	4	1
CXCL5	3	1
CXCL6	4	3
CXCL8	47	37
CXCL10	1	3

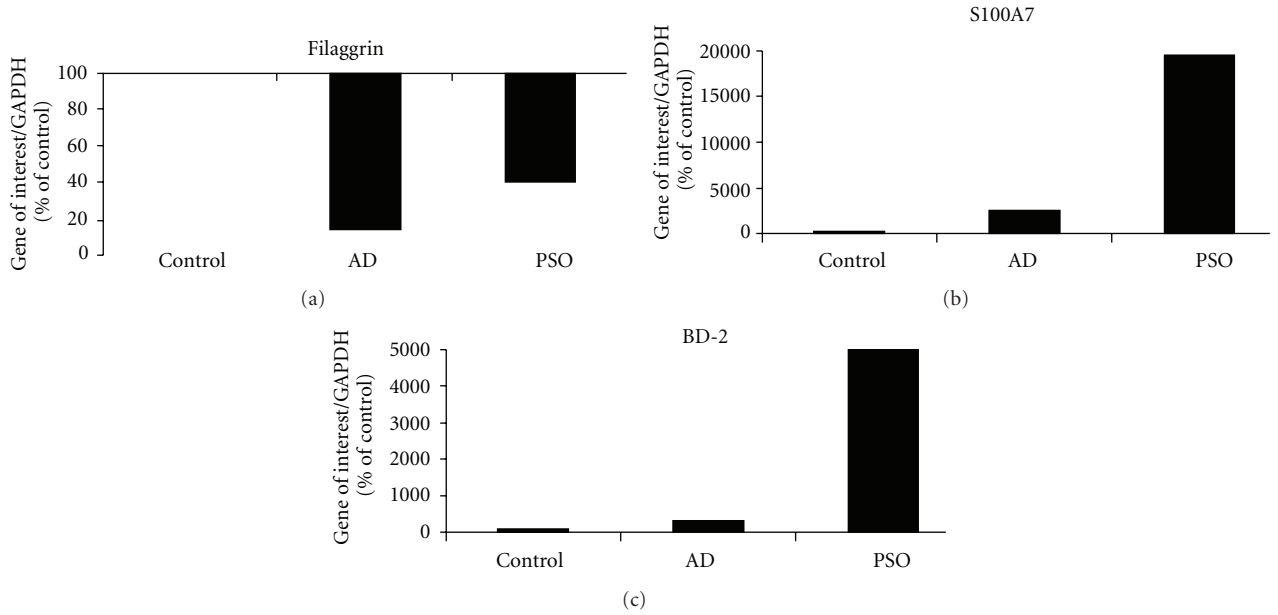

Figure 4: RT-qPCR analysis of the expression of the transcripts for filaggrin, S100A7, and BD-2 after 24 h treatment of RHE by AD or PSO mixes, as in Figure 3. Analyses were performed as in Figure 2.

the immununohistological variations of filaggrin, S100A7, and BD-2 are confirmed at the mRNA level (Figure 4).

Taken together, these different patterns of *in vitro* expression were also found in chronic AD and psoriatic skin lesions. Interestingly, we demonstrate that by using exclusively keratinocytes and sets of cytokines, in the absence of other epidermis or dermis skin cells, resident or infiltrating immune cells, we can offer in vitro models that approach AD and psoriasis skin lesions. Obviously, this is downstream events that we model with keratinocytes and cytokines, depending of upstream mechanisms of recruitment and activation of other innate adaptive immune cells. Anyway, cytokine are sufficient to induce phenotypic features of chronic AD and psoriasis on keratinocytes. If still necessary, it highlights the fact that specific cytokines or combinations of cytokines are key targets for biotherapies. This approach is already largely developed for psoriasis treatments, not so far for AD treatments. Whatever, the effect of combination of cytokine are complex to analyze, and also depends on other parameters such as dose or kinetic of expression. Of course these simple keratinocyte models can be improved, but however, one lesson from these experiments is that on a common background, the presence of one additional cytokine can shift the tissue response toward a given phenotype, mutually exclusive such as in AD and psoriasis.

5. Concluding Remarks

In recent years, an increasing number of reports have led to define a panel of proinflammatory cytokines able to play a central role in the induction and maintenance of chronic skin inflammation. The presence of specific subsets of Th lymphocytes polarizes the response, and keratinocyte are downstream targets of these cytokines. This cytokine environment plays a central role on the skin morphology and innate immunity, giving to keratinocytes a status of bona fide actors of the innate immunity.

The specific cytokine environment in chronic AD includes an IL-22, TNFα, IL-4, and IL-13 specific combination. Altogether, these cytokines are able to mimic an "atopic dermatitis like" state on NHEK and RHE. IL-22 is especially involved in the development of the epidermal hyperplasia and hypogranulosis. TNFα is especially involved in the induction of the innate response, in synergy with IL-22, whereas IL-4 and IL-13 inhibits the BD-2 production. A large number of chemokines are also produced. Despite the production of neutrophil-attractive chemokines, the absence of neutrophils in AD lesions remains to be further studied. In psoriasis lesions, over expression of IL-17 is observed whereas IL-4 and IL-13 were not detected. Interestingly, the replacement of IL-4 and IL-13 by IL-17 from this cocktail is able to mimic in vitro a "psoriasis-like" status on keratinocytes.

These studies open the way to new therapeutic strategies focusing on more specific and downstream targets, that is, keratinocytes targeting cytokines rather than on a systemic inhibition of T lymphocytes overproducing cytokines. The specific blockade of new cytokines or their receptors is an alternative approach for the treatment of AD. In any case, cytokines are very potent factors, with the advantages (or the defects, depending on the case) to be pleiotropic and redundant, and to be involved in cascade. Such treatment could reduce the biological effects of an overproduced cytokine and/or disrupt the vicious circle involving it. Targeting more than one cytokine could be a valuable strategy to assure a complete and sustained clinical improvement.

Obviously, the *in vitro* models of inflammatory epidermis induced by a specific set of cytokines could be useful tools to screen new drugs.

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Review Article

The Contribution of Allergen-Specific IgG to the Development of Th2-Mediated Airway Inflammation

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In both human asthmatics and animal models of allergy, allergen-specific IgG can contribute to Th2-mediated allergic inflammation. Mouse models have elucidated an important role for IgG and Fc-gamma receptor (FcγR) signaling on antigen presenting cells (APC) for the induction of airway inflammation. These studies suggest a positive feedback loop between IgG produced by the adaptive B cell response and FcγR signaling on innate immune cells. Studies of IgG and FcγRs in humans with asthma or allergic lung disease have been more controversial. Some reports have identified associations between allergen-specific IgG and severity of allergic responses, while other studies have found associations of IgG subclass IgG4 with allergic tolerance. In this paper, we review the literature to help define the nature of IgG and FcγR signaling on innate immune cells and how it contributes to the development of allergic immune responses.

1. Atopic Asthma Is Commonly Associated with Th2 Responses

Asthma is a chronic inflammatory disease of the lungs marked by recurrent episodes of airway hyperresponsiveness resulting in chest tightness, wheezing, and shortness of breath. Allergic or atopic asthma is the most common form of asthma, and allergic sensitization occurs in about 80% of asthmatic children and 60% of asthmatic adults [1]. Although there are now multiple phenotypes for atopic asthma, it has been classically associated with an excessive Th2-driven inflammatory response [2]. Development of an aberrant Th2 response leads to production of several cytokines including IL-4, IL-5, IL-9, and IL-13 that results in eosinophilia, goblet cell hyperplasia, mast cells activation, and smooth muscle hypertrophy [3]. In addition to these cellular effects, there is an important humoral response generated during primary sensitization that leads to production of allergen-specific IgE and IgG1. Much of the interest in dissecting the pathogenesis of asthma has focused on allergen-specific IgE which is well known to induce allergic hypersensitivity [4]. However, it was found that $IgE^{-/-}$ mice were still able to develop anaphylaxis and airway hyperreactivity suggesting that other mediators including allergen-specific IgG could be playing an important role in disease pathogenesis [5, 6]. Moreover, the total allergen-specific IgG response is greater in magnitude and has a significantly increased half-life compared to the total allergen-specific IgE response [7]. The complex nature of IgE in promoting allergy can be reviewed in a variety of recent articles [8–10]. In this review, we discuss current research on IgG, FcγRs, and allergy in order to better identify the role of IgG during allergic airway inflammation.

Based on research discussed in this paper, we propose a model whereby allergen-specific IgG promotes the expansion of secondary Th2 responses through ligation of FcγRs on innate immune cells (Figure 1). Allergen-specific IgG can be detected in the airways of sensitized individuals, and we propose that during secondary exposure to inhaled allergens, immune complexes (ICs) consisting of antigen and allergen-specific IgG are formed. These ICs can interact with both activating and inhibitory FcγRs on innate immune cells and

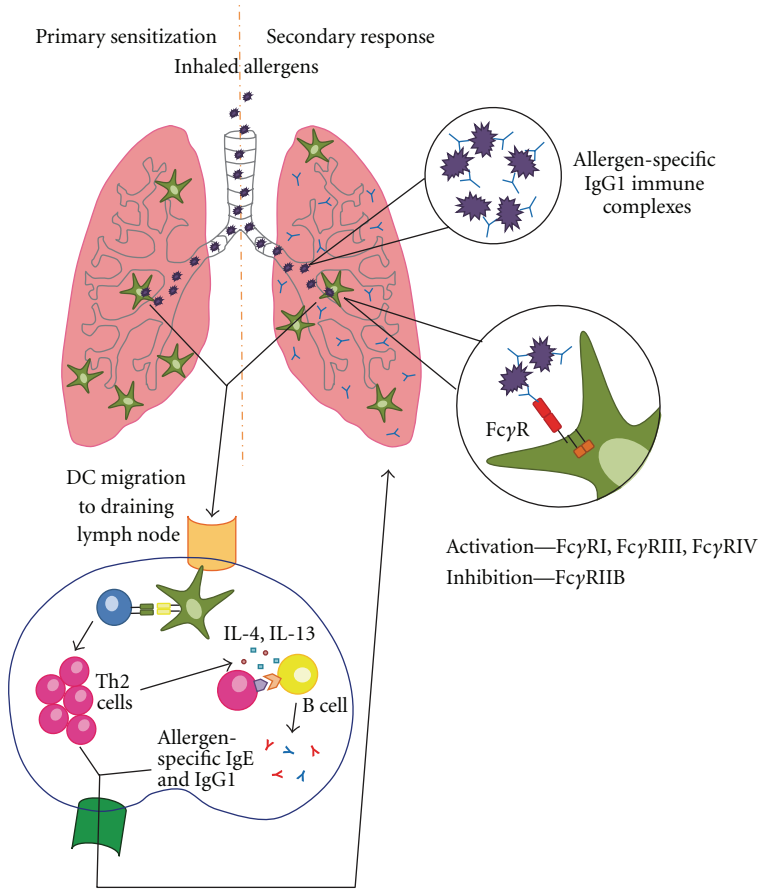

Figure 1: Model of IgG-mediated DC activation during secondary allergic responses in the lung. During primary sensitization, inhaled allergens are taken up by dendritic cells that then migrate to the draining lymph node to promote differentiation of T cells. Skewing towards a Th2 phenotype results in production of IL-4 and IL-13 that can promote IgE and IgG1 class switching in B cells. The IgG1 can complex with inhaled allergen during a secondary exposure to form ICs that signal through FcγRs on hematopoietic cells to promote allergic responses.

affect their activation and function. In our model, crosstalk between the IgG generated by the adaptive immune system and ligation of FcγRs on innate immune cells can contribute to the pathogenesis of Th2 inflammation during secondary responses to inhaled allergens.

2. FcγR Expression and Function on Hematopoietic Cells

FcγRs have emerged as an important bridge between the innate and adaptive arms of the immune system as they are primarily expressed on innate cells and their function can be affected by IgG ligation of FcγRs. Thus far, there have been four FcγRs identified in mice: FcγRI (CD64), FcγRIII (CD16), FcγRIV (CD16-2), and FcγRIIb (CD32) [11]. The family of FcγRs is more complex in humans because multiple isoforms exist: FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB [12]. In mice, FcγRI, FcγRIII, and FcγRIV are activating receptors that signal primarily through the Fc common γ-chain (FcRγ, noted as γ_2 in Figure 2), and in humans, FcγRI, FcγRIIA, FcγRIIC, and FcγRIIIA are activating Fc receptors although only FcγRI and FcγRIIIA use FcRγ [13]. FcRγ contains immunoreceptor tyrosine-based activation motifs (ITAMs) that when phosphorylated allow Syk kinases to dock and become activated [14]. In contrast to mice, human FcγRIIA and FcγRIIC contain an intracellular ITAM, and human FcγRIIIB is a GPI-linked receptor only expressed on human neutrophils [12]. On the other hand, FcγRIIB in both humans and mice is an inhibitory receptor with an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytosolic portion that will recruit and activate the SH2-domain containing inositol 5′ phosphatase (SHIP) upon phosphorylation [15]. A schematic of both mouse and human receptors along with their signaling chains is shown in Figure 2.

In addition to the numerous FcγRs, there are four IgG subclasses present in mice (IgG1, IgG2a, IgG2b, and IgG3) and humans (IgG1, IgG2, IgG3, and IgG4). Each FcγR has a varying affinity for the monomeric IgG subclasses. FcγRI in mice and FcγRI in humans have the highest affinity for IgG and can bind monomeric IgG2a in mice or IgG1 and IgG3 in humans [17, 18]. The other FcγRs have a significantly

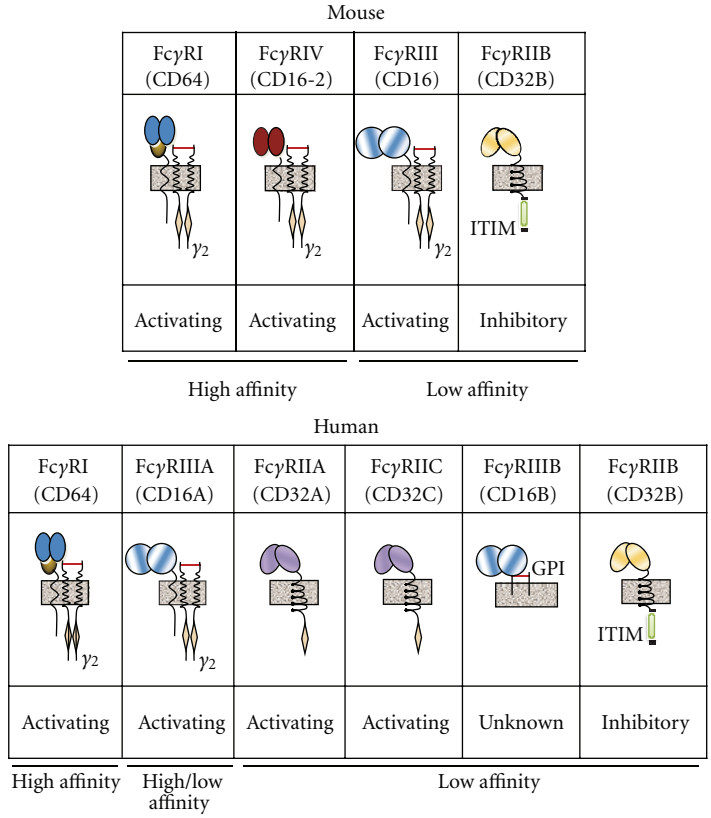

Figure 2: Mouse and human FcγR structure diagram. Receptors are labeled as "activating," "inhibitory," or "unknown." Relative affinity of each receptor to monomeric (free) IgG is listed below each receptor [12, 16]. Signaling chains associated with each receptor are labeled; beige diamonds represent ITAMs, green box represents ITIM, and grey box represents cell membrane.

lower affinity for IgG and primarily bind to IgG-ICs [16]. In mice, FcγRIIB and FcγRIII are able to bind IgG1, IgG2a, and IgG2b while FcγRIV has been shown to bind IgG2a, IgG2b, and IgE [19–22]. In humans, IgG1 and IgG3 can be found bound by all the FcγRs; IgG2 binds to allelic variants of FcγRII and FcγRIII; IgG4 binds to FcγRI, the FcγRII family, and an allelic variant of FcγRIII [12]. In both mice and human, IgG1 and IgG2 are found in the highest abundance in the serum. Human IgG4 is found at very low concentrations and is the only IgG subtype unable to form immune complexes or activate complement through binding C1q [23, 24]. Differences between IgG subtype expression and their affinity for activating or inhibitory FcγRs may be important mechanisms for the regulation of allergic disease.

Both activating and inhibitory FcγRs can be expressed on hematopoietic cells, primarily innate immune cells, and the expression patterns of these receptors vary between mice and humans as highlighted in Figure 3. Most of the innate immune cells express both activating and inhibitory FcγRs resulting in a certain threshold that will lead to the activation or inhibition of the immune response based on the ratio of these receptors and which IgG subclasses are present. This added level of control allows antibodies generated by the humoral immune system to play a critical role in affecting activation of innate immune cells through ligation of specific FcγRs to induce immunogenic or tolerogenic responses, which will be discussed below.

3. Murine Models of Allergy Suggest an Important Role in IgG Signaling in the Development of Allergy

Preliminary studies investigating the contribution of FcγRs in IgG-mediated disease models demonstrated that they helped regulate the immune response. The first knockout mouse model developed to address the role of IgG and activating FcγRs in inflammatory responses was the FcR$\gamma^{-/-}$ mouse which had an attenuated Arthus reaction, an IC-mediated type III hypersensitivity response [25]. These results were confirmed in several other studies highlighting that FcR$\gamma^{-/-}$ mouse had significantly decreased IgG-mediated cellular responses [26–28]. However, the specific FcγRs involved in mediating this response could not be identified because the FcR$\gamma^{-/-}$ mouse lacked all activating FcγRs, so other studies developed specific FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRIV$^{-/-}$ mice to further clarify their role during in vivo immune responses. Studies in mice deficient for activating FcγRs, particularly FcγRI and FcγRIII, showed decreased IgG-mediated responses in the knockout mice in several

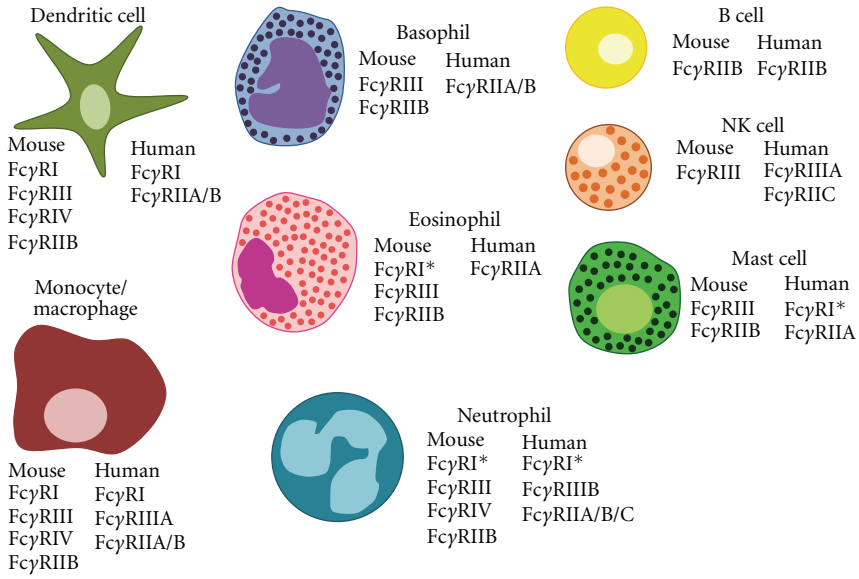

Figure 3: Human and murine FcγRs are expressed on a variety of hematopoietic cells. Cells of the innate immune system express both activating and inhibitory FcγRs: monocytes, macrophages, DCs, basophils, eosinophils, neutrophils, NK cells, and mast cells. On the other hand, B cells uniquely express FcγRIIb both in mice and humans. *denotes that expression can be induced upon activation.

disease models: passive cutaneous anaphylaxis, complement-independent Arthus reactions, arthritis, IgG-dependent anaphylaxis, experimental autoimmune hemolytic anemia, bacterial infections, and glomerulonephritis [29–35]. FcγRIV is the most recently identified FcγR, and studies have demonstrated that it can contribute to IgG2b-mediated inflammatory responses in mice [36–40]. Recent studies of IgG1 and IgG2 signaling through FcγRIIIA and FcγRIV showed a neutrophil-dependent mechanism of anaphylaxis in both mice and humans [41]. The balance between activating and inhibitory FcγRs has been shown to play a critical role in mediating inflammatory responses, so it was also important for investigators to analyze the role of the inhibitory receptor FcγRII. Early studies with the FcγRIIB$^{-/-}$ mouse helped to confirm its role as an inhibitory inflammatory signal because these mice developed increased humoral and anaphylactic responses [42, 43]. Furthermore, it was found that FcγRIIB$^{-/-}$ mouse developed increased Th2 responses in murine models of allergic airway inflammation [44–46]. Thus, the opposing effects of activating and inhibitory FcγRs demonstrate the significant role that IgG and FcγRs can play in modulating the immune response. Taken together, the results from the FcγR knockout mice indicate an ongoing interaction between the adaptive and innate immune system to shape the immune response.

The crosstalk between the humoral response and FcγRs on innate immune cells suggests that allergen-specific IgG can contribute to the development and augmentation of Th2 responses in the lung during secondary responses to inhaled allergens. It was first seen that allergen-specific IgG could affect allergic airway inflammation on its own in the absence of a memory response utilizing passive transfer models. These studies highlighted that administration of antigen-specific IgG followed by antigen challenge could lead to the development of immediate hypersensitivity, airway hyperresponsiveness, and anaphylaxis [47, 48]. As outlined in Figure 1, formation of allergen-specific ICs could be one of the mechanisms by which allergen-specific IgG participates in the pathogenesis of allergic lung disease. This hypothesis

is supported by studies investigating whether ICs alone could mediate Th2 responses in the lungs. It was shown that intranasal administration of anti-OVA IgG-ICs resulted in increased airway inflammation, eosinophilia, Th2 cytokine production, and antigen-specific T cell proliferation in an FcRγ-dependent manner [49]. In another study using airway hyperreactivity as a readout, mice were given polyclonal anti-BSA IgG intratracheally followed by an intravenous injection of BSA, and they developed severe airway hyperreactivity that peaked one hour after antigen administration and had resolved by 24 hours suggesting an immediate response to IgG-ICs [50]. These results indicate that allergen-specific IgG could exacerbate allergic lung diseases by forming allergen-specific IgG-ICs that promote activation of innate immune cells through interactions with FcγRs.

Although several innate cells contribute to allergic lung disease, there has been a great deal of interest in understanding how DCs contribute to this process. It is well established that DCs are important in promoting Th2 inflammation in the lungs and can direct differentiation of $CD4^+$ T cells into specific T cell lineages, and FcγRs on DCs have been identified as being a potential mediator in affecting DC and Th2 responses in the lungs [49, 51–54]. Studies in our lab highlighted a role for FcγRIII in regulating Th2 responses; we demonstrated that when TLR4-stimulated DCs received an additional signal through FcγRIII, it led to augmented Th2 responses in murine models of allergic airway inflammation in an IL-10-dependent manner [53]. Our studies are supported by the finding that activation by IgG-ICs on macrophages and neutrophils results in heterodimerization of TLR4 and FcγRIII [55]. These conclusions suggest that IgG-ICs could provide a link between the adaptive and innate immune responses by modulating TLR signaling. Thus, a positive feedback loop between allergen-specific IgG and FcγRs on DCs could drive established Th2 responses and exacerbate the development of allergic lung diseases. However, it should be noted that several studies have recently argued that free IgG1, not in immune complexes, and IVIg can bind FcγRIII and induce "inhibitory" ITAM signaling [56, 57]. The relevance of this effect may not be applicable to the development of allergic lung diseases because during secondary responses, ICs that bind to FcγRIII at a much higher affinity than free monomeric IgG are formed [16]. Other studies investigating the role of FcγRIIB on APCs have pointed to an inhibitory role for FcγR in Th2 responses. Sensitized wild-type mice receiving an intranasal aeroallergen challenge had an increase in FcγRIIB expression on respiratory $CD14^+$/$MHCII^+$ mononuclear cells and $CD11c^+$ cells [44]. Furthermore, another study suggested that FcγRIIB on DCs contributed to tolerance induction against mucosal antigens [58]. One possible mechanism by which FcγRIIB on DCs ameliorates allergic airway inflammation is through inhibition of antigen uptake and DC activation [45]. Collectively, the studies in mice suggest that allergen-specific IgG generated during primary sensitization complexes with inhaled antigen during secondary responses and depending on the balance of FcγRs signaled on DCs, can either positively or negatively affect the development of Th2 inflammation in the lungs.

4. FcγR and IgG in Human Allergy and Asthma

The complexity of the IgG subclasses and FcγRs in humans has made it more difficult to determine the effect of allergen-specific IgG on the development of Th2 responses in allergic diseases. Most studies in humans have investigated the correlation between different IgG subclasses, their specificities, and association with allergic phenotypes, while only a few studies have investigated the role of IgG signaling and their receptors in human allergic disease. Correlations between atopy and FcγR expression levels have provided conflicting results yet overall suggest that increased expression of activating FcγRs is augmented in allergic individuals [59–62]. Interestingly, one study investigating the ability of human FcγRIII$^-$ and FcγRIII$^+$ monocyte-derived DCs (mDCs) to reactivate memory responses found that FcγRIII$^+$ mDCs stimulated stronger T cell responses in vitro [63]. In general, determining the function of human FcγRs has proven difficult as there are limited reagents and variable populations being studied. Currently, studies have utilized transgenic mice overexpressing human FcγR genes to study function, but these studies are often limited by cell expression and binding affinity to mouse IgG. To examine the role of human FcγRs in models of allergic disease, human FcγRIIA which binds mouse IgG1, IgG2a, and IgG2b was overexpressed in an FcγR$^{-/-}$ mouse and assayed for anaphylactic responses. Expression of only human FcγRIIA in mice was sufficient to induce both active and passive anaphylaxis, as well as acute allergic responses [64]. Similarly, an FcγR humanized mouse that expresses the entire human FcγR family and lacks all mouse FcγRs was generated. These mice were able to mount comparable immune responses to wild-type mice during immune-complex mediated anaphylaxis, NP-OVA sensitization and challenge, FcγRIIB-dependent vaccination, and antitumor immunity; thus, they could prove to be a valuable tool in clarifying the role of human FcγRs [65]. While these studies utilized human FcγRs expressed in mice, together they confirm the hypothesis that FcγRs contribute to the development of allergic disease and demonstrate that human receptors display similar phenotypes to their murine counterparts.

Several studies have found a contributing role for antigen-specific IgG in the pathogenesis of asthma in humans. Notably in 2005, the German Multicentre Allergy Study published results from a longitudinal study of cat exposure and asthma development in children from age 6 months to 10 years. As expected this study found that cat Fel d 1 antigen-specific IgE levels were associated with increased asthma risk but surprisingly found that children with both antigen-specific IgE and IgG antibodies showed the greatest risk for asthma. This study and other studies did not find a correlation between allergen-specific IgG alone and increased asthma risk [66]. A similar study investigated childhood exposure and antibody responses to both house dust mites (HDM) and cats and determined that both total antigen-specific IgG and IgG4 to cat Fel d 1 and HDM Der f 1 paralleled the level of total antigen exposure. Analysis of these populations showed a strong association with HDM-specific IgG, but not cat-specific IgG, for increased risk of asthma

[67]. These studies suggesting a role for allergen-specific IgG in the promotion of Th2-mediated allergic disease are further supported by data showing elevated levels of IgG1 and IgG4 in the bronchoalveolar lavage fluid (BALF) of individuals with asthma [68, 69]. Furthermore, the induction of IgG during allergic Th2 responses is not limited to asthma; in other Th2-mediated diseases individuals with allergic bronchopulmonary aspergillosis and allergic alveolitis (e.g., farmer's lung and bird fancier's disease) are associated with increased levels of antigen-specific IgG [70, 71]. Together, these studies point to an important association between allergic IgG response and the perpetuation of airway inflammation in allergic individuals and support our model of antigen-specific IgG influencing the innate response to promote allergy.

It has been proposed that in some contexts IgG may be able to block normal IgE antigen binding and thereby inhibit the allergic response. IgG-mediated tolerance induction is thought to result from an accumulation of IgG4 and loss of IgE responsiveness. One study investigating the role of IgG in HDM responses found that allergic children were strongly associated with increased HDM-specific IgG1 and IgG4 levels [72]. However, supporting an inhibitory role of IgG4 in human allergic responses, this study compared children needing hospitalization as a result of acute asthmatic exacerbation and found a dramatic loss of both antigen-specific IgG1 and IgG4 compared to nonhospitalized allergic children, while HDM-specific IgE remained similar in magnitude and specificity between groups [72]. These findings emphasize the relationship between the presence of IgG4 and controlling asthma in children, suggesting that IgG4 could be ameliorating asthmatic disease in children. The prevalent hypothesis for IgG4 in asthma is that antigen-specific IgG4 can block IgE binding on allergens to reduce hyperresponsiveness. To test the ability of IgG4 to block IgE binding to antigen, $IgG4^+$ B cells were isolated from human patients undergoing allergen immunotherapy and successfully cloned IgG4 antibodies. One cloned IgG4 antibody was found to be specific against the grass pollen allergen Phl p 7, from *Phleum pratense*, and was shown to be able to modify IgE responses and inhibit basophil activation in vitro [73]. These results show that IgG4 in some context can block IgE binding to specific epitopes on antigen; however, the authors did not emphasize the low percentage of antigen-specific IgG4 found in their sample population [73]. A similar study analyzing Birch pollen IgG1 found similar inhibitory effects suggesting that IgG1 could be overlapping epitopes with IgE and may not be unique to IgG4 [74]. Additionally, it has also been shown that the diversity and affinity of the antigen-specific IgE response may be an important part in the activation of human Th2 responses and could explain in part why IgG4 may be able to block responses in some patients and not others [75]. These studies argue a possible role of IgG4 antibodies in displacing IgE binding to allergen epitopes and thereby reducing allergic susceptibility.

In contrast, multiple studies have found that elevated levels of IgG4 had no effects on allergic disease [76–78]. A recently published study investigating newly exposed laboratory-animal workers revealed that IgG4 levels did not change over two years of tracking and no correlation between newly sensitized IgE producing individuals to rodents and levels of antigen-specific serum IgG4 [77]. Similarly, school children who owned cats were associated with increased IgG4 levels but were not protected from developing asthma [76]. Moreover, allergic patients treated with grass pollen immunotherapy for two years had augmented antigen-specific IgG during therapy but returned to pretreatment IgG levels quickly following the end of therapy without a loss of tolerance [79]. Further, it is well known that IgG4 is unable to form immune complexes, activate complement, nor does it have a high affinity for FcγRs. Together, these findings suggest that even though IgG4 is induced in response to constant antigen exposure, it may act as a bystander and not a bona fide mechanism for mediating allergic tolerance. Overall, data has suggested that IgG in some context may be capable of overlapping epitope binding with IgE responses in order to dampen allergic sensitivity; however, it appears that it may not be a common mechanism and more research will be needed to elucidate these contradicting results.

One mechanism of immune regulation that might reconcile the seemingly contradictory findings presented above is recent studies suggesting a role for regulatory T cell populations (Treg or Tr1) in the development of both tolerance and IgG4 following allergen-specific immunotherapy [80–82]. Treg-produced IL-10 may contribute to the effects of immunotherapy by enhancing the survival and proliferation of previously differentiated IgG4 B cells [81]. Additionally, IL-10 treatment of IL-4-treated peripheral blood mononuclear cells (PBMCs) can promote the production of IgG4 and decreased IgE antibodies from peripheral B cells [81]. Finally, purified IL-10-producing Tr1 cells or Treg cells co-cultured with PBMCs from allergic individuals can modulate the B cell response to HDM antigen Der p 1 away from IgE and towards IgG4 [82]. Together these human studies identify an important role for regulatory T cell subsets on the development of tolerance in allergy. Importantly, these results suggest that the correlation of elevated levels of IgG4 with tolerance during immunotherapy may actually be a side effect of increased IL-10 in the serum and not a direct effector mechanism.

5. Discussion

Although the mechanisms by which IgG contributes to the pathogenesis or tolerance of allergic responses remain controversial, we believe that the evidence from both mouse and human studies points towards an important role for IgG in the regulation of allergic phenotypes. Clinical studies have demonstrated that in addition to elevated levels of allergen-specific IgE, allergic individuals also have elevated levels of allergen-specific IgG. Our previously published results showed that the activating receptor FcγRIII was necessary for potent induction of Th2 responses [53]. Further we found that FcγRIII is able to modulate TLR signaling on DCs in order to drive Th2 responses [53]. Based on these results and the data presented above, we propose a model (Figure 1)

whereby primary sensitization to an allergen results in B cell class switching and the production of allergen-specific IgG1. IgG1 can be found present in the lungs of sensitized individuals and will be able to complex with inhaled allergens during secondary responses. Allergen-ICs in the lung can signal through the activating FcγRs on innate immune cells, in particular FcγRIII. We have found that signaling through this receptor induces DC that specifically augments Th2 development through the expression of multiple genes. These migratory respiratory DCs can provide a milieu supportive of allergic responses in the airways thereby providing a positive feedback loop between the development of B cells that produce allergen-specific IgG, and the innate response of the respiratory DCs in the lungs. While we propose a positive feedback of allergen-specific IgG, the expression levels and affinity of different FcγRs on innate cells in the lungs may explain the differential results found between some studies. The presence of different FcγRs and different types of innate cells in normal versus atopic individuals could determine whether antigen-specific IgG contributes to the development of allergy or tolerance. Specifically, inhibitory FcγR receptors may explain how in some murine studies FcγRIIB seems to be able to overcome the activating FcγRs to ameliorate disease. It is known that FcγRIIB has lowered affinity to IgG compared to the activating receptors, therefore suggesting that results studying FcγRIIB in mice may not be the same in human allergy. Thus to thoroughly understand the importance of the IgG response in allergy, questions remain such as the differential expression of IgG subtypes, differences in IgG-affinity, and expression levels of FcγRs on innate cells between allergic and nonallergic individuals.

Authors' Contribution

J. W. Williams and M. Y. Tjota contributed equally to this work.

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Review Article

Regulatory T Cells and the Control of the Allergic Response

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The study of immune regulation and tolerance has been traditionally associated with self/nonself-discrimination. However, the finding that dominant tolerance, a model that puts in evidence the active role of regulatory T cells, can develop to nonself-antigens suggests that the imposition of tolerance can be context dependent. This paper reviews the emerging field of acquired immune tolerance to non-self antigens, with an emphasis on the different subsets of induced regulatory T cells that appear to specialize in specific functional niches. Such regulatory mechanisms are important in preventing the onset of allergic diseases in healthy individuals. In addition, it may be possible to take advantage of these immune regulatory mechanisms for the induction of tolerance in cases where pathological immune responses are generated to allergens occurring in nature, but also to other immunogens such as biological drugs developed for medical therapies.

1. Introduction

For many decades the self/nonself-discrimination by the immune system was assumed to be a consequence of clonal selection of effector T cells. Compelling evidence has, however, imposed a revised view of self/nonself-discrimination: dominant regulatory mechanisms, where regulatory T (Treg) cells play a central role, are essential for maintenance of self-tolerance [1]. But recently it is becoming apparent that the importance of dominant regulation goes beyond the discrimination of self and nonself: it also discriminates between harmful and innocuous. In fact, cellular mechanisms, as detailed below, persistently patrol the organism preventing the onset of inflammation, namely, allergic inflammation. The biological significance of this active tolerance-imposing mechanism is well demonstrated by the severity of the allergic and autoimmune syndrome that arises in individuals that lack these ability to tolerate self- and harmless antigens.

Indeed, the organism is constantly exposed to non-pathogenic antigens that, in healthy individuals, are tolerated. It is, however, common (and becoming increasingly frequent) that an overzealous immune system will activate and develop effector responses to such harmless antigens developing allergy and other inflammatory diseases. Over the last decades allergic diseases, including allergic asthma, atopic dermatitis, and food allergy, have become a major health problem in developed countries [2]. Despite the advances in the understanding of the pathophysiology of allergy and in its clinical management, allergic pathology remains a significant burden on the quality of life and economy of western society. Several strategies have been devised to overcome the pathological immune response by inducing immune tolerance. This paper reviews the impact of dominant regulatory mechanisms in the maintenance of tolerance to foreign antigens, including allergens.

A major cellular mechanism in maintaining immune tolerance is the population of natural (or thymic-derived) Foxp3$^+$ Treg cells [3, 4]. Indeed these have been clearly implicated as potent inducers of a nonresponsive state in several immune-mediated pathologies like autoimmunity, transplantation, graft-versus-host disease, and allergy [5–9]. It has been shown, in allergy, that regulatory T cells can be transferred conferring specific tolerance to subsequent challenges with the allergen [10, 11]. In addition, depletion of the regulatory T cells can have a detrimental effect in allergic airway hyperreactivity [12]. Importantly Foxp3 deficiency, in

mice and human beings, leads to a severe immune disregulation syndrome characterized by allergic and autoimmune manifestations that are rapidly fatal [13]. In addition to the important role of natural Foxp3$^+$ Treg cells (nTreg) in preventing autoimmunity, it has become established that Foxp3 expression can be peripherally induced following T-cell activation in presence of TGF-β [14]. These peripherally induced Treg cells (iTreg) are believed to be important for tolerance induction to nonselfantigens, including allergens [14].

2. Induction of Regulatory T Cells

The study of peripheral induction of Treg cells was greatly facilitated with the use of *Rag*-insufficient TCR-transgenic mice, with the TCR specific for a nonselfantigen. In these mice nTregs cannot be formed in the thymus due to the absence of a selecting thymic antigen. In 2003 it was shown that conventional T cells can be converted into iTreg *in vitro* when activated in presence of TGF-β [15]. In addition those iTreg cells were fully capable of controlling airway hyperreactivity (AHR) in previously sensitized mice [15–19]. It was subsequently found that reducing or blocking the available amount of TGF-β exacerbates AHR [20, 21], while the local delivery of this cytokine or adoptive transfer of T cells engineered to express latent TGF-β rescue mice from antigen sensitization and therefore prevent AHR [22, 23]. Interestingly, suboptimal TCR signaling together with TGF-β greatly enhances iTreg conversion [24], which is in agreement with *in vivo* data showing that repeated low doses of allergen exposure promotes the emergence of Foxp3$^+$ iTregs expressing TGF-β on the membrane [25]. Under suboptimal TCR stimulation, which can be obtained by using a low dose of plate bound anti-CD3 or DCs pulsed with a low dose of agonist peptide or with downmodulation of the TCR with nondepleting anti-CD4, iTreg conversion is promoted in the absence of exogenous TGF-β [26]. Under those conditions Foxp3 expression still requires TGF-β, but the T cells can produce TGF-β and benefit from the presence of this cytokine for conversion to Treg [26].

In addition to the importance of TGF-β for iTreg conversion, some studies showed that TGF-β can directly inhibit GATA3 expression thus impairing Th2 differentiation [27–29]. Because the Th2 response is impaired, the production of IL-4 is diminished, and this has a direct impact on B-cell class switch preventing IgE and favoring IgA production [30].

It is also becoming apparent that the environment influences the outcome of T-cell activation and the decision to induce Foxp3 and regulatory properties. Several reports have shown that the mucosal surfaces have a role in establishing an iTreg population: alveolar epithelial cells have been reported to participate in iTreg induction in a mechanism dependent of MHC class II expression and TGF-β [31]. Both alveolar and gut epithelia have been shown to depend on retinoic acid together with TGF-β to induce tolerance [32, 33]. It was also found that retinoic acid in the presence of TGF-β impaired STAT6 binding to the Foxp3 promoter therefore enhancing histone acetylation and reverting the repressive effect of IL-4 on the Foxp3 promoter [34].

Despite the critical role of TGF-β in iTreg induction and henceforth tolerance, this cytokine can also have some adverse effects since it is instrumental in the differentiation of Th17 and Th9 together with IL-6 and IL-4, respectively [35, 36]. Note that although Treg cells can prevent allergic autoimmune encephalomyelitis (EAE), mice with T cells with a dominant negative receptor for TGF-β1 do not develop EAE as Th17 cells are not induced [37]. Moreover, TGF-β has also been implicated in tissue remodeling, by induction of collagen expression in fibroblasts, as well as goblet cell proliferation and mucus production [38].

3. Regulatory T Cells and IL-10

Although TGF-β is the major known driver of iTreg differentiation, IL-10 has been shown to be another key player that has been vastly described in protection from allergic diseases [39].

Studies with bee venom-specific immunotherapy have shown that tolerance to the allergen can be induced in a process that is IL-10 mediated [40]. In addition, respiratory exposure tolerance induction to OVA relied on antigen specific CD4$^+$ regulatory cells that produced IL-10 [41]. Tolerance was transferrable and abrogated when IL-10 or ICOS ligand was blocked. Interestingly, those regulatory cells shared some features with effector Th2 cells: both populations expressed IL-4 and IL-10 although in different amounts. While the regulatory cells primarily release IL-10, the effectors rely on IL-4 as the main cytokine. It has been suggested that different types of effector cells, including Th2, produce IL-10 at the end of the immune response in a mechanism that is important in limiting their inflammatory behavior [42]. IL-10 producing T cells has been described able to control the late response in allergic asthma by reducing neutrophilia [43]. It has been suggested that Foxp3-negative IL-10 producing T cells can be induced following activation in presence of IL-10 and constitute a population of regulatory cells different from Foxp3$^+$ Tregs that are named TR1 [44, 45]. TR1 cells have been identified in mice and humans, and there are currently clinical trials [9, 46]. There are several other lines of evidence demonstrating the crucial role of IL-10 in the prevention of airway inflammation: IL-10-deficient mice have an exacerbated allergic airways response with high levels of proinflammatory cytokines like IL-5 and IFN-γ in the BAL [47]. Furthermore, intranasal administration of rmIL-10, concurrently with OVA, inhibited both airway neutrophilia and eosinophilia [48]. It was also shown that allergen-specific CD4$^+$CD25$^+$ Tregs can suppress allergic airway disease *in vivo* through an IL-10-dependent mechanism [18]. In this study, adoptive transfer of Treg cells reduced AHR, Th2 and eosinophil recruitment into the airways, and secretion of Th2-type cytokines. The effect was IL-10 mediated, since neutralizing anti-IL-10R abrogated suppression. In addition, these effects were independent of IL-10 production by the CD4$^+$CD25$^+$ regulatory cells themselves [18].

Unlike TGF-β, IL-10 does not directly influence B-cell class switch [49]. However, it is possible that indirectly, by inhibiting the inflammatory response, IL-10 shapes the

humoral outcome. Indeed, it was proposed that IL-10 may favor the ratio of IgG4/IgE ratio [50]. In fact immunotherapy studies show that Th2 responses can be suppressed by IL-10 secreting regulatory cells accompanied by an increase of circulating IgG4 [51, 52].

4. Different Subsets of Regulatory T Cells

Foxp3$^+$ Treg cells, despite an apparent phenotypic uniformity and immunosuppressive function, can have different subtypes with distinct genetic signatures. The first major division was identified between nTreg and iTreg, where the first are enriched in Helios, a transcription factor that is primarily expressed in T-lineage cells and early precursors [53, 54]. While nTreg cells have epigenetic mechanisms that stabilize Foxp3 expression allowing them to be a stable differentiated cell lineage, TGF-β induced Tregs lack those mechanisms having incomplete demethylation [55]. Therefore, although iTreg cells have high levels of Foxp3, the expression of Foxp3 is less stable [55–57]. In addition, conserved noncoding DNA sequence (CNS) elements at the Foxp3 locus encode information defining the size, composition, and stability of the Treg cell population [58]. CNS3, which binds c-Rel, has a drastic effect on the frequency of Treg cells generated in the thymus. Contrary to CNS3, CNS1 has no effect on thymic generation of Treg cells but is essential for induction of iTregs [58]. CNS1 contains a TGF-β-NFAT response element, so these results could represent the requirement of TGF-β and NFAT for Treg induction in the periphery [58–60]. Although CNS2-deficient T cells can acquire Foxp3 expression, they fail to maintain Foxp3 expression on their progeny due to the failure on recruitment of Foxp3-Runx1-Cbf-β complexes to CNS2 after demethylation of the CNS2 CpG island [58, 61]. Interestingly CNS1 deficient mice had no lymphoproliferative disorder. However, it can be argued that these animals kept in clean facilities have a minimal exposure to foreign antigens and thus nTreg may be sufficient to maintain homeostasis in such conditions. In effector T cells, GATA-3 is a hallmark of the Th2 cells, but Treg cells can also express GATA-3, that binds both to the Th2 cell locus and to the CNS2 of Foxp3 locus [62]. In fact, there is a dramatic increase of GATA-3 binding to CNS2 compared to conventional T cells, suggesting that GATA-3 regulates CNS2 activity in Treg cells [62].

There is strong evidence that the CCR7-dependent continuous migration of DC from the lung to its draining LNs is required for the transport of inhaled Ag and thereby for the proper composition of APCs in the LN. These processes are essential to induce peripheral tolerance of T cells [63]. The costimulation with ICOS, crucial for regulatory phenotype polarization in allergy [64], promotes the downregulation of CCR7 and CD62L after activation, leading to a reduced return of activated CD4 T cells to the lymph nodes and a more efficient entry into the lungs [65]. Regulatory T cells express CCR4 and CD103 induced by antigen-driven activation in the lymph nodes. In addition, the accumulation of Tregs in the skin and lung airways is impaired in the absence of CCR4 expression [66]. Mice without CCR4 in the Treg compartment develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs [66]. Some studies suggest that CCR4 has a prominent role in effector Th2 homing [67]. Despite their differences it seems both regulatory and effector T cells share the response to homing factors [68, 69].

But GATA-3 is not the only transcription factor characteristic of effector T-cell responses that can be expressed by Foxp3$^+$ Treg cells. Under the influence of IFN-γ, Foxp3$^+$ Treg cells can express the Th1-defining transcription factor T-bet [70]. T-bet expression by Foxp3$^+$ Treg cells induces the expression of the chemokine receptor CXCR3, necessary for these Treg cells to accumulate at the site of type 1 inflammation. T-bet expression was thus required for the homeostasis and function of Treg cells during type-1 inflammation [70].

It is likely that the regulation of different types of immune response requires the participation of specialized subsets of regulatory cells. This way, iTreg cells induced in an environment favorable to Th1 or Th2 type of immune responses require the appropriate chemokine receptors to give them access to the same locations as effector T cells (Figure 1).

Th17 cells that have been implicated in autoimmunity and allergy share with iTreg cells the need for TGF-β to differentiate [71]. The decision of antigen-stimulated cells to differentiate into either Th17 or iTreg depends on the cytokine balance of IL-6, IL-21, and IL-23 that relieve Foxp3-mediated inhibition of RORγt [72]. These results indicate that Foxp3 and RORγt are transcription factors that antagonize each other in the lineage differentiation.

Another subset of T cells, the follicular T helper cells (Tfh), is mostly spatially confined to secondary lymphoid organs, more precisely to the B-cell follicles [73]. Tfh cells express high levels of the transcription factor Bcl-6, that impairs the expression and function of other transcription factors specific for other CD4 subsets: Tbet, GATA3, and RORγt, thereby regulating cytokine production by Tfh cells [74, 75]. Tfh cells differentiate under the influence of ICOS:ICOSL and IL-21 but independently of any other cytokine [76]. In addition, the characteristic anatomical distribution of Tfh cells is dependent of CXCR5 that endows access to the B-cell follicle [73, 77, 78]. We and others have recently found that also this subset of effector T cells has a specialized regulatory counterpart [69, 79, 80]. It was found that Foxp3$^+$ Treg cells can be found within the B-cell follicle [81], sharing many characteristics of Tfh and Treg cells [69, 79, 80]. Importantly, Bcl-6 can be coexpressed with Foxp3 as it seems Foxp3 expression is not inhibited by Bcl-6. These follicular regulatory T cells (Tfr) are immunesuppressive and can control de magnitude of the germinal center response [69, 79, 80]. In addition, they exhibit a CTLA4^{hi}GITR^{hi}IL-10^{hi} phenotype that is the characteristic of activated Tregs [69, 79, 80]. However, the Tfr origin is quite distinct from the other induced Treg cells previously described. Tfr cells do not derive from the commitment of conventional CD4 T cells, but result from acquisition of "follicular" characteristics (viz. Bcl-6 expression) by natural Foxp3$^+$ Treg cells [69, 79, 80]. In fact sorted Tfh cells

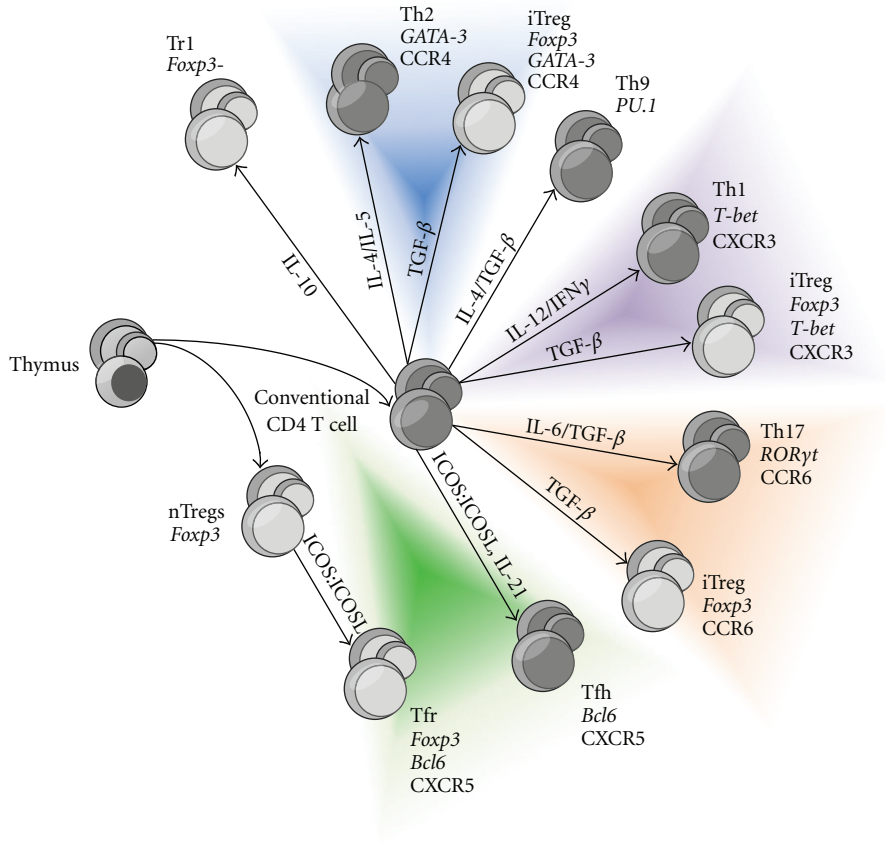

Figure 1: Functional specialization of effector and regulatory T cells. Different types of immune responses carry different cytokine microenvironments that can influence both effector and regulatory T-cell differentiation. In the same way effector T cells when activated in specific cytokine environment acquire specialized functions, induced regulatory cells (iTreg) can also activate the expression of different transcription factors (italics) that endow them access to different anatomic compartments on the basis of the chemokine receptors they express. Follicular regulatory cells (Tfr) represent an exception among peripherally induced Foxp3$^+$ cells, as they are derived from natural regulatory cells (nTreg) that acquire Bcl-6 expression, rather than from conventional CD4 T cells.

exposed to optimal conditions to induce Foxp3 expression in conventional T cells (including TGF-β) resist conversion to Tfr [79]. Given the importance of the germinal center response for allergy, it is likely that Tfr cells can play an important role in regulating IgE production.

Besides conventional T cells, also natural killer T (NKT) cells are important players in defining the outcome of immune responses. Notably, invariant NKT (iNKT) cells were found able to help B-cell differentiation, germinal-center formation, affinity maturation, and immunoglobulin response that was uniquely dependent on iNKT cell-derived IL-21, although the GCs maintain a small size throughout the reaction [82, 83]. This contribution of iNKT cells for humoral responses can be added to their ability to contribute to allergic airways diseases by producing IL-4 and IL-13 [84, 85], or IL-17 [86, 87]. But iNKT cells can also have a regulatory role, namely, in preventing EAE following administration of its TCR agonist [88, 89]. We and others recently described that activation of murine or human iNKT cells in presence of TGF-β induces Foxp3 expression and acquisition of suppressive function [88, 90].

5. Influencing Regulatory T Cells in Allergy

The understanding of the mechanisms involved in regulatory T-cells generation and function may lead to novel strategies to restore immune tolerance where it has been lost. As TGF-β and IL-10 play a crucial role in tolerance induction, several studies on immune tolerance induction took advantage of environments rich in those anti-inflammatory cytokines. To our advantage the mucosa itself is an anatomical location rich in these immune mediators [91].

Airborne antigens can be transferred from the mother to the newborn through milk [92]. Breastfeeding-induced tolerance was found to be mediated by induced Foxp3$^+$ Treg cells and dependent on TGF-β [92]. It has been proposed that metallomatrix proteases, derived from commensal bacteria in the gut, can facilitate the conversion of latent TGF-β to its active form, thus favoring iTreg differentiation [93]. In addition, CD103$^+$ dendritic cells in the mucosa-draining lymph nodes have been shown effective in promoting conversion of iTregs in the gut, mediated by TGF-β and the synthesis of retinoic acid, a powerful inducer of Foxp3 expression [32, 94, 95]. Furthermore, vitamin D receptor

deficient mice were associated with a reduction in tolerogenic $CD103^+$ dendritic cells favoring the development of effector type T cells [96]. Vitamin D3 can be used to induce human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into regulatory cells that produced only IL-10, but no IL-5 and IFN-γ, and furthermore retain strong proliferative capacity [97]. Several other studies put vitamin D3 in relevance as acting directly on T cells to induce IL-10^+ regulatory cells and also influencing levels of TGF-β [98–100]. These data suggest that the mucosa, in particular the gut, has several mechanisms that can favor immune tolerance. Sublingual immunotherapy (SLIT) and oral immunotherapy (OIT) are becoming more relevant as effective tolerance-inducing strategies to treat inhalant as well as food allergies [101].

Allergen specific immunotherapy (SIT) which comprehends SLIT, OIT, and subcutaneous immunotherapy (SCIT) has been in clinical use for around 100 years [102] and consists on the administration of increasing doses of an allergen [103]. It has been shown that both $Foxp3^+$ and IL-10 positive regulatory T cells can be induced during the course of SIT protocols [104, 105]. Furthermore, allergen-specific TR1 cells, in healthy individuals, have been suggested to play a key role in preventing pathologic responses [52, 102, 106]. While the presence of IL-10 leads B cells to produce IgG4 in detriment of IgE [107, 108], TGF-β drives B cells to switch to IgA production [106]. Another approach to direct the organism towards a tolerant state arises from the results that suggest that reduced TCR stimulation favors the induction of a regulatory phenotype on the T cells [26, 109, 110]. Blockade of molecules involved in the immune synapse has been suggested as an approach to achieve suboptimal TCR activation [26, 110]. Blockade of CD4 was shown a robust approach to achieve Treg-mediated dominant tolerance in transplantation [111–113]. We recently showed that a nondepleting anti-CD4 monoclonal antibody can induce in mice robust, antigen-specific tolerance to house dust mite, even in presensitized animals [16]. In addition, a similar strategy was effective to prevent peanut-induced anaphylaxis in mice [114]. Costimulation blockade was also shown effective in preventing allergic sensitization in mice [115]. Based on previous studies of tolerance induction to alloantigens following costimulation blockade, it is likely the mechanism also relies on Treg cells [116, 117]. Regarding the different modalities for costimulation blockade, on one hand CTLA4Ig was shown able to greatly reduce the secretion of IL-4 but not enough to impair Th2 response [118]. On the other hand, treatment with OX40L-blocking mAbs inhibited to some extent allergic immune responses induced by TSLP in the lung and skin, preventing Th2 inflammatory cell infiltration, cytokine secretion, and IgE production in mice and nonhuman primate models of asthma [119].

6. Final Remarks

The realization that active regulatory mechanisms, such as the ones mediated by Treg cells, can prevent pathological immune responses to harmless antigens is changing the way immunotherapy is perceived. In very diverse fields of immunology, ranging from cancer immunotherapy to autoimmunity and allergy, regulatory mechanisms need to be considered when therapeutic interventions are designed to boost or dampen the immune response. The realization that different subsets of regulatory T cells exist may offer the possibility to fine tune such interventions in order to achieve optimal therapeutic benefit with limited immunosuppressive consequences in unrelated immune responses.

At a time when therapeutic interventions rely increasingly on potentially immunogenic drugs, such as recombinant proteins to correct genetic diseases or monoclonal antibodies, where even the human antibodies can be immunogenic due to their unique idiotypes [120, 121], the issue of tolerance induction to nonselfantigens will not be restricted to allergy and transplantation, but a growing concern for drug efficacy.

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