

A New Mechanism for Inhalational Priming: IL-4 Bypasses Innate Immune Signals¹

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Signaling via innate immune mechanisms is considered pivotal for T cell-mediated responses to inhaled Ags. Furthermore, Th2 cells specific for one inhaled Ag can facilitate priming of naive T cells to unrelated new inhaled Ags, a process we call “Th2 collateral priming”. Interestingly, our previous studies showed that collateral priming is independent of signals via the innate immune system but depends on IL-4 secretion by CD4⁺ T cells. We thus hypothesized that IL-4 can bypass the need for signals via the innate immune system, considered essential for pulmonary priming. Indeed, we were able to show that IL-4 bypasses the requirement for TLR4- and MyD88-mediated signaling for responses to new allergens. Furthermore, we characterized the mechanisms by which IL-4 primes for new inhaled allergens: “IL-4-dependent pulmonary priming” relies on IL-4 receptor expression on hematopoietic cells and structural cells. Transfer experiments indicate that within the hematopoietic compartment both T cells and dendritic cells need to express the IL-4 receptor. Finally, we were able to show that IL-4 induces recruitment and maturation of myeloid dendritic cells in vivo and increases T cell recruitment to the draining lymph nodes. Our findings bring new mechanistic knowledge to the phenomenon of polysensitization and primary sensitization in asthma. *The Journal of Immunology*, 2008, 181: 7307–7315.

While the past decades have produced a wealth of knowledge with regard to the effector phase of an immune response in the context of allergic airway diseases, the sensitization phase of these pathologies is much less understood. This is due, in part, to the lack of suitable models. Most research on allergic airway disease is done in models where sensitization is achieved via intraperitoneal application of suitable proteins, a route hardly representative of sensitization processes in humans (1, 2). Our laboratory has recently developed a model of allergic airway disease where sensitization is achieved via inhalational application of OVA, thus providing a tool to dissect immunological pathways involved in priming for airway disease. In this model we have demonstrated that signals from the innate immune system, via TLR4 and MyD88, are required for inhalational priming, a finding that was later confirmed and extended by other investigators (3–8).

Consecutively, our laboratory has shown that inhalational sensitization can also occur independently of signaling via TLR4 or MyD88, provided that it takes place during an ongoing inflammation of the lung (9). We have shown that a Th2-polarized immune

response of the lung facilitates priming to new, unrelated Ags. This phenomenon, which we call “collateral priming”, depends largely on IL-4 secretion by T cells (9), a finding that has also been reported by other investigators (10). IL-4 has been known for decades to be a cytokine with a pivotal role in the development of Th2-polarized T cells (11, 12), and hence Th2-polarized immune responses (13–17), resulting from its pleiotropic effects, which encompass hematopoietic and nonhematopoietic cells (18). We and others have shown that cytokines such as TNF- α (3) or GM-CSF (19) can act as adjuvant in the induction of allergic airway disease. Given the known properties of IL-4, it seemed like an ideal novel candidate for “adjuvant” properties in the context of inhalational priming.

Combining our recent observations, we hypothesized that IL-4 can bypass the need for signals via the innate immune system, normally required for inhalational priming, through mechanisms not yet described. Indeed, we were able to show that IL-4 bypasses the requirement for TLR4- and MyD88-mediated signaling for responses to new allergens. We were therefore interested in determining the mechanism through which IL-4 bypasses the need for signals of the innate immune system in inhalational priming. We were able to show that signaling via the α -chain of the IL-4 receptor (IL-4R α)⁵ (8) in hematopoietic and nonhematopoietic (structural) cells is required for “IL-4-dependent pulmonary priming.” Furthermore, we showed that IL-4 acts on dendritic cells by increasing their recruitment to the lungs, Ag uptake and migration to the draining lymph nodes, and on T cells by increasing their recruitment to the draining lymph nodes. Our findings provide new insights into the lesser studied field of allergic sensitization and might therefore provide important starting points for the further

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⁵ Abbreviations used in this paper: IL-4R α , α -chain of the IL-4 receptor; BAL, bronchoalveolar lavage; BALF, BAL fluid; BM, bone marrow; BMDC, BM-derived dendritic cell; LN, lymph node; mLN, mediastinal LN; TLR4d, mouse strain defective in signaling via TLR4; WT, wild type; KO, knockout.

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dissection of the initiation mechanisms of this misdirected immune response.

Materials and Methods

Mice

BALB/cJ (wild type (WT)), BALB/c-*Il4ra^{tm1Sz}*/J (IL-4R α knockout (KO)), and C.C3H-*Tlr4^{Lps-d}* (TLR4 defective, TLR4d) (10) mice (both on a BALB/cJ background) were purchased from The Jackson Laboratory. MyD88-deficient mice were generated as previously described (20) and kindly provided by R. Medzhitov on a BALB/cJ background (Yale University, New Haven, CT). TCR-transgenic CD4⁺ T cell donors were DO11.10 mice backcrossed onto a BALB/c $\alpha\beta^{-/-}$, IL-4, or IL-13 negative background that were bred in our facility (21). Six- to 10-wk-old female mice were used in all experiments. All experimental methods described herein were performed as approved by the Institutional Animal Care and Use Committee.

Bone marrow (BM) transfers

BM cells were isolated from both femur and tibia of male mice. Cells were treated with rbc lysis medium (1.6 g NH₄Cl, 0.2 g KHCO₃, and 0.03 g EDTA) for 5 min, washed, and injected at 5–8 $\times$ 10⁶ BM cells/mouse into congenic, irradiated (2 $\times$ 600 rad, 4 h apart) female recipient mice. Eight weeks were allowed for reconstitution. Reconstitution was analyzed via FISH (fluorescence in situ hybridization) analysis of Y chromosome staining of hematopoietic cells.

Airway sensitization and challenge

For sensitization mice were exposed to 5 μ g (see Figs. 2 and 3) or 100 μ g (see Figs. 1B–D and 4E) of OVA (grade V; Sigma-Aldrich) with or without 1 μ g IL-4 or IL-13 (BD Biosciences) intranasally on days 0, 1, and 2. Collateral priming (see Fig. 1A) was induced by treatment of mice with OVA (5 μ g) + KLH (5 μ g) for primary challenge after adoptive transfer and OVA (5 μ g) or KLH (5 μ g) for secondary challenge, as described previously (9). For LPS treatment (see Fig. 1B–D), 0.05 μ g *Escherichia coli* LPS O55:B5 (Sigma-Aldrich) was instilled together with OVA. Challenge was performed with 25 μ g of OVA on days 14, 15, 18, and 19. For analysis of bronchoalveolar lavage (BAL), OVA-specific IgE, and mediastinal lymph node cytokine production, mice were sacrificed on day 21. Sensitization with fluorochrome-labeled OVA (see Fig. 4, A–D and F–H) was performed on days 0, 1, and 2 with 5 μ g of DQ-OVA or OVA-Alexa 647 (both from Molecular Probes) with or without 1 μ g IL-4 intranasally for analysis of lung or mediastinal lymph node dendritic cells (DCs), respectively. Sacrifice was performed on day 3 with methods outlined below. For Fig. 5, sensitization was performed only once, 24 h after transfer of Ag-specific T cells, with 5 μ g OVA + 1 μ g IL-4. Mice were sacrificed for analysis of Ag-specific T cell recruitment and proliferation 24, 48 (data not shown), and 72 h after sensitization. Control mice for all experiments were treated with OVA alone or with OVA + LPS (TLR4d and MyD88 KO mice in Fig. 1B only), challenge performed as above.

Analysis of BAL

BAL inflammatory cells were obtained by lavage of the airway lumen with PBS as previously described (17). Cytospin slides were stained with Diff-Quick (Dade Behring), and 200 cells/sample were differentiated microscopically.

Determination of serum Ab concentration

For measurement of OVA-specific IgE Abs, plates were coated with 100 μ l of 2 μ g/ml anti-mouse IgE (R35-72, BD Biosciences) at room temperature for 4 h. Assay diluent buffer (ADB; BD Biosciences) was used to block plates. Sera were incubated overnight at 4°C followed by digoxigenin-OVA (made in our own laboratory) and digoxigenin-conjugated peroxidase (Hoffmann-LaRoche) at room temperature, followed by tetramethylbenzidine substrate (Dako). IgE standard was a kind gift of E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO); the highest standard dilution was set at 500 U/ml with detection limit being 31.25 U/ml.

Lymph node (LN) preparation and cytokine production

Mediastinal LNs (mLNs) were harvested and pooled from each group at time of sacrifice. Single-cell suspensions were obtained by mechanical disruption. Cells were either immediately subjected to FACS analysis or stimulated in vitro with 200 μ g/ml OVA and syngeneic T cell-depleted splenocytes. We measured cytokines in culture supernatants using Multiplex-based bead technology (Millipore).

Lung cell preparation

Mice were subjected to intranasal sensitization with 5 μ g DQ-OVA or OVA-Alexa 647 (both from Invitrogen) with or without IL-4 on days 0, 1, and 2 and were sacrificed on day 3. Lungs were perfused with 10 ml PBS through the right ventricle. After removal from animals, lungs were digested with 150 U/ml collagenase (Sigma-Aldrich), 20 μ g/ml type IV DNase I (Sigma-Aldrich) for 30 min at 37°C before further disruption via a metal sieve and removal of debris via a cell strainer (100 μ m, BD Biosciences), followed by lysis of RBC (see above). Lung cells were then subjected to FACS analysis with the indicated Abs (BD Biosciences). Lungs were processed individually and at least three lungs per group were analyzed independently.

Analysis of CCL20 in lung homogenates

Lungs were harvested, weighed, and homogenized in PBS containing protease inhibitor and 0.05% Triton X-100. Cell-free supernatant was obtained by 15 min of centrifugation at 10,000 $\times$ g. CCL20 content of lung homogenates was analyzed via ELISA (R&D Systems) according to the manufacturer's instructions and calculated on a per lung basis by multiplying results from the ELISA with the amount of fluid obtained from centrifugation of lung homogenates.

Isolation of CD4⁺ T cells and adoptive transfer

CD4⁺ T cells were isolated from the spleens of BALB/c or transgenic mice by negative selection using Abs to MHC class II I-A^d (212.A1), CD8 (TIB 210), B220 (TIB 164), and FcR (24G2) followed by anti-Ig-coated magnetic beads (Polysciences). Cells (10 $\times$ 10⁶) were transferred for reconstitution experiments (Fig. 3). For tracking of Ag-specific CD4⁺ T cells (Fig. 5), 0.2 $\times$ 10⁶ cells were injected i.v. into BALB/cJ mice. A fraction of CD4⁺ T cells were subjected to FACS analysis for purity control before transfer. Th2 cells (see Fig. 1A) were generated from CD4⁺ T cells isolated from WT or IL-4- or IL-13-deficient DO11.10 mice and transferred into syngeneic hosts as described previously (9).

BM-derived dendritic cell (BMDC) preparation and transfer

For generation of BMDCs, BM cells were isolated by perfusion of femur and tibia. Cells were cultured in RPMI 1640 (Invitrogen) in the presence of 1% culture supernatant from a cell line transfected with the murine GM-CSF gene. After 6 days, cells were harvested and 1 $\times$ 10⁶ cells/mouse were transferred intranasally under light anesthesia. A fraction of DCs were subjected to FACS analysis for purity control before intranasal transfer.

Flow cytometry analysis

All staining procedures were performed on ice. Cells were incubated with anti-FcR (24G2) Ab in combination with mouse Ig for 20 min on ice, then stained with Abs against CD11c, CD11b, CD86, CD40, MHC class II, CD4, CD8, B7-H1, or B7-DC and appropriate isotype controls (BD Biosciences) for 30 min on ice. The presence of OVA-transgenic T cells was determined before i.v. transfer and from mLNs after sensitization by staining with an anti-clonotypic, self-grown mAb (KJ1-26) (22). Cells were analyzed by a FACSCalibur or LSRII flow cytometer (BD Biosciences) in association with FlowJo (Tree Star) software. DCs in lung were determined as autofluorescence negative, CD11c^{high}-positive, and CD11b-positive cells, and DCs in mLNs were determined as CD11c^{high}-positive cells. Total cell counts for lung and lymph node DCs were determined by multiplication of percentages obtained via flow cytometry times total cell numbers obtained via microscopic enumeration of single-cell suspensions of respective organs.

Statistical analysis

Statistical significance was determined using the Mann-Whitney *U* test. *p* < 0.05 was considered to be significant and is designated by an asterisk in the graphs. Unless indicated otherwise, five mice were used for each condition studied in an individual experiment. Each treatment condition was repeated at least three times.

Results

IL-4 bypasses the need for innate immune signals in pulmonary priming

We have previously shown that an ongoing Th2-polarized airway inflammation facilitates priming toward new, unrelated

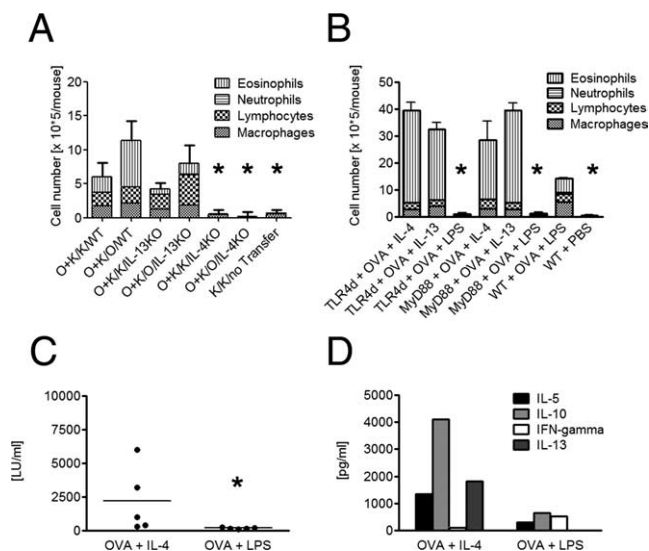


FIGURE 1. IL-4 bypasses the need for innate immune signals in pulmonary priming. WT (BALB/c) mice received Th2 cells derived from DO11.10 mice with a WT, IL-4 KO, or IL-13 KO background and were challenged intranasally with OVA and KLH (O+K) and rechallenged with OVA (O) or KLH (K) (A), as described previously (9). WT, TLR4d, and MyD88 KO mice were sensitized with OVA plus LPS or with OVA plus IL-4 intranasally. Differential cell counts in BALF (B), serum OVA-specific IgE titers (C), and Ag-specific local cytokine secretion in mLN following in vitro restimulation (D) were measured via microscopic cell differentiation, ELISA, and Multiplex bead assays, respectively, following challenge with OVA. C and D were obtained using TLR4d mice exclusively.

Ags. This phenomenon, which we call Th2 collateral priming, is dependent on IL-4 production by adoptively transferred Th2 cells ((9) and Fig. 1A), but, contrary to our previous findings in a different mouse model of pulmonary priming (3), independent of signaling via TLRs (9). We therefore asked whether IL-4 alone can bypass signals of the innate immune system normally required for priming via the airways. To this end we sensitized mice unable to signal through TLR4 (C3H-*Tlr4*^{Lps-d} strain, which has a loss of function mutation in the TLR4 gene (23, 24), referred to as TLR4d mice) or mice lacking the common TLR adaptor protein MyD88 (further referred to as MyD88 KO mice) (20) with Ag with or without IL-4 intranasally and challenged with Ag alone (Fig. 1B). We found that exogenously added IL-4 is sufficient for priming of naive T cells to new Ags. Moreover, these experiments determined that IL-4 bypasses the need for innate immune signaling in priming of naive T cells. As expected from our previous studies, treatment of mice unable to signal through TLR4 (TLR4d mice) with Ag and LPS failed to induce an inflammatory response in the lung that was readily observed in WT mice (3). Only WT mice showed significant airway inflammation in response to OVA and LPS, measured by counting and differentiating cellular BAL infiltrate. WT mice showed a strong increase in total cell numbers with a predominance of eosinophils and lymphocytes, characteristic of a Th2 response, while TLR4d mice showed baseline cell numbers within the bronchoalveolar lavage fluid (BALF), which consisted almost exclusively of macrophages. However, treatment of the same mouse strain with Ag and IL-4 induced a robust pulmonary inflammatory response within the BALF with a significant number of eosinophils (Fig. 1B). Additionally, we observed Ag-specific Th2-polarized systemic sensitization only if OVA was administered in conjunction with IL-4. First,

TLR4d mice only mounted significant serum levels of OVA-specific IgE if treated with IL-4 in addition to Ag (Fig. 1C). Second, significant local Th2-cytokine secretion (namely IL-5 and IL-13) upon restimulation of mLN cells with OVA was also only observed if TLR4d mice were treated with Ag and IL-4 (Fig. 1D). Moreover, studies in mice deficient in the expression of the adaptor protein MyD88 (20), which is involved in signaling through most common TLRs, were also able to mount airway responses to OVA only if it was coadministered with IL-4 (Fig. 1B). These results demonstrate that IL-4 bypasses the need for signals via the innate immune response in the induction of allergic airway inflammation.

To better understand the contribution of IL-13 vs IL-4, molecules known to have some redundant properties (16), we tested whether IL-13 also plays a role in Th2 collateral priming. Th2 collateral priming still occurred when IL-13-deficient transgenic Th2 cells were transferred to induce collateral priming (IL-13KO, Fig. 1A), although decreased recruitment of eosinophils in the absence of IL-13 was evident. In contrast, IL-4-deficient Th2 cells were not able to induce collateral priming anymore (IL-4KO, Fig. 1A), as we had published earlier (9). In contrast, we found that exogenously added IL-13 primed TLR4d mice to new Ag (OVA) in a manner similar to the effects of IL-4 (Fig. 1B). These results show that, compared with the role of IL-4, IL-13 plays a secondary role in Th2 collateral priming and we therefore concentrated further studies on the effects of IL-4.

The induction of three hallmark phenotypical changes in a mouse model of allergic airway disease (e.g., pulmonary inflammation, systemic sensitization, and local Th2-polarized recall cytokine production) denoted that priming with Ag and IL-4 induces a sustained, systemic, Th2-polarized immune response. Finally, rechallenge with Ag alone after 8 wk established that immunological memory was generated through priming with Ag and IL-4 (data not shown).

IL-4-dependent pulmonary priming depends on signaling via IL-4Rα in structural and hematopoietic compartments

In a next step, we wanted to close in on the cell types required for the induction of a Th2-polarized pulmonary inflammatory response to IL-4. In our model, we were specifically interested in the dissection between hematopoietic cells (i.e., cells originating in the BM) and structural cells (i.e., cells constituting the parenchyma) because several hematopoietic cells such as T cells, B cells, and DCs express the IL-4Rα (18), and one or several of these cell types could be solely responsible for the induction of the phenotype we observed. On the other hand, several different structural cell types such as epithelial cells, endothelial cells, and fibroblasts (18) also express the IL-4Rα and could therefore be solely responsible or contribute to the phenotype we observed.

To determine which cell type is required to express the IL-4Rα for IL-4-mediated Th2 pulmonary priming, we created BM chimeras from IL-4Rα-deficient and WT mice. Transferring BM lacking expression of IL-4Rα into WT recipients, WT BM into IL-4Rα-deficient recipients, WT BM into WT recipients, and IL-4Rα-deficient BM into IL-4Rα-deficient recipients, we created mice selectively deficient in IL-4Rα expression in either the hematopoietic compartment (SC⁺/HP⁻), structural compartment (SC⁻/HP⁺), or neither (SC⁺/HP⁺) as well as mice deficient in both compartments (SC⁻/HP⁻). We were thus able to dissect the compartment that IL-4 needs to address to induce the full phenotype of IL-4-dependent pulmonary priming. As expected from an earlier publication, mice lacking the IL-4Rα in both compartments (SC⁻/HP⁻) were resistant to the induction of Th2 polarization, systemic sensitization, and allergic airway inflammation (16). Interestingly, we found that lack of

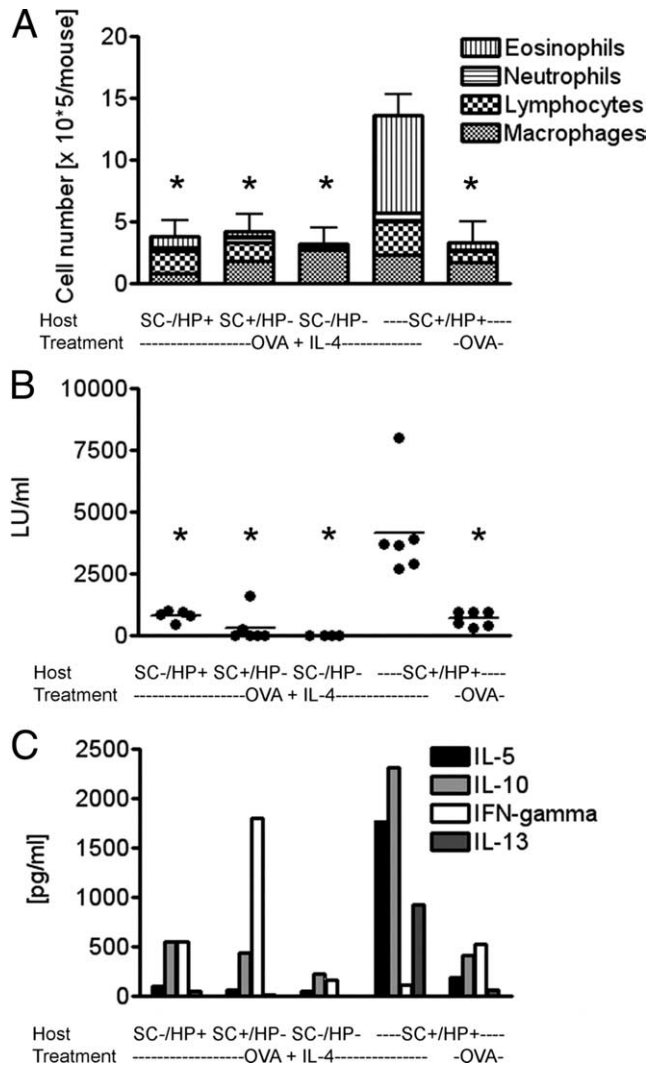


FIGURE 2. IL-4-dependent pulmonary priming depends on signaling via IL-4R α in structural and hematopoietic compartments. Lethally irradiated WT and IL-4R α -deficient mice were reconstituted with BM from WT or IL-4R α -deficient mice to generate mice expressing IL-4R α in hematopoietic cells only (SC⁻/HP⁺), structural cells only (SC⁺/HP⁻), or both (SC⁺/HP⁺) or neither (SC⁻/HP⁻) compartments before sensitization with OVA plus IL-4. Sensitization and challenge were performed as in Fig. 1. However, subimmunogenic doses of OVA (5 μ g/mouse) were used, as titrated in previous experiments, to eliminate adjuvant effects through LPS contamination of OVA in mice capable of responding to LPS. Therefore, mice primed with OVA without IL-4 constitute negative controls. Differential cell counts in BALF (A) and OVA-specific IgE titers (B) were measured following challenge with OVA as in Fig. 1. Cytokine production was measured after challenge following *in vitro* restimulation of mLN cells with OVA via Multiplex bead assays (C).

IL-4R α expression in either hematopoietic or structural compartment also resulted in the loss of Th2 priming and induction of allergic airway inflammation, as evidenced by decreased cell counts in BALF, with eosinophilic infiltration being particularly abrogated in mice lacking IL-4R α expression on hematopoietic cells (Fig. 2A). Concomitantly, we observed a blunted induction of OVA-specific IgE (Fig. 2B) and Ag-specific cytokine production by the cells from mLNs upon restimulation with OVA (Fig. 2C). In this context, signaling via both compartments seemed to be particularly important for up-regulation of IL-5 and IL-10 secretion and down-regulation of IFN- γ secretion by mLN cells. This full induction/repression could only be ob-

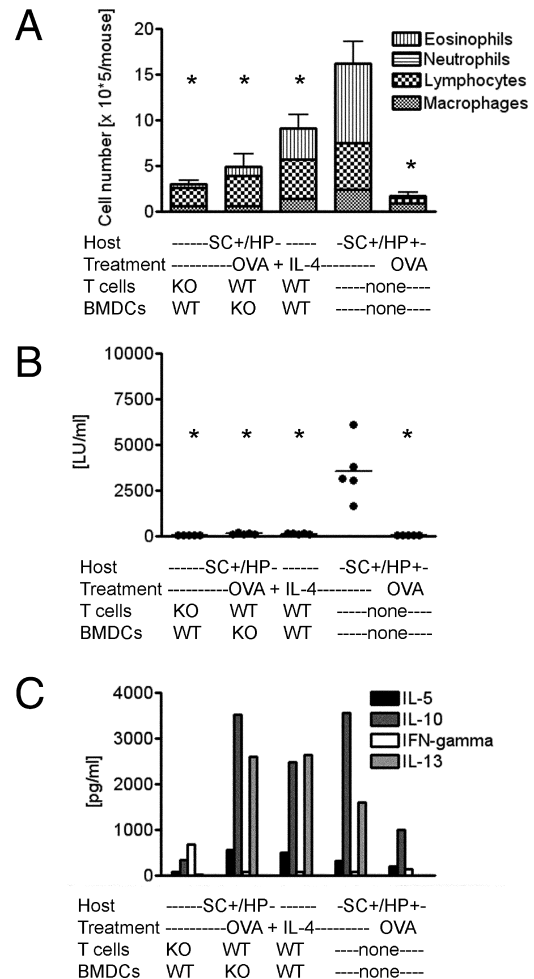


FIGURE 3. Signaling via IL-4 in CD4⁺ T cells and myeloid DCs partially restores allergic airway inflammation, systemic sensitization, and Th2 polarization. To test which hematopoietic cells are necessary to induce the IL-4-dependent phenotype in pulmonary priming, we performed transfer experiments. BM chimeras lacking IL-4R α expression in the hematopoietic compartment (SC⁺/HP⁻) were reconstituted with BMDs and/or T cells from WT or IL-4R α -deficient mice before sensitization with OVA plus IL-4 and compared with BM chimeras sufficient in both compartments (SC⁺/HP⁺). Sensitization and challenge were performed as in Fig. 1, apart from the use of 5 μ g of OVA/mouse for sensitization for reasons explained in Fig. 2. Differential cell counts in BALF (A) and OVA-specific IgE titers (B) were measured following challenge with OVA as in Fig. 1. Cytokine production was measured after challenge following *in vitro* restimulation of mLN cells via Multiplex bead assays (C).

served in the phenotype sufficient for signaling via IL-4 in both compartments (SC⁺/HP⁺) upon treatment with OVA and IL-4. The failure to suppress IFN- γ secretion was even more pronounced in the SC⁺/HP⁻ chimeras compared with SC⁻/HP⁺ chimeras, suggesting that IL-4 signaling in hematopoietic cells directly controls Th2 polarization, as it has been reviewed earlier (25). Thus, both compartments need to be able to respond to IL-4 to develop the complete phenotype of IL-4-dependent allergic airway inflammation. Similar results were obtained using STAT6-deficient mice (data not shown).

Signaling via IL-4 in CD4⁺ T cells and myeloid DCs partially restores allergic airway inflammation, systemic sensitization, and Th2 polarization

Given the crucial role of myeloid DCs as professional APCs in the initiation of the immune response in the respiratory tract

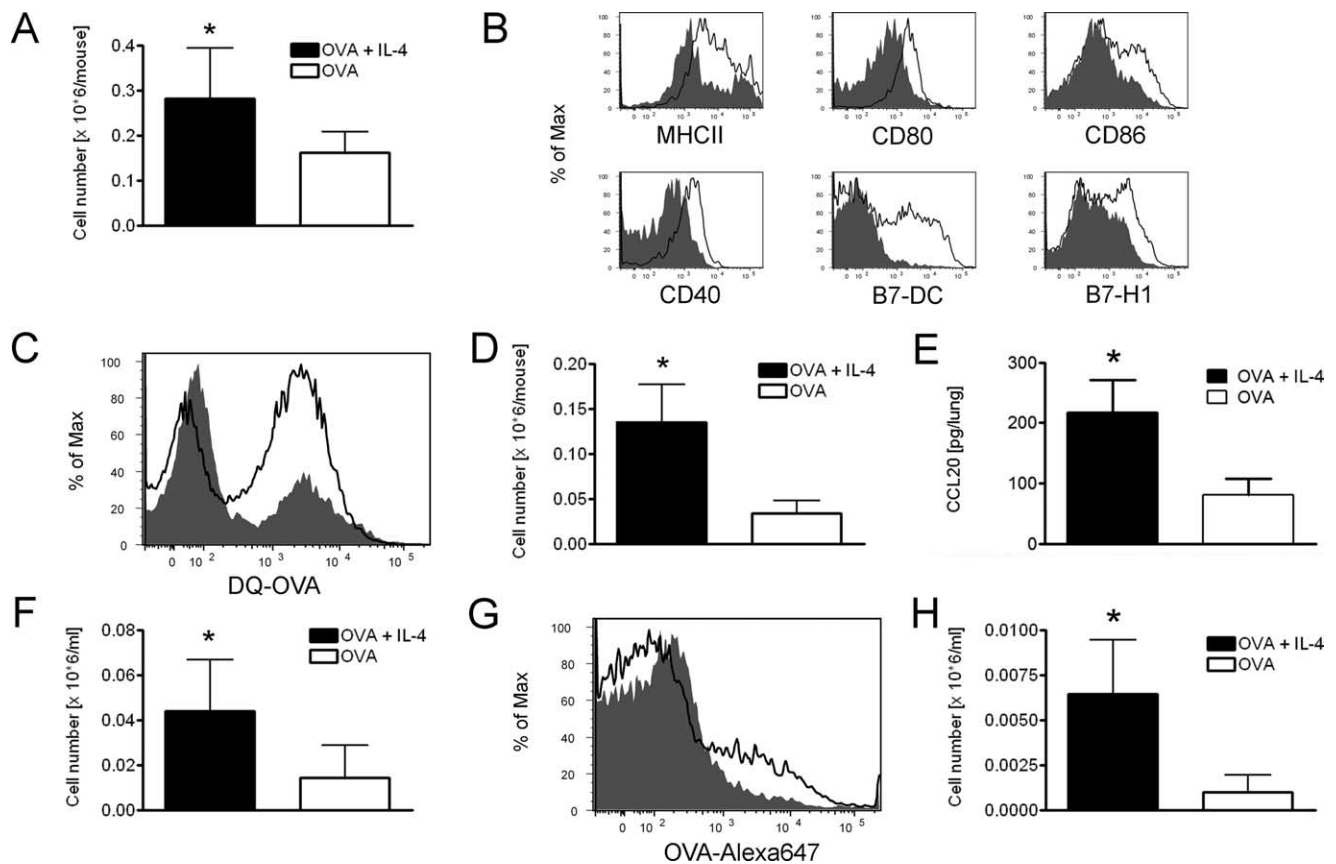


FIGURE 4. IL-4 increases recruitment, costimulatory molecule expression, and Ag presentation by DCs in vivo. WT mice were sensitized with OVA, conjugated to the fluorochromes DQ (lung) or Alexa 647 (mLN) with or without IL-4 to assess the effect of IL-4 on DC Ag uptake, processing, and presentation, as described in *Materials and Methods*. Twenty-four hours after the last sensitization, lungs were digested and mLNs were mechanically dispersed to obtain single-cell suspensions. Cell counts were assessed microscopically. Total cell numbers were calculated by multiplying total cell counts from single-cell suspensions with respective percentages from flow cytometric analysis. Pulmonary myeloid DCs were identified via flow cytometric staining for nonautofluorescent, CD11c^{high}-positive, CD11b-positive cells (A). Additionally, the above-referenced costimulatory molecules (B) and uptake of DQ-OVA (C and D) were analyzed on these cells. Lungs were harvested from TLR4d mice treated with OVA with or without IL-4 24 h after last sensitization, and homogenates were obtained and analyzed as described in *Materials and Methods* (E). Mediastinal lymph node myeloid DCs were identified via flow cytometric staining for CD11c^{high}-positive cells (F). In addition to CD11c staining, Ag uptake was determined by analysis of Alexa 647⁺ cells (G and H). Filled histograms represent results from animals treated with fluorochrome-labeled OVA alone; unfilled histograms represent animals treated with fluorochrome-labeled OVA plus IL-4.

(reviewed in Ref. 26; see also Ref. 4) and information from earlier studies that had identified CD4⁺ T cells (15) to be the single hematopoietic cell type necessary to induce an asthmatic response in mouse models, we sought to clarify the contribution of these two cell types in our model through transfer of IL-4R α -competent or -deficient cells of these two cell types. We thus transferred WT or IL-4R α -deficient BMDC and/or CD4⁺ T cells to hosts lacking IL-4R α expression in the hematopoietic compartment (HP⁻/SC⁺ phenotype) before priming. Although we were never able to restore the full phenotype (as seen in SC⁺/HP⁺ mice), possibly attributable to the contribution of yet unidentified other HP cells expressing the IL-4R α , we found that expression of IL-4R α on both BMDC and T cells led to partial restoration of airway inflammation and had an additive effect with regard to pulmonary inflammation (Fig. 3A). Transfer of IL-4R α -sufficient BMDCs alone never induced even partial restoration of the IL-4-dependent phenotype, presumably due to the failure of naive T cells to develop into Th2 cells without IL-4-dependent signaling. However, transfer of IL-4R α -competent T cells alone was able to do so, which was augmented even further by additional (co-)transfer of IL-4 R α -competent BMDCs (Fig. 3A). As expected, Ag-specific IgE se-

cretion could only be observed in mice fully competent in IL-4R α expression in all hematopoietic (and structural) cells (SC⁺/HP⁺ phenotype, Fig. 3B) given its known role in B cell isotype switching to IgE (27). The induction of IL-5, IL-10, and IL-13 seemed to be mainly controlled by IL-4R α -dependent signaling in CD4⁺ T cells since their transfer alone induced significant levels of these cytokines, which were not further augmented by (co-)transfer of IL-4 R α -competent BMDCs (Fig. 3C), suggesting that Ag presentation by APCs deficient in IL-4R α -signaling was sufficient to allow CD4⁺ T cell priming and was not required for restoring IL-5, IL-10, or IL-13 production.

IL-4 increases recruitment, costimulatory molecule expression, and Ag presentation by DCs in vivo

Subsequently, we evaluated the in vivo effects of IL-4 on myeloid DCs. To this end, WT mice were sensitized with fluorochrome-labeled OVA (DQ-OVA for analysis of lung DCs, OVA-Alexa 647 for mLN DCs) with or without IL-4 on 3 consecutive days. Twenty-four hours after the last sensitization, lungs were digested and mLN cells were mechanically dispersed to obtain single-cell

Table I. *IL-4 does not increase proliferation of Ag-specific T cells^a*

	% Proliferating CFSE ⁺ KJ1-26 ⁺ T Cells	% Nonproliferating CFSE ⁺ KJ1-26 ⁺ T Cells	% All CFSE ⁺ KJ1-26 ⁺ T Cells	Ratio Proliferating/ Nonproliferating
24 h				
OVA + IL-4	0.08557	0.014	0.09957	6:11
OVA	0.03304	0.00601	0.03905	5:49
72 h				
OVA + IL-4	0.265	0.00569	0.27069	46:57
OVA	0.139	0.00297	0.14197	46:80

^a CFSE-labeled CD4⁺ T cells from DO11.10 TCR transgenic mice were transferred to BALB/cJ mice 1 day prior to priming with OVA (5 μ g) with or without IL-4, as described in *Materials and Methods*. To analyze effects of IL-4 on T cell priming, T cell proliferation in mLNs was determined via CFSE dilution at the indicated time points. Ratios of proliferating vs nonproliferating cells were determined by dividing percentages of total proliferating cells (*left and middle boxes* in Fig. 5) through percentages of cells without CFSE dilution (*right boxes* in Fig. 5).

suspensions. Cell counts of individual mice were assessed microscopically. Cells were further analyzed for Ag uptake and expression of costimulatory molecules via flow cytometric analysis. Multiplication of microscopic cell counts with corresponding percentages from flow cytometric analyses led to assessment of total cell numbers of respective cell subpopulations.

For lung DCs gates were set on nonautofluorescent cells initially, and among these CD11c⁺ and CD11b⁺ cells were considered to be myeloid DCs (Fig. 4A). Consecutively, the expression of costimulatory molecules was assessed on these cells (Fig. 4B), as well as the uptake and processing of DQ-OVA (Fig. 4, C and D). Lung homogenates were analyzed for CCL20 content to provide mechanistic insight into DC recruitment (Fig. 4E). For mLNs, DCs were considered CD11c⁺ cells (Fig. 4F). In addition to CD11c staining, Ag uptake was determined by analysis of Alexa 647⁺ cells (Fig. 4, G and H).

IL-4 led to increased recruitment of myeloid DCs to the lung following priming with OVA and IL-4, compared with OVA alone (Fig. 4A). Additionally, IL-4 increased expression of MHC class II and costimulatory molecules on lung DC (Fig. 4B) and facilitated Ag uptake and processing of lung DCs (Fig. 4, C and D). Mechanistically, we found increased levels of CCL20 in lung homogenates of animals treated with OVA plus IL-4 compared with animals treated with OVA alone (Fig. 4E), suggesting that this chemokine might mediate the increased recruitment of DCs to the lung.

Finally, IL-4 increased migration of Ag-carrying myeloid DCs to mLNs when comparing mice treated with fluorochrome-labeled OVA and IL-4 to mice treated with fluorochrome-labeled OVA alone (Fig. 4F–H). Taken together with the results from the BMDC transfer experiments, these results suggest that IL-4 acts on DCs by increasing numbers and Ag presentation capacity, thereby facilitating priming of naive T cells.

IL-4 treatment increases T cell recruitment to draining LNs

To dissect the effect of IL-4 on naive T cells, we transferred Ag-specific CFSE-labeled DO11.10 CD4⁺ T cells before priming and analyzed the effects of adding IL-4 to Ag in the sensitization phase. Interestingly, we were not able to observe an effect of IL-4 on T cell proliferation. We consistently observed a trend toward higher total percentages of KJ1-26⁺ CFSE-labeled cells in the draining LNs of animals treated with OVA and IL-4. However, the ratio of proliferating vs no-proliferating Ag-specific T cells among total CFSE-labeled Ag-specific T cells within the draining LN did not differ significantly between animals treated with OVA and IL-4 vs animals treated with OVA alone (Table I). In contrast, total cell numbers differed considerably when comparing the two groups, an effect valid for Ag-specific T cells, as well as for CD4⁺ and CD8⁺

T cells in draining LNs in general (Fig. 5B). These findings indicate that recruitment of Ag-specific T cells to the mLNs was increased via IL-4, but not their proliferative potential. Addition of IL-4 to Ag increased recruitment of Ag-specific and total T cells at all time points analyzed (24 and 48 h not shown for total cell numbers), suggesting that increased recruitment of T cells specific for Ags other than the Ag inducing the immune response might

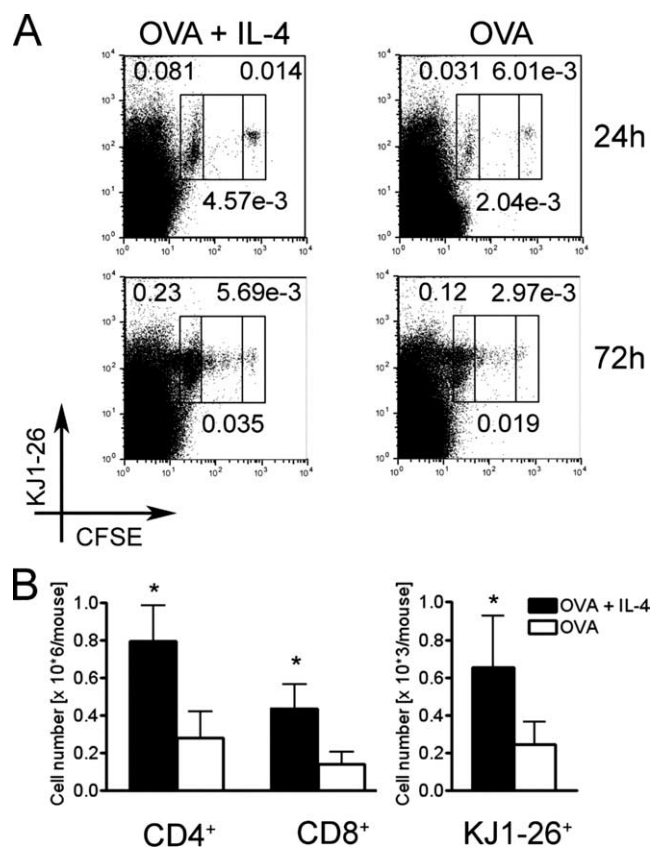


FIGURE 5. IL-4 treatment increases T cell recruitment to draining LNs. CFSE-labeled CD4⁺ T cells from DO11.10 TCR transgenic mice were transferred to BALB/cJ mice 1 day before priming with OVA (5 μ g) with or without IL-4, as described in *Materials and Methods*. To analyze the effects of IL-4 on T cell priming, T cell proliferation in mLNs was determined via CFSE dilution at the indicated time points (A), while T cell recruitment to draining lymph nodes was assessed by staining for CD4⁺ and CD8⁺ and the TCR-specific Ab KJ1-26⁺ at 72 h (B). Total cell counts were obtained by multiplying respective percentages from flow cytometric analysis with corresponding total cell counts obtained from microscopic cell counts from single-cell suspensions from mLNs.

indeed be an important mechanism by which IL-4 facilitates (colateral) priming.

Discussion

In the past decades, tremendous progress has been made in the understanding of the effector phase of allergic airway diseases. However, the sensitization phase of these pathologies is far less understood, partly due to the lack of suitable animal models (1, 2). In recent years our laboratory has contributed to the understanding of the priming phase of allergic airway disease by developing different models suited to analyze this part of the immune response in which we show 1) a requirement for TLR and MyD88 signaling in priming via the airways (3, 4), and 2) a promotion of naive T cell priming through an ongoing Th2-polarized airway inflammation (9), findings that have since been confirmed by other investigators (5–8).

Combining these two major findings, we show herein that IL-4 can bypass the need for signals via the immune system in pulmonary priming, a process we call IL-4-dependent pulmonary priming. Similar to what has been described for other cytokines, for example, TNF- α (3) or GM-CSF (19), IL-4 induces CD4⁺ T cell priming toward new Ags. Through consecutive studies, we were able to determine that this process depends on intact expression of IL-4R α on CD4⁺ T cells and myeloid DCs but also on yet undefined cells in the structural compartment of the lung or mLN. While the identification of the cell types and mechanisms involved in IL-4-dependent pulmonary priming in the structural compartment was beyond the scope of this study, our results suggest that IL-4 contributes to Th2 priming via the hematopoietic compartment by 1) an autocrine pathway directed toward homing of naive T cell to local LNs, as suggested by other studies (10), and 2) by activating lung DC and facilitating their interaction with T cells in the draining LNs, a finding that had also been suggested by older studies (28–31).

It is well known that IL-4 and the IL-4 receptor play a central role in the allergic airway response and Th2 differentiation in general (11–17), crucially regulating the occurrence and intensity of airway inflammation by expression of the IL-4 receptor on hematopoietic and nonhematopoietic cells (32). Until recently, however, it was not clear if IL-4 can directly influence naive T cell priming in vivo. In vitro studies had shown that IL-2 is necessary for the initial clonal expansion during priming and/or differentiation of Th2 cells, and its autocrine secretion was thought to be crucially dependent on two-signal-dependent T cell activation by fully activated APCs (33–36). Activation of APCs, on the other hand, was thought to depend on the recognition of stimuli directed toward the innate immune system (3–5, 37). Previously, only our collateral priming system (9) demonstrated that IL-4 can facilitate T cell priming toward new Ags independent of signals via the innate immune system.

Our new data assert that priming via the airways can occur independently of signals via pattern recognition receptors. Other authors have determined that priming within the airways is driven by pathogen-associated molecular patterns recognized by TLR2 (6), TLR3 (38), and TLR4 (3). However, our new results confirm and extend the data from Eisenbarth et al. (9) and Hayashi et al. (10) that priming at this site can also occur independently from such signals and therefore establish IL-4-dependent pulmonary priming as a novel mechanism of sensitization via the airway.

Furthermore, by showing a direct effect of IL-4 on naive T cells and via DCs in naive mice, we establish the main cellular players in this process. Previous studies have shown that the facilitation of priming toward new Ags through an ongoing airway inflammation crucially depends on T cell recruitment to the draining LN (10), an

observation we retrace with our findings that recruitment of naive T cells to the mLN is affected by IL-4 but not proliferation per se (Fig. 5). By showing that within the hematopoietic compartment IL-4R α expression on CD4⁺ T cells alone is sufficient to induce secretion of Th2 cytokines and low-grade airway inflammation (Fig. 3), two aspects of the asthmatic phenotype, but that is insufficient to fully induce these two features (e.g., systemic sensitization and airway inflammation), whereas IL-4R α expression on DCs alone is insufficient to induce any of the hallmark phenotypical changes of our asthma model, we also recapitulate older data showing that CD4⁺ T cells are the single hematopoietic cell type sufficient for development of the main features of the asthmatic phenotype and crucially depend on IL-4 in this context (15). Considering that more recent studies show that IL-4 renders naive T cells resistant to T regulatory-dependent suppression (39) and down-regulates IDO expression (40), it is tempting to speculate that the modulation of regulatory mechanisms contributes to IL-4-dependent pulmonary priming.

Moreover, various studies have shown that airway inflammation and IL-4 can activate immature DCs in vivo and in vitro (29, 30, 41). Data on the contribution of the IL-4 receptor has indicated that cells with a phenotype also compatible with immature DCs play a crucial role in regulating the severity of airway inflammation (32), a finding compatible with our observations. Our transfer experiments (Fig. 3) suggest that DCs capable of responding to IL-4 are necessary to induce the maximal asthmatic phenotype but are insufficient to induce any of the phenotypical changes of our model by themselves, contrary to our observations when transferring T cells alone (Fig. 3). Furthermore, our results suggest that increased DC recruitment to the lung is mediated, at least partially, to an increase in CCL20 secretion, which is known to be secreted by epithelial cells in response to IL-4 (42) and that has been shown to play an important role in DC migration (43). The dissection of these mediators with particular emphasis on the contribution of the structural, nonhematopoietic compartment and the recruitment of T cells and DCs to the draining LNs is the topic of ongoing studies in our laboratory.

Our results demonstrating that proliferation of Ag-specific transgenic T cells was not affected (Table I, Fig. 5) despite increased Ag-presenting capacities of DCs within the draining LNs (Fig. 4) are difficult to reconcile. Our present data are not sufficient to explain these discrepancies. However, our data showing that increased numbers of CD4⁺ and CD8⁺ T cells are present in the lymph nodes of animals treated with OVA and IL-4 evoke the possibility that the small number of transgenic T cells is not so much affected by a pro-proliferative property of IL-4 as the proliferation of endogenous T cells. More detailed experiments are needed to address this issue.

In summary, our studies extend the known findings pertaining to DCs and IL-4 by suggesting that through increased recruitment, maturation, and Ag presentation IL-4 might influence DC-dependent priming of naive T cells for new Ags. Th2 differentiation by DCs has been shown to be crucially dependent on NF- κ B induction in DCs (44), which in turn can be induced by maturation with IL-4 (45), suggesting a molecular mechanism for our observations, which await analysis in future studies.

In vitro and in vivo studies have shown disparate results with regard to the effects of IL-4 on the capacity of DCs to influence the balance of Th1 or Th2 polarization (44–49). They reveal a complex regulatory role of IL-4 depending on the timing, culture conditions, mediators, and cell types studied. In our system, similar to that of Sriram et al. (49), the data point toward a positive role for IL-4 in driving an immune response toward Th2 polarization. Our in vivo data showed an increase in Th2 cytokines (Fig. 1) as an

endpoint read-out parameter influenced by the capacity of DCs to prime T cells toward Th1 or Th2 polarization. Similarly, in vitro data confirmed an increased capacity of DCs subjected to priming with IL-4 in vivo to prime naive T cells (data not shown). However, future studies will have to determine molecular pathways and mediators utilized by these DCs to induce the phenomena that we observed.

The clinical implications of our findings relate to how susceptible individuals might acquire new sensitizations to environmental proteins. Although the interaction between genetic susceptibility and environmental factors during the development of allergic diseases in childhood is recognized to play a pivotal role in the development of these diseases (50), this concept places little emphasis on the contribution of the local microenvironment. Our data suggest that this environment might provide key factors to the future direction of the disease once a susceptible individual has become sensitized. Furthermore, it might also determine an individual's susceptibility to develop an allergic response in the first place. Clinical data show that sensitization to one Ag is a major risk factor for subsequent sensitizations to other Ags (51–53), a process that is associated with an increase in IL-4 production from PBMC (54). We suggest that the initiation of Th2-polarized airway disease and the subsequent clinical outcome of an individual crucially depend on the genetic propensity for IL-4 production as well as environmental factors triggering IL-4 production in the organ where allergen contact occurs. Our model clearly shows that IL-4 facilitates priming of CD4⁺ T cells for new Ags, suggesting that interventions aimed at reducing IL-4 production such as immunotherapy (55–57) might take up the pathological immune response at a crucial point and would therefore be effective in the long-term inhibition of disease progression.

Disclosures

The authors have no financial conflicts of interest.

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