

Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice

Denis Bedoret, ... , Pierre Lekeux, Fabrice Bureau

J Clin Invest. 2009;119(12):3723-3738. <https://doi.org/10.1172/JCI39717>.

Research Article

Pulmonology

The respiratory tract is continuously exposed to both innocuous airborne antigens and immunostimulatory molecules of microbial origin, such as LPS. At low concentrations, airborne LPS can induce a lung DC-driven Th2 cell response to harmless inhaled antigens, thereby promoting allergic asthma. However, only a small fraction of people exposed to environmental LPS develop allergic asthma. What prevents most people from mounting a lung DC-driven Th2 response upon exposure to LPS is not understood. Here we have shown that lung interstitial macrophages (IMs), a cell population with no previously described *in vivo* function, prevent induction of a Th2 response in mice challenged with LPS and an experimental harmless airborne antigen. IMs, but not alveolar macrophages, were found to produce high levels of IL-10 and to inhibit LPS-induced maturation and migration of DCs loaded with the experimental harmless airborne antigen in an IL-10-dependent manner. We further demonstrated that specific *in vivo* elimination of IMs led to overt asthmatic reactions to innocuous airborne antigens inhaled with low doses of LPS. This study has revealed a crucial role for IMs in maintaining immune homeostasis in the respiratory tract and provides an explanation for the paradox that although airborne LPS has the ability to promote the induction of Th2 responses by lung DCs, it does not provoke airway allergy under normal conditions.

Find the latest version:

<https://jci.me/39717/pdf>





Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice

Denis Bedoret,¹ Hugues Wallemacq,¹ Thomas Marichal,¹ Christophe Desmet,¹ Florence Quesada Calvo,² Emmanuelle Henry,³ Rodrigue Closset,¹ Benjamin Dewals,⁴ Caroline Thielen,⁵ Pascal Gustin,⁶ Laurence de Leval,⁵ Nico Van Rooijen,⁷ Alain Le Moine,⁸ Alain Vanderplasschen,⁴ Didier Cataldo,² Pierre-Vincent Drion,⁹ Muriel Moser,³ Pierre Lekeux,¹ and Fabrice Bureau¹

¹Laboratory of Cellular and Molecular Physiology, GIGA-Research, University of Liège, Liège, Belgium. ²Laboratory of Biology of Tumors and Development, GIGA-Research, Centre Hospitalier Universitaire (CHU) de Liège, Liège, Belgium. ³Laboratory of Animal Physiology, Institute of Molecular Biology and Medicine, Université Libre de Bruxelles, Gosselies, Belgium. ⁴Laboratory of Immunology and Vaccinology, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium. ⁵Laboratory of Pathology, GIGA-Research, CHU de Liège, Liège, Belgium. ⁶Department for Functional Sciences, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium. ⁷Department of Molecular Cell Biology, Faculty of Medicine, Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands. ⁸Institute for Medical Immunology, Université Libre de Bruxelles, Gosselies, Belgium. ⁹Animal Facility (B23), University of Liège, Liège, Belgium.

The respiratory tract is continuously exposed to both innocuous airborne antigens and immunostimulatory molecules of microbial origin, such as LPS. At low concentrations, airborne LPS can induce a lung DC-driven Th2 cell response to harmless inhaled antigens, thereby promoting allergic asthma. However, only a small fraction of people exposed to environmental LPS develop allergic asthma. What prevents most people from mounting a lung DC-driven Th2 response upon exposure to LPS is not understood. Here we have shown that lung interstitial macrophages (IMs), a cell population with no previously described *in vivo* function, prevent induction of a Th2 response in mice challenged with LPS and an experimental harmless airborne antigen. IMs, but not alveolar macrophages, were found to produce high levels of IL-10 and to inhibit LPS-induced maturation and migration of DCs loaded with the experimental harmless airborne antigen in an IL-10-dependent manner. We further demonstrated that specific *in vivo* elimination of IMs led to overt asthmatic reactions to innocuous airborne antigens inhaled with low doses of LPS. This study has revealed a crucial role for IMs in maintaining immune homeostasis in the respiratory tract and provides an explanation for the paradox that although airborne LPS has the ability to promote the induction of Th2 responses by lung DCs, it does not provoke airway allergy under normal conditions.

Introduction

Respiratory mucosal surfaces are constantly exposed to a broad range of nonpathogenic environmental antigens. In the absence of proinflammatory signals, inhalation of harmless antigens results in immunological tolerance. Indeed, a subset of pulmonary myeloid DCs is able to produce the tolerogenic cytokine IL-10 after innocuous antigen uptake and, therefore, stimulate the development of antigen-specific Tregs (1, 2). Similarly, lung plasmacytoid DCs protect against aberrant immune responses to inhaled antigens by inducing Tregs (3).

Epidemiological studies have shown that ambient air contains not only inert antigens but also immunostimulatory molecules of microbial origin (4–9). Of particular interest is LPS (endotoxin), a cell wall component of Gram-negative bacteria that is ubiquitous in the environment (4, 5, 9). Airborne LPS activates cells of the respiratory innate immune system, such as DCs, through CD14 and TLR4 (10, 11). When the respiratory tract is stimulated with airborne LPS, lung DCs lose their tolerogenic properties and instead promote the development of either Th1 or Th2 cells directed against concomitant aeroantigens (11, 12). In spite of the fact that high or very high levels of endotoxin exposure in early life protect against Th2 sensitization by enhancing Th1 immunity (13–15), most evidence indicates that exposure to house dust endotoxin is a signifi-

cant risk factor for increased asthma prevalence and severity (4, 6, 9, 15–19). For example, the National Survey of Endotoxin in United States Housing has clearly demonstrated relationships between household endotoxin and diagnosed asthma, occurrence of asthma symptoms, current use of asthma medication, and wheezing (18).

Although LPS is omnipresent in the environment and favors airway allergy, only a minority of people develops asthma. These contradictory observations imply the existence of mechanisms capable of preventing LPS-triggered Th2 responses to inhaled antigens. We report here that LPS-induced airway allergy is tightly controlled by lung interstitial macrophages (IMs), a cell population that remains largely uncharacterized. IMs can be distinguished from alveolar macrophages (AMs) by their unique capacity to inhibit lung DC maturation and migration upon LPS stimulation, thereby preventing sensitization to concomitant aeroantigens. We furthermore demonstrate that this functional paralysis of lung DCs involves IL-10 production by IMs. We conclude that in the presence of LPS, IMs, but not AMs, break the link between innate and adaptive immunity, allowing harmless inhaled antigens to escape from T cell-dependent responses.

Results

Characterization of IMs. Although AMs and lung DCs have been described in detail, IMs have not yet been fully characterized, and their *in vivo* function remains unknown. It has been shown that AMs are positive for both the macrophage marker F4/80 and the DC marker CD11c, whereas IMs and lung DCs are F4/80⁺CD11c⁻

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 119:3723–3738 (2009). doi:10.1172/JCI39717.

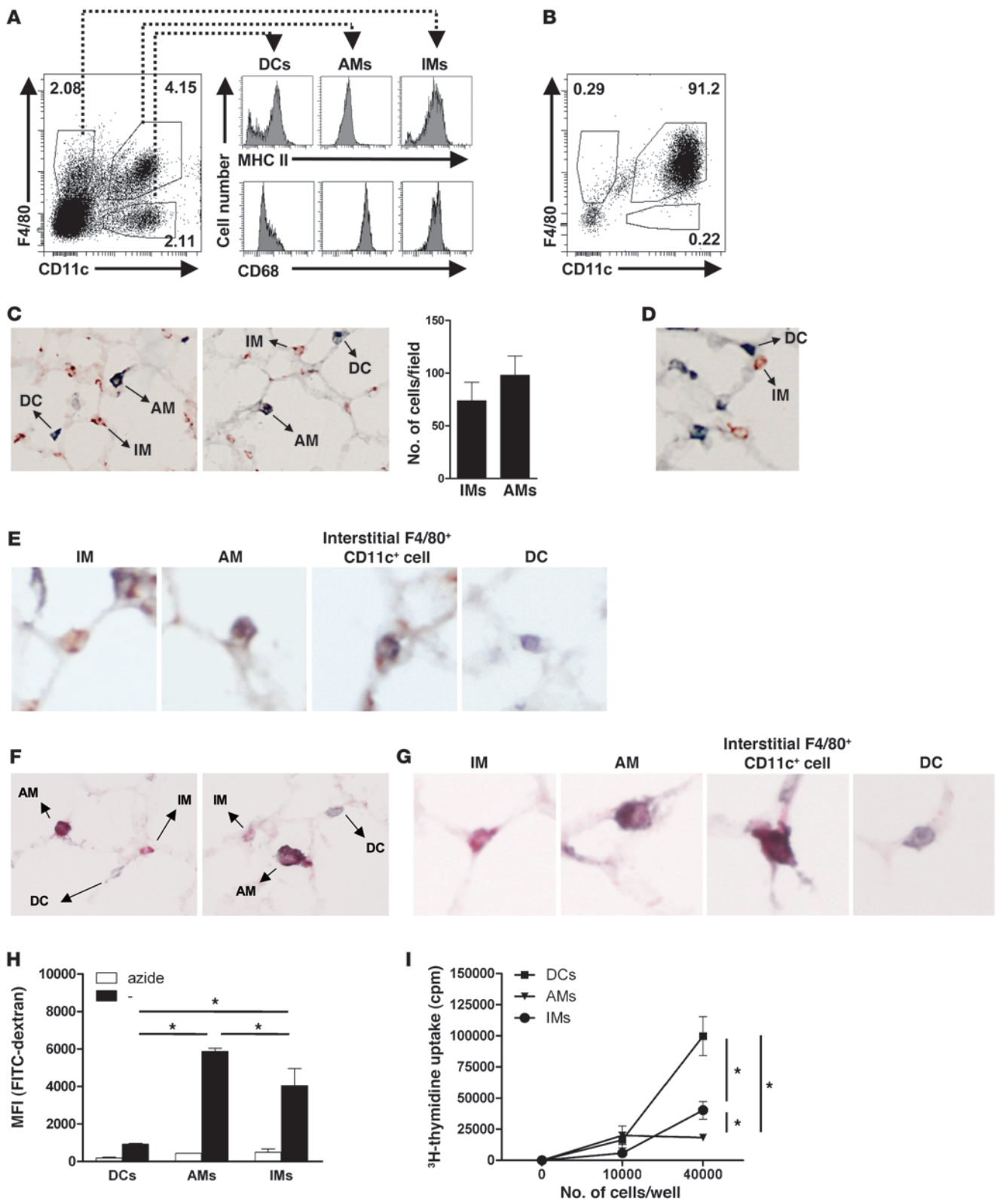




Figure 1

Phenotypic and functional comparison of IMs, AMs, and lung DCs. (A) The percentage of IMs (F4/80⁺CD11c⁻), AMs (F4/80⁺CD11c⁺), and DCs (F4/80⁻CD11c⁺) in whole lung from BALB/c mice was determined by flow cytometry. IMs, AMs, and DCs were also stained for MHC II and CD68. (B) The percentage of F4/80⁺CD11c⁻, F4/80⁺CD11c⁺, and F4/80⁻CD11c⁺ cells in BALF from BALB/c mice. (C–E) Lung cryosections were double stained for CD11c (blue) and F4/80 (red). IMs (F4/80⁺CD11c⁻) are stained red, DCs (F4/80⁻CD11c⁺) are stained blue, and AMs (F4/80⁺CD11c⁺) are double stained. (C) Representative photographs showing some IMs, AMs, and DCs (original magnification, ×100). The average number of IMs and AMs per field was calculated. (D) Image of IMs in the vicinity of DCs (original magnification, ×100). (E) Photographs at higher magnification showing IMs, AMs, interstitial F4/80⁺CD11c⁺ macrophages, and DCs (original magnification, ×200). (F and G) Alternatively, F4/80 was stained pink rather than red. (F) Representative photographs showing some IMs (pink), DCs (blue), and AMs (purple) (original magnification, ×100). (G) Photographs at higher magnification (original magnification, ×200). (H) FACS-sorted APCs were cocultured with FITC-labeled dextran in the absence or presence of sodium azide. After 45 minutes, the uptake of fluorescent dextran was determined by flow cytometry. (I) FACS-sorted APCs (1 × 10⁴ or 4 × 10⁴ cells/well) were loaded with the DO11.10 OVA peptide and cocultured for 3 days with naive DO11.10 CD4⁺ T cells. DO11.10 T cell proliferation was assessed by [³H]thymidine uptake in a 16-hour pulse. *P < 0.05 (H and I).

and F4/80⁺CD11c⁺, respectively (20). To further characterize IMs and to compare them with AMs and lung DCs, whole lungs from naive BALB/c mice were digested and stained for F4/80 and CD11c. We found that IMs were about two times less abundant than AMs (~2.1 vs. ~4.2%) and were present at a frequency similar to that of lung DCs (Figure 1A). Further phenotype analysis of IMs revealed that these cells express high levels of MHC class II (Figure 1A). Indeed, MHC II expression in IMs was equivalent to that found in lung DCs and significantly higher than that observed in AMs (Figure 1A). Finally, we showed that AMs and IMs were all positive for the pan-macrophage marker CD68, whereas lung DCs did not express this molecule (Figure 1A).

To gain further insight into the localization of lung macrophage subsets, extensive bronchoalveolar lavages (BALs) were performed and BAL cells were examined for F4/80 and CD11c expression by flow cytometry. Lungs were lavaged with PBS-EDTA to dislodge strongly adherent airway cells. More than 90% of BAL cells were positive for both F4/80 and CD11c (Figure 1B), which is consistent with the fact that AMs express these two markers and that they are the most abundant cells found in the airway lumen (20). Some cells displaying the phenotype of IMs (F4/80⁺CD11c⁻) were found in the BAL fluid (BALF). However, these cells represented only approximately 0.2% of total BAL cells (Figure 1B). Double immunostaining of lung tissues confirmed that F4/80⁺CD11c⁻ macrophages are confined to the interstitial compartment (Figure 1, C–G), where they could be detected in the vicinity of lung DCs (Figure 1, D and F). Immunohistochemistry also revealed the presence in the interstitium of some macrophages harboring the AM phenotype (F4/80⁺CD11c⁺) (Figure 1, E and G). However, most F4/80⁺CD11c⁺ cells were found in the airway lumen, as expected (Figure 1, C and E–G). Although some DCs (F4/80⁻CD11c⁺) were observed in the lumen (Figure 1C), most of them were located in the interstitium (Figure 1, C–G).

Macrophages, unlike DCs, have a high capacity to phagocytose but are poor inducers of T cell proliferation (21, 22). We exam-

ined these two functions in FACS-sorted IMs, AMs, and lung DCs. Phagocytosis was determined by flow cytometry evaluation of FITC-labeled dextran uptake. Although lung DCs were able to take up dextran, only IMs and AMs displayed strong phagocytic activity (Figure 1H). To assess their ability to induce T cell proliferation, IMs, AMs, and lung DCs were loaded with the DO11.10 chicken OVA peptide 323–339 and cultured with CD4⁺ T cells from DO11.10 mice (i.e., OVA₃₂₃₋₃₃₉-specific, MHC II-restricted, TCR transgenic mice; ref. 23). Figure 1I shows that lung DCs were much more effective than IMs and AMs in inducing DO11.10 T cell proliferation. As previously reported by others (24), IMs, compared with AMs, had a lower phagocytic potential but were more effective in stimulating T cell proliferation (Figure 1, H and I).

IMs, but not AMs, have the capacity to prevent airway allergy induced by LPS-stimulated, OVA-pulsed DCs. Stimulation of the respiratory tract by low LPS doses enables lung DCs to induce asthmatic responses to concomitantly inhaled antigens (11). However, only a small fraction of people exposed to environmental LPS suffer from atopic asthma, indicating that protective mechanisms exist that can counteract the pro-Th2 effects of LPS. We postulated that IMs, which are found in the vicinity of lung DCs (Figure 1D), might alter the function of lung DCs and therefore play a role in the control of airway allergy. The following experiments were conducted to verify this hypothesis.

Lambrecht et al. have shown that bone marrow-derived dendritic cells (BMDCs) pulsed with OVA from batches containing low quantities of LPS undergo maturation in vitro and are subsequently endowed with the capacity to induce Th2 sensitization to OVA when injected intratracheally (i.t.) (25). As the number of DCs obtained from the lungs of mice was too small to perform large-scale experiments, BMDCs were cocultured with either IMs or AMs to determine whether these macrophage subsets may impact the function of LPS-stimulated DCs. To generate BMDCs, bone marrow cells from naive BALB/c mice were cultured for 8 days in the presence of GM-CSF. As GM-CSF may alter macrophage function (26), the BMDC culture medium was replaced on day 8 with fresh medium devoid of GM-CSF. FACS-purified AMs or IMs were then added to BMDC cultures, and cocultured cells were pulsed 1 hour later with OVA combined with low-dose LPS (OVA_{LPS}; 100 μg OVA and 10 ng LPS/ml; the LPS dose was analogous to levels commonly found in extracts from household dust; ref. 14). At day 9, OVA_{LPS}-stimulated cocultured cells (hereafter referred to as OVA_{LPS}-BMDCs/AMs and OVA_{LPS}-BMDCs/IMs) were collected and injected i.t. into syngeneic recipients. Unpulsed BMDCs (PBS-BMDCs) and OVA_{LPS}-pulsed BMDCs (OVA_{LPS}-BMDCs) were used as negative and positive controls of Th2 sensitization, respectively. Ten days after adoptive transfer, mice were challenged with OVA aerosol during a 30-minute challenge on 5 consecutive days to induce allergic airway inflammation. Twenty-four hours after the last challenge, the severity of pulmonary allergy was assessed. As expected in this model, mice that received OVA_{LPS}-BMDCs, but not those injected with PBS-BMDCs, developed BALF and peribronchial lung tissue eosinophilia and lymphocytosis, accompanied by goblet cell hyperplasia and increased mucus production (Figure 2, A–C). All these inflammatory signs were strongly attenuated in mice administered OVA_{LPS}-BMDCs/IMs, an effect not observed in mice receiving OVA_{LPS}-BMDCs/AMs. Furthermore, serum levels of OVA-specific IgE, as well as Th2 cytokine production and T cell proliferation in mediastinal lymph nodes (MLNs), were substantially decreased in mice injected with OVA_{LPS}-BMDCs/

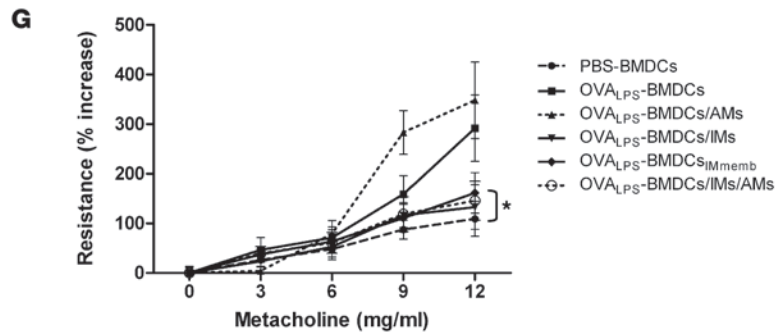
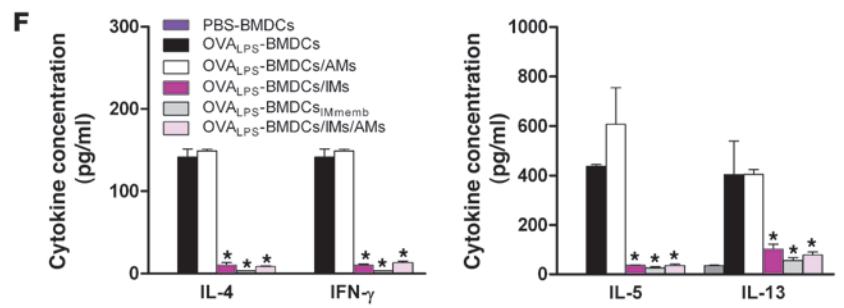
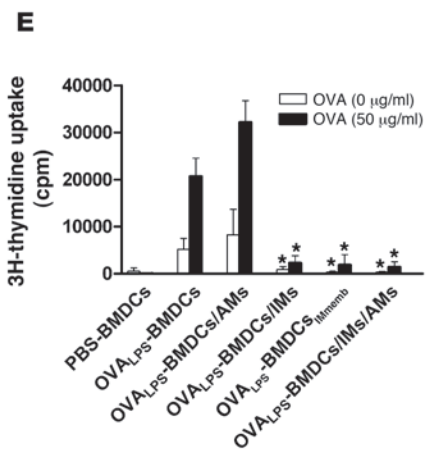
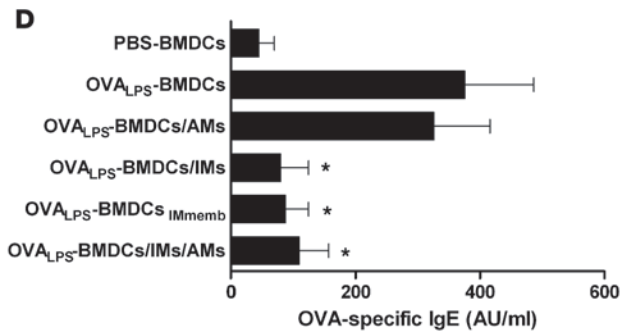
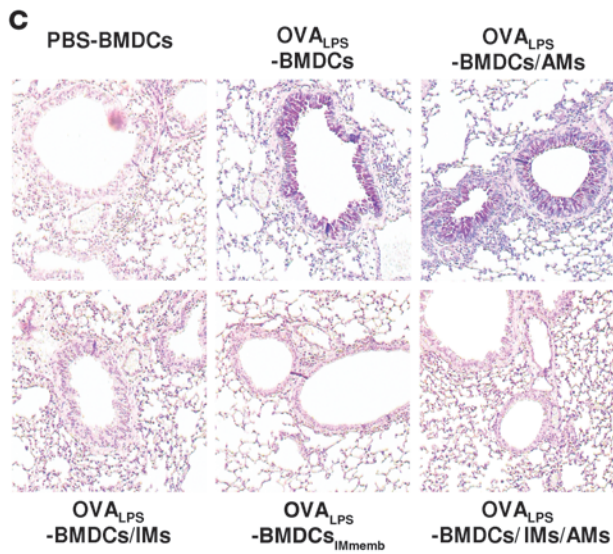
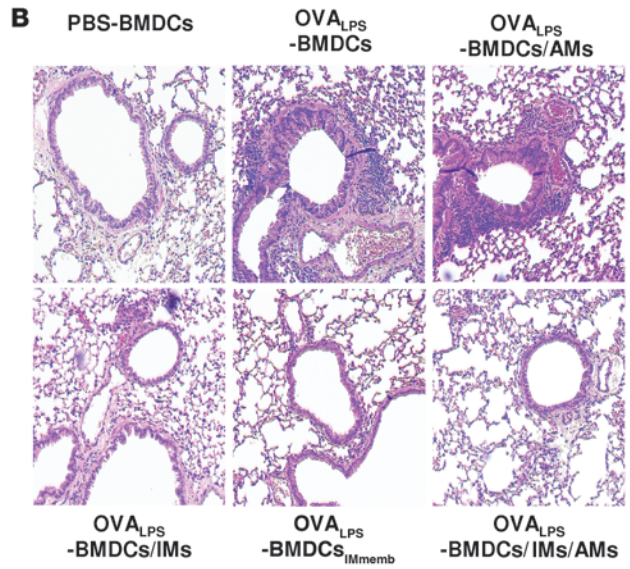
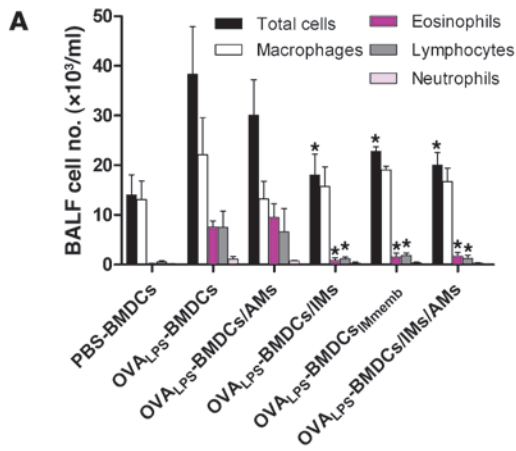




Figure 2

IMs are able to suppress the induction of airway allergy by OVA-pulsed, LPS-stimulated DCs. (A–G) Naive BALB/c mice were injected i.t. with PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/AMs, OVA_{LPS}-BMDCs/IMs, OVA_{LPS}-BMDCs_{IMmemb}, or OVA_{LPS}-BMDCs/IMs/AMs. From day 10 to day 14, mice were exposed to OVA aerosols. Twenty-four hours after the last challenge, the severity of airway allergy was evaluated. (A) BALF was subjected to total and differential cell counts. (B and C) Lung sections were stained with either H&E (B) or PAS (C) (original magnification, ×100). (D) Levels of OVA-specific IgE were measured in serum samples by ELISA. OVA-specific IgE levels are expressed as AU. (E and F) MLN cells were restimulated in vitro for 3 days with 50 μg/ml OVA. (E) The proliferation was measured as [³H]thymidine incorporation during the last 16 hours. (F) Culture supernatants were assayed for IL-4, IFN-γ, IL-5, and IL-13 by ELISA. (G) AHR to various doses of methacholine was assessed by invasive measurement of dynamic resistance. *P < 0.05 versus OVA_{LPS}-BMDCs and OVA_{LPS}-BMDCs/AMs (A and D–G).

IMs compared with those administered either OVA_{LPS}-BMDCs or OVA_{LPS}-BMDC/AMs (Figure 2, D–F). We also measured airway hyperreactivity (AHR) to nonspecific stimuli (methacholine), a cardinal feature of allergic asthma. As shown in Figure 2G, mice injected with OVA_{LPS}-BMDCs or OVA_{LPS}-BMDCs/AMs displayed a considerable increase in responsiveness to methacholine compared with mice receiving PBS-BMDCs, as assessed by invasive measurement of dynamic resistance in mechanically ventilated mice. By contrast, mice that received OVA_{LPS}-BMDCs/IMs did not show hyperreactivity to methacholine (Figure 2G). Together, these results indicate that IMs, but not AMs, have the capacity to prevent airway allergy induced by OVA_{LPS}-BMDCs.

Next, experiments were performed in which IMs and BMDCs were cocultured across a semipermeable membrane during stimulation with OVA_{LPS}. In these experiments, BMDCs (hereafter referred to as OVA_{LPS}-BMDCs_{IMmemb}) were injected alone into recipients, and immune responses were assessed after OVA challenge as described above. OVA_{LPS}-BMDCs_{IMmemb} were unable to

induce significant asthmatic responses in recipient mice (Figure 2, A–G). This result confirms that IMs exert their effects by interfering with DC function rather than by acting directly on the adaptive immune system and demonstrates that soluble rather than membrane-bound molecules are responsible for IM actions.

In another series of experiments, BMDCs, IMs, and AMs were cocultured and pulsed with OVA_{LPS} (OVA_{LPS}-BMDCs/IMs/AMs) before being injected together into naive recipients. Mice that received OVA_{LPS}-BMDCs/IMs/AMs did not develop overt asthmatic reactions after OVA challenge (Figure 2, A–G), showing that IMs can exert their inhibitory effects in the presence of AMs.

Of note, i.t. administration of BMDCs pulsed with LPS-free OVA did not result in Th2 responses (data not shown), confirming that activation of DCs by proinflammatory mediators is required for induction of airway allergy. Finally, neither AMs nor IMs induced any inflammatory response when pulsed with LPS-free OVA or OVA_{LPS} and injected alone into recipient mice (data not shown).

To make sure that LPS was required for inducing the immunosuppressive effects of IMs, we performed additional experiments with TLR4-deficient IMs. We observed that TLR4-deficient IMs were unable to prevent allergic sensitization induced by cocultured WT OVA_{LPS}-BMDCs. Indeed, once reinjected into the trachea of WT recipient mice, OVA_{LPS}-BMDCs that were cocultured with *Tlr4*^{-/-} IMs induced airway eosinophilia of the same intensity as did OVA_{LPS}-BMDCs that were cultured alone (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI39717DS1). Consistently, *Tlr4*^{-/-} IMs also did not prevent Th2 cytokine production and T cell proliferation in MLNs following i.t. injection of cocultured OVA_{LPS}-BMDCs (Supplemental Figure 1, C and D). These data demonstrate the importance of TLR4, and hence LPS, in promoting the immunosuppressive activity of IMs.

IMs can inhibit primary T cell activation triggered by OVA_{LPS}-pulsed DCs. Next, we tested the effects of IMs (and AMs) on primary T cell activation induced by OVA_{LPS}-BMDCs. To enhance the frequency of OVA-specific CD4⁺ T lymphocytes in vivo, naive BALB/c mice

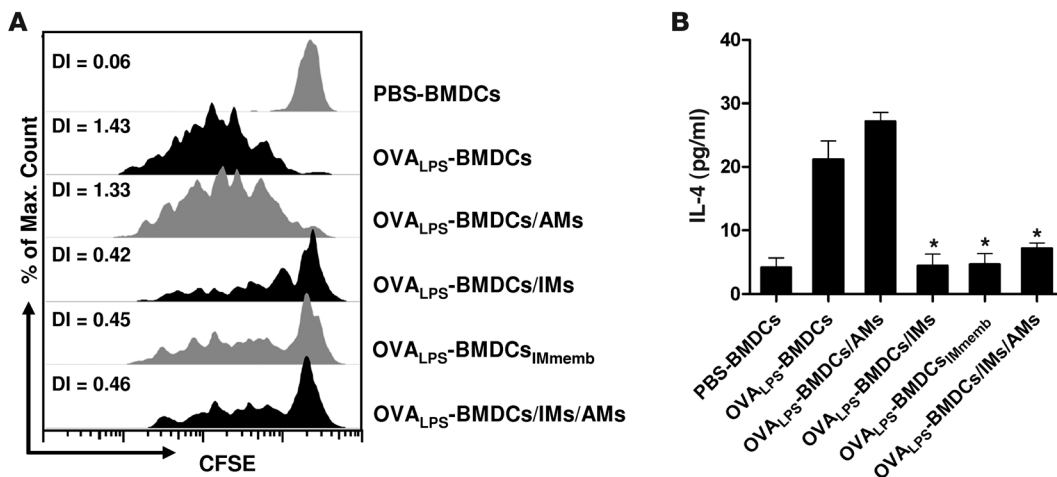


Figure 3

IMs can inhibit DC-mediated priming of Th2 cells. (A and B) Naive BALB/c mice were injected i.v. with 10⁷ CFSE-labeled DO11.10 T cells (day –1). Twenty-four hours later (day 0), mice received an i.t. administration of PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/AMs, OVA_{LPS}-BMDCs/IMs, OVA_{LPS}-BMDCs_{IMmemb}, or OVA_{LPS}-BMDCs/IMs/AMs. On day 3, MLNs were collected. (A) Proliferation of CFSE-labeled OVA-specific T cells was measured by flow cytometry. DI, division index; Max, maximum. (B) MLN cells were restimulated in vitro for 3 days with 25 μg/ml OVA, and supernatants were assayed for IL-4 by ELISA. *P < 0.05 versus OVA_{LPS}-BMDCs and OVA_{LPS}-BMDCs/AMs.

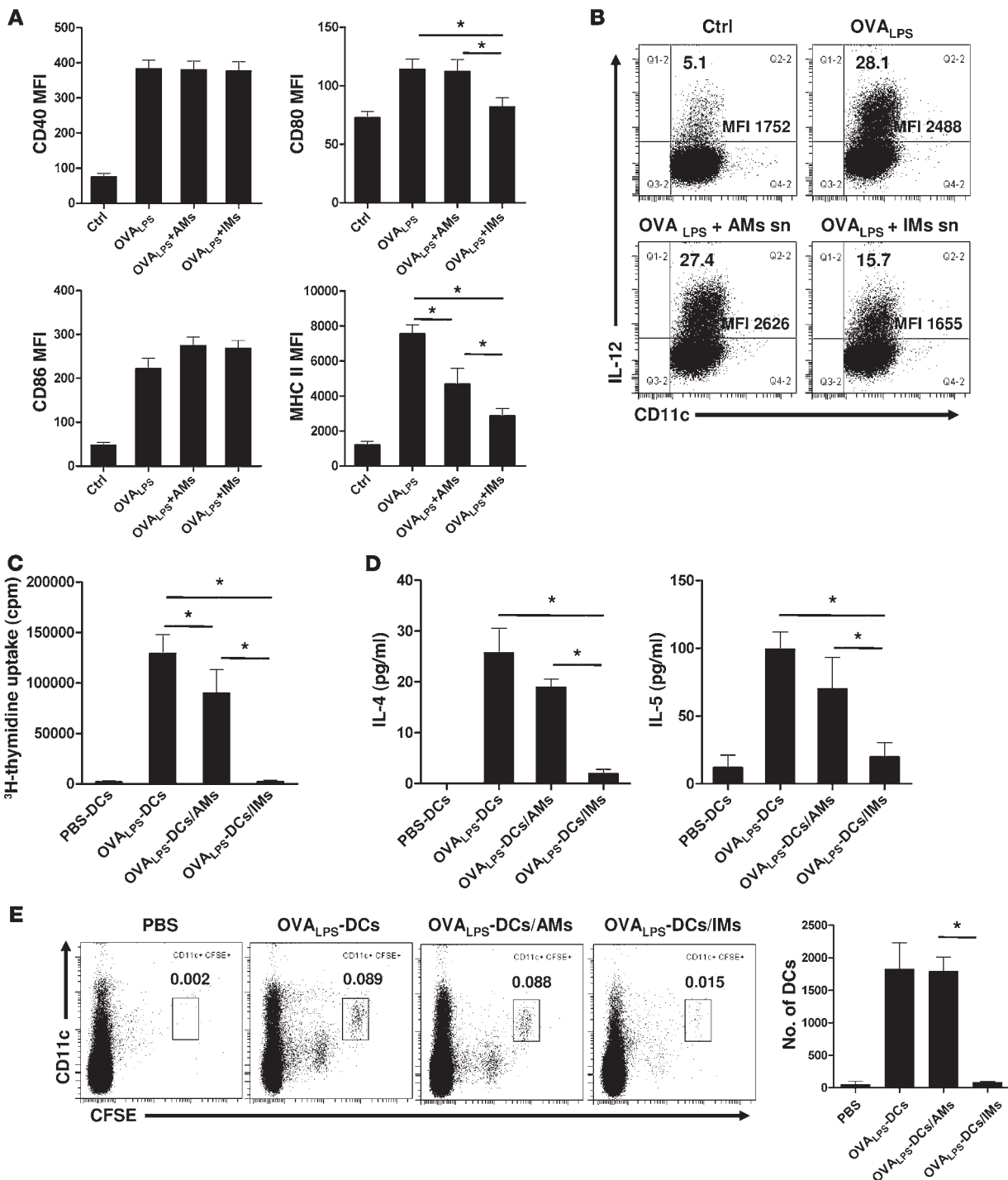


Figure 4

IMs have the capacity to attenuate LPS-induced maturation and migration of antigen-loaded DCs. **(A)** Lung DCs (10^6 cells) from BALB/c mice were stimulated for 16 hours with OVA_{LPS} in the presence or absence of AMs (2×10^6 cells) or IMs (1×10^6 cells). DCs were then assayed for expression of CD40, CD80, CD86, and MHC II by FACS. Freshly isolated lung DCs served as control (Ctrl). MFIs are shown. **(B)** Lung DCs were placed in fresh medium or in the supernatant (sn) of OVA_{LPS}-stimulated IMs or AMs. DCs were then treated with OVA_{LPS} for 2 hours, incubated with Brefeldin A for an additional 5 hours, and finally stained for IL-12p40. The percentage of IL-12-positive cells was measured by FACS. **(C and D)** Lung DCs (2×10^4 cells) were stimulated for 16 hours with OVA_{LPS} in the presence or absence of AMs (4×10^4 cells) or IMs (2×10^4 cells). APCs were then cocultured for 72 hours with 2×10^5 DO11.10 CD4⁺ T cells. Unpulsed DCs were used as controls. **(C)** DO11.10 T cell proliferation was measured. **(D)** IL-4 and IL-5 were measured in the supernatants (ELISA). **(E)** CFSE-labeled OVA_{LPS}-DCs, OVA_{LPS}-DCs/AMs, or OVA_{LPS}-DCs/IMs were injected in the trachea of naive mice. Control mice received PBS. Twenty-four hours later, MLNs were digested and stained for F4/80 and CD11c. The percentages of migrating DCs (CFSE⁺F4/80-CD11c⁺) among total MLN cells were determined by FACS. The total numbers of migrating DCs were calculated. * $P < 0.05$ (A and C–E).



were transferred with CFSE-labeled DO11.10 transgenic T cells. Twenty-four hours later, transferred mice were injected i.t. with PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/AMs, OVA_{LPS}-BMDCs/IMs, OVA_{LPS}-BMDCs_{SI_{mem}}, or OVA_{LPS}-BMDCs/IMs/AMs. Seventy-two hours after DC injection, MLNs were collected and analyzed by flow cytometry for the proliferation of CFSE-labeled DO11.10 T cells. OVA_{LPS}-BMDCs induced strong proliferation of OVA-specific T cells, whereas no cell division was observed in mice injected with PBS-BMDCs (Figure 3A). IMs, but not AMs, significantly, but not totally, reduced T cell expansion when injected together with OVA_{LPS}-BMDCs (Figure 3A). It has to be noted that adoptive transfer of large numbers of antigen-specific T cells artificially amplified the T cell proliferative response. This may explain why the residual stimulatory activity of OVA_{LPS}-BMDCs/IMs observed here seems of higher magnitude than the residual inflammation developing in the lungs of mice receiving OVA_{LPS}-BMDCs/IMs (Figure 2). The inhibitory effect of IMs on OVA_{LPS}-BMDC-induced T cell proliferation was congruently reflected in the decreased IL-4 production by OVA-restimulated MLN cells from mice that received OVA_{LPS}-BMDCs/IMs, as compared with those from mice sensitized with either OVA_{LPS}-BMDCs or OVA_{LPS}-BMDCs/AMs (Figure 3B). IMs retained their inhibitory potential when cultured with BMDCs across a semipermeable membrane or when cultured with both BMDCs and AMs (Figure 3, A and B). Of note, OVA_{LPS}-stimulated IMs and AMs neither induced significant DO11.10 proliferation nor stimulated IL-4 production in MLNs when injected alone (data not shown).

Finally, we performed experiments to determine whether IMs were able to induce the differentiation of Foxp3⁺ Tregs. In these experiments, DO11.10 CD4⁺ T cells were cultured with FACS-sorted lung DCs, IMs, or AMs; stimulated or not with OVA_{LPS}; and assessed for the differentiation of Foxp3⁺ Tregs by intracellular staining and flow cytometry analyses. Supplemental Figure 2 shows that none of the conditions tested led to the differentiation of Tregs.

These data show that IMs have the ability to suppress DC-mediated priming of Th2 cells, without inducing Tregs.

Maturation and migration of LPS-activated DCs is impaired in the presence of IMs. Additional experiments were undertaken to more precisely define the mechanisms by which IMs affect DC function. As only small numbers of DCs were required to perform these experiments, we used lung DCs rather than BMDCs. DCs stimulated with proinflammatory molecules undergo maturation, a prerequisite for induction of robust immune responses (27). As expected, OVA_{LPS}-DCs, compared with freshly isolated lung DCs, displayed increased expression of the maturation markers CD40, CD80, CD86, and MHC II (Figure 4A). Expression of MHC II by OVA_{LPS}-DCs was substantially decreased when these cells were cocultured with either AMs or IMs, with a more pronounced effect of IMs. Furthermore, IMs, but not AMs, abrogated the increase in CD80 expression in OVA_{LPS}-DCs. The effects of IMs on CD80 and MHC II expression and of AMs on MHC II expression by OVA_{LPS}-DCs were statistically significant. Neither AMs nor IMs affected the levels of CD40 and CD86 expression. Thus, there were only small differences in costimulatory molecule expression between DCs cocultured with IMs and DCs cocultured with AMs, suggesting that IMs induced other, more specific DC changes. We therefore performed additional experiments to determine whether IMs also affected cytokine production by lung DCs. Figure 4B shows that OVA_{LPS}-DCs, compared with freshly isolated lung DCs, produced much higher amounts of the immunostimulatory cytokine IL-12.

The production of IL-12 by OVA_{LPS}-DCs decreased by about 50% when these cells were incubated with the culture supernatant of OVA_{LPS}-stimulated IMs. This effect was specific. Indeed, the culture supernatant of OVA_{LPS}-activated AMs had no effect on IL-12 production by DCs. These data on DC maturation suggested that IMs were able to compromise the ability of lung DCs to stimulate T cells. To investigate this possibility, we incubated OVA_{LPS}-DCs, OVA_{LPS}-DCs/AMs, and OVA_{LPS}-DCs/IMs with DO11.10 T cells. We observed that, while lung DCs alone induced robust T cell proliferation and AMs only marginally reduced it, coculture of lung DCs with IMs abolished T cell proliferation (Figure 4C). Moreover, while DO11.10 T cells stimulated by OVA_{LPS}-treated lung DCs expressed IL-4 and IL-5, indicative of Th2 differentiation, levels of these cytokines were significantly reduced when the DCs were cultured in the presence of IMs (Figure 4D). By contrast, AMs had only slight effects on Th2 cytokine production by DO11.10 T cells (Figure 4D). The loss of lung DC function in the presence of IMs can be explained, at least partially, by the combined effects of IMs on costimulatory molecule expression and cytokine production by DCs. However, further investigation will be required to formally identify all of the mechanisms involved in the inhibition of DC function by IMs.

We next sought to determine whether IMs could also interfere with the ability of LPS-stimulated DCs to migrate to the MLNs. OVA_{LPS}-DCs, OVA_{LPS}-DCs/AMs, and OVA_{LPS}-DCs/IMs were stained with CFSE before being injected i.t. into recipients. Twenty-four hours later, MLNs were collected and assayed by flow cytometry for the percentage of CFSE-positive DCs (F4/80⁺CD11c⁺). As shown in Figure 4E, OVA_{LPS}-DCs were able to reach the MLNs. However, when they were cocultured with IMs, OVA_{LPS}-DCs lost their migratory properties, an effect not seen with AMs. This effect of IMs on DC migration was statistically significant. Finally, flow cytometry analyses revealed that neither IMs (F4/80⁺CD11c⁺) nor AMs (F4/80⁺CD11c⁺) were able to migrate from peripheral tissues to MLNs following i.t. instillation (data not shown). It is interesting to note that a CD11⁻CFSE^{lo} cell population was found in the MLNs following i.t. injection of CFSE-labeled APCs (Figure 4E). This population most likely corresponds to resident lymph node cells that have endocytosed debris originating from dying CFSE-labeled DCs. It is indeed known that mature DCs die rapidly after reaching the draining lymph nodes. As we assessed DC migration 24 hours after injection, it is probable that a fraction of the DCs died after arriving in the MLNs and before analysis.

Taken together, these results show that IMs have the capacity to significantly affect maturation and migration of LPS-activated DCs, thereby preventing DC-driven Th2 priming.

IMs exert their inhibitory effects through IL-10 production. The results presented in Figures 2 and 3 showed that soluble rather than cell-associated molecules are responsible for the effects of IMs. We therefore sought to identify these soluble factors. As it has been shown that IL-10 and TGF- β can alter DC function (28–30), we first determined whether IMs are able to produce these immunomodulatory cytokines. IMs, AMs, and lung DCs spontaneously produced IL-10 (Figure 5A). This constitutive IL-10 production was 4-fold and 8-fold higher in IMs than in AMs and lung DCs, respectively. Stimulation with OVA_{LPS} had no significant effect on IL-10 secretion by lung DCs and AMs. By contrast, OVA_{LPS}-treated IMs produced 4-fold more IL-10 than untreated counterparts. Figure 5A also shows that lung DCs, AMs, and IMs produced only negligible amounts of TGF- β in the conditions tested. We therefore hypothe-

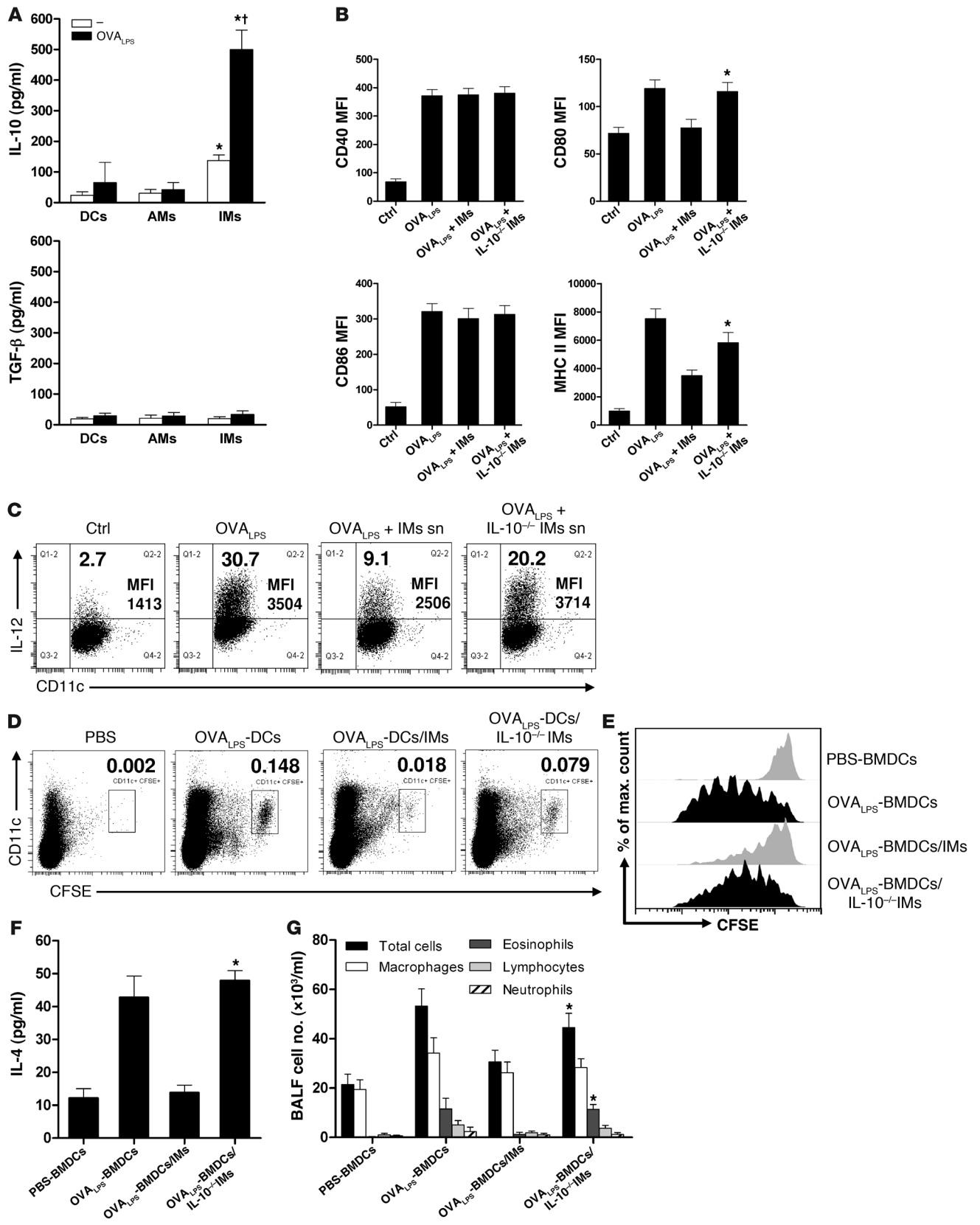




Figure 5

IL-10 secretion by IMs is required for functional paralysis of LPS-stimulated DCs. **(A)** IMs, AMs, and lung DCs were stimulated with OVA_{LPS}. Sixteen hours later, supernatants were assayed for IL-10 and TGF- β . * $P < 0.05$ versus AMs and DCs. † $P < 0.05$ versus unstimulated IMs. **(B)** Lung DCs were stimulated for 16 hours with OVA_{LPS} in the presence or absence of WT or *Il10*^{-/-} IMs. DCs were assayed for expression of CD40, CD80, CD86, and MHC II by FACS. MFIs are shown. Ctrl, freshly isolated DCs. * $P < 0.05$ versus Ctrl and OVA_{LPS} plus IMs. **(C)** Lung DCs were placed in fresh medium or in the supernatant of OVA_{LPS}-stimulated WT or *Il10*^{-/-} IMs. DCs were treated and stained as in Figure 4B. The percentage and MFI of IL-12–positive cells were measured by FACS. **(D)** CFSE-labeled OVA_{LPS}-DCs, OVA_{LPS}-DCs/IMs, and OVA_{LPS}-DCs/*Il10*^{-/-} IMs were injected i.t. to recipients. Control mice received PBS. Twenty-four hours later, the percentages of migrating DCs (CFSE⁺F4/80-CD11c⁺) among total MLN cells were determined by FACS. **(E and F)** Mice were injected with CFSE-labeled OT-II T cells. Twenty-four hours later, mice received PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/IMs, or OVA_{LPS}-BMDCs/*Il10*^{-/-} IMs. Three days later, proliferation of CFSE-labeled OVA-specific T cells in MLNs was measured by FACS **(E)**. Alternatively, MLN cells were restimulated with OVA, and supernatants were assayed for IL-4 **(F)**. **(G)** Mice received PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/IMs, or OVA_{LPS}-BMDCs/*Il-10*^{-/-} IMs. From days 10 to 14, mice were exposed to OVA aerosols. On day 15, BALF cell numbers were determined. * $P < 0.05$ versus PBS-BMDCs and OVA_{LPS}-BMDCs/IMs **(F and G)**.

sized that IL-10 production could account for the inhibitory effects of IMs. To verify this hypothesis, we repeated most of the coculture experiments described above with IL-10–deficient IMs. Of note, all the experiments described hereafter were performed in the C57BL/6 background. *Il10*^{-/-} IMs, compared with wild-type ones, had reduced ability to inhibit maturation and migration of OVA_{LPS}-treated lung DCs (Figure 5, B–D). Furthermore, BMDCs that were cocultured with *Il10*^{-/-} IMs, unlike those that were cocultured with wild-type IMs, induced strong primary and secondary Th2 responses when pulsed with OVA_{LPS} and injected i.t. into recipient mice (Figure 5, E–G). The intensity of these responses was similar to that measured after i.t. administration of OVA_{LPS}-BMDCs alone. Results similar to those presented in Figure 5 were obtained when wild-type IMs and BMDCs were cocultured in the presence of neutralizing anti-IL-10 antibodies (Supplemental Figure 3). These data show that IL-10 production by IMs contributes to functional paralysis of LPS-stimulated DCs.

IMs prevent Th2 responses to harmless inhaled antigens combined with LPS. To determine whether the observed properties of IMs were of physiological relevance, the immune responses induced by i.t. administration of OVA combined with a low LPS dose were measured in IM-depleted mice. IMs were specifically depleted by i.p. injection of anti-F4/80 antibodies (31–33). Figure 6A shows that anti-F4/80 antibodies efficiently depleted IMs but did not target AMs. In this figure, only living cells were gated. In Figure 6B, we did not gate on living cells but considered all events. In this case, events could be detected in the quadrant of IMs (F4/80⁺CD11c⁻) after treatment with anti-F4/80 antibodies. However, IMs displayed altered morphology (reduced side scatter, SSC), whereas AMs were unaffected. Staining of the cells with DAPI, which only penetrates dead cells, revealed extensive DAPI staining of the IMs, but not the AMs, of mice receiving depleting antibodies. As a second control to confirm selective depletion of IMs by the anti-F4/80 antibody, lung sections of isotype control– or anti-F4/80 antibody–treated mice were immunostained for the pan-macro-

phage marker CD68. Preliminary FACS analysis indicated that both IMs and AMs, but not DCs, express CD68 (Figure 1A). These immunostaining experiments confirmed that the anti-F4/80 antibody effectively depleted macrophages located in the interstitium, without affecting macrophages in the airway lumen (Figure 6C). Finally, anti-F4/80 antibodies were administered i.t. to assess for their possible effects on AM viability in the case of direct exposure. As shown in Supplemental Figure 4, i.t. instillation of the antibody did not result in depletion of AMs, although it still mildly affected IMs. This result confirms that AMs are protected from antibody-mediated lysis and that anti-F4/80 antibodies selectively deplete IMs. The protection of AMs from antibody-induced cell death might be due to insufficient amounts of complement proteins or the low abundance of cytotoxic cells in the airway lumen of healthy mice. Taken together, these data clearly demonstrate a selective effect of anti-F4/80 antibodies on IMs.

Intranasal instillation of OVA with LPS doses in the 100-ng range (11, 34) results in allergic airway inflammation. As expected from the fact that mice do not spontaneously develop airway allergy, i.t. administration of OVA with LPS doses that more closely approximated common environmental LPS concentrations (OVA_{LPS}; OVA: 100 μ g/mouse; LPS: 10 ng/mouse) did not induce marked Th2 cell priming (Figure 7, A and B). In contrast, inhalation of OVA_{LPS} promoted Th2 cell differentiation and proliferation in IM-depleted mice. In another set of experiments, IMs were depleted during a first exposure to instilled OVA_{LPS}, and the secondary immune response was assessed 10 days later following challenge with intranasal OVA. Figure 7C clearly shows that after OVA challenge, IM-depleted mice, but not control mice, developed overt eosinophilic airway inflammation. Finally, we examined the effects of IM depletion on in vivo DC migration. For that, 100 μ g FITC-labeled OVA and 10 ng LPS were given i.t. to normal or IM-depleted naive BALB/c mice, and migrating lung DCs were counted in MLNs 24 hours later by flow cytometry (FITC⁺F4/80-CD11c⁺ cells). As shown in Figure 7D, the number of FITC⁺ DCs was substantially higher (2-fold increase) in IM-depleted mice than in controls. This effect was statistically significant.

A limiting factor in the aforementioned experiments comes from the fact that off-target effects of depleting antibodies never can be ruled out. To confirm that depleting anti-F4/80 antibodies had no indirect or unspecific effects on T and B cell number and function, we first analyzed the proportions of T (CD3 ϵ ⁺, CD4⁺, or CD8 α ⁺ cells) and B (CD19⁺ cells) cells in the draining lymph nodes of animals treated with the depleting antibody or PBS. We did not observe any quantitative difference in T and B cell populations in the two experimental groups (Figure 7E). We next isolated B cells from the draining lymph nodes of both groups, stimulated them with agonist anti-CD40 antibodies, and assessed cell proliferation. We observed no difference in B cell proliferation in cells coming from mice treated with the depleting antibody compared with controls (Figure 7F). We performed comparable experiments with isolated T cells stimulated with anti-CD3 and anti-CD28 antibodies, with similar outcome (Figure 7G).

To formally prove that AMs were not required for protection against allergic sensitization in the airways, AMs were depleted using liposomal clodronate prior to primary exposure to i.t. instilled OVA_{LPS}. Intratracheal administration of liposomal clodronate allowed specific depletion of AMs, as determined by flow cytometry (Supplemental Figure 5A). As shown in Supplemental Figure 5B, depletion of AMs did not result in airway eosinophilia upon

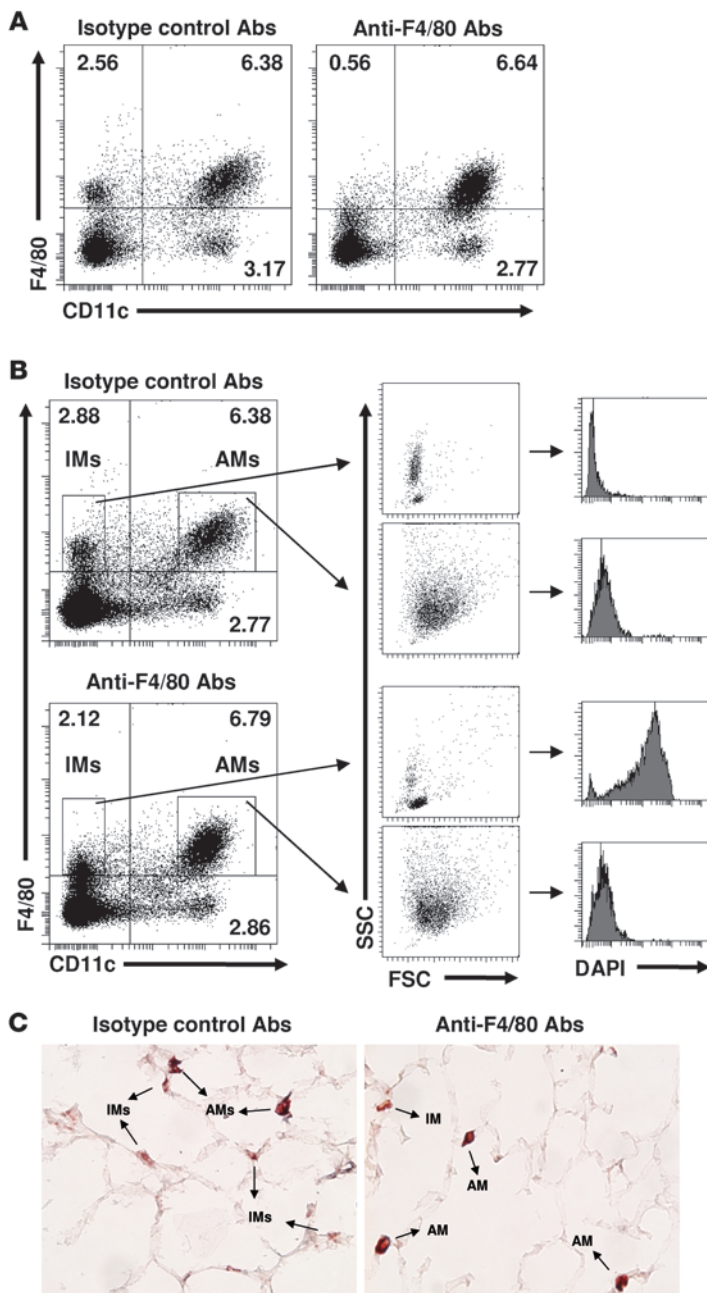


Figure 6

Specific depletion of IMs by i.p. injection of anti-F4/80 antibodies. (A–C) Naive BALB/c mice were injected i.p. on 3 consecutive days with 250 µg of depleting anti-F4/80 or control isotype antibodies. (A and B) On day 4, lungs were digested and stained for F4/80 and CD11c. (A) Percentages of lung DCs (F4/80⁻CD11c⁺; lower-right quadrant), AMs (F4/80⁺CD11c⁺; upper-right), and IMs (F4/80⁺CD11c⁻; upper-left) among living cells. (B) Percentages of lung DCs, AMs, and IMs among dead and living cells (all events were considered; left panels). FACS analysis of the forward scatter (FSC) and side scatter (SSC) is provided to show the specific effects of depleting anti-F4/80 antibodies on IM morphology (middle panels). Cells were incubated with DAPI in order to stain dead cells (right panels). Only IMs were dead (DAPI-positive) following anti-F4/80 treatment. (C) Lung cryosections from isotype antibody- and anti-F4/80 antibody-treated mice were stained for the pan-macrophage marker CD68 (original magnification, ×100).

observations is still lacking. In the present report, we provide clear evidence that LPS-triggered airway allergy is tightly controlled by IMs. Indeed, IMs have the capacity to inhibit lung DC maturation and migration upon LPS stimulation, thereby preventing Th2 sensitization to concomitant inhaled antigens. Our results thus reveal a previously unknown role for IMs in regulating respiratory immune reactions and explain why the pulmonary immune system normally does not respond to harmless antigens in the presence of environmental LPS.

AMs and IMs represent the two main lung macrophage subsets. Although AMs have been extensively studied (35), the phenotype and in vivo function of IMs have been incompletely characterized. Our findings confirm and extend previous reports that IMs are phenotypically and functionally distinct from AMs (20, 24). First, IMs, unlike AMs, do not express the DC marker CD11c but express high levels of MHC II (20) (Figure 1A). Second, IMs, compared with AMs, have a lower phagocytic potential but are more efficient in stimulating T cell proliferation in vitro (24) (Figure 1, D and E). Finally, IMs and AMs differentially affect DC function and pulmonary immune responses. It has been shown that AMs can limit the production of antigen-specific antibodies during the effector phase of pulmonary immune responses (namely, in sensitized animals exposed to aerosolized antigens; refs. 36–38). During this phase, AMs exert their immunosuppressive effects by interfering with lung DC function (36,

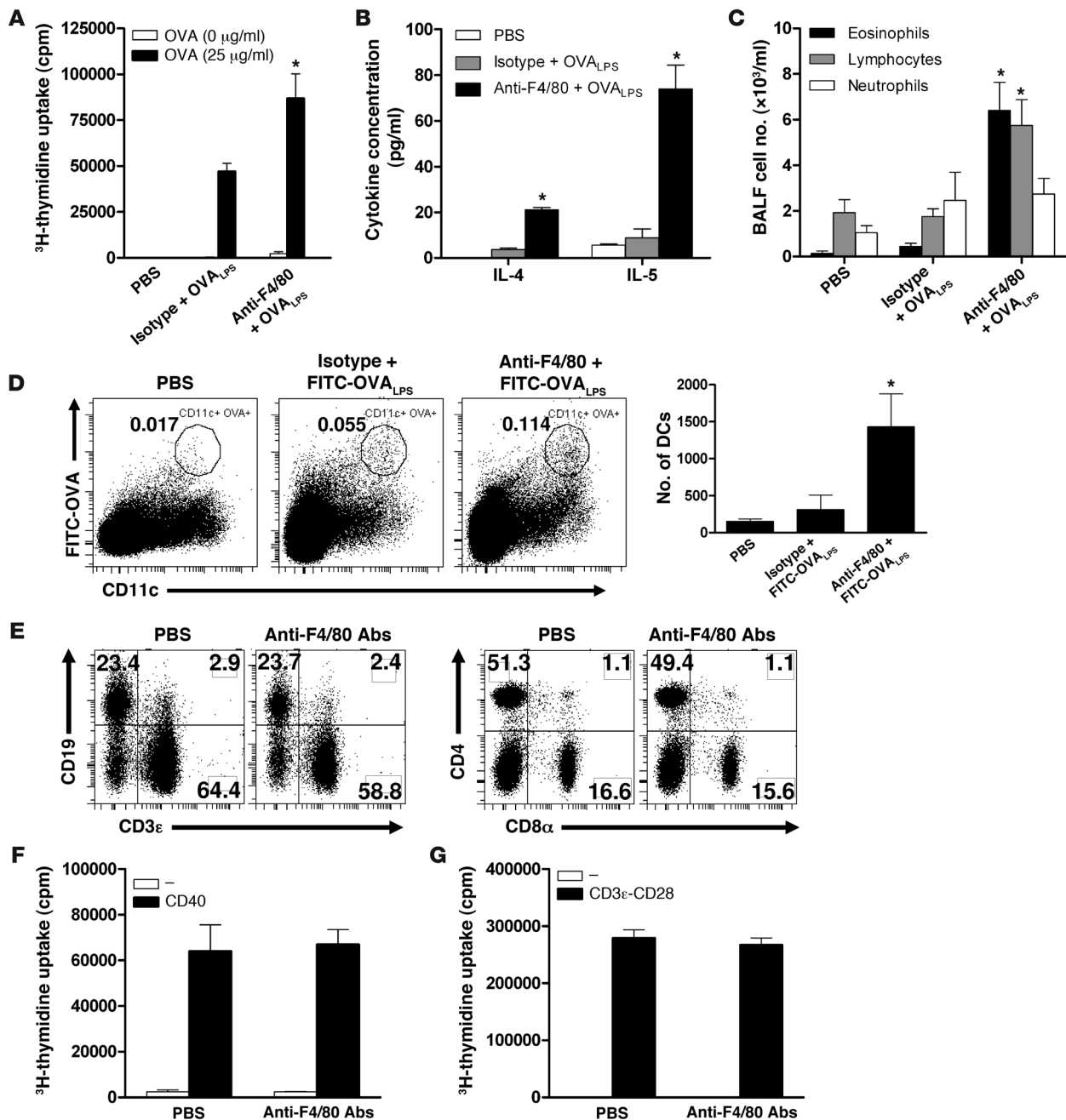
subsequent intranasal OVA_{LPS} challenge, ruling out the hypothesis that AMs exert immunosuppressive effects in this model.

With due consideration for possible technical limitations, all these results strongly support that IMs, but not AMs, play a crucial role in preventing LPS-induced asthmatic responses to harmless inhaled antigens.

Discussion

LPS is ubiquitous in the environment and is found at low levels in ambient air (5, 6, 9). Airborne LPS, at low concentrations, has the ability to promote the induction of DC-driven Th2 cell responses to harmless inhaled antigens (10, 11, 17). However, only a minority of people exposed to environmental LPS develop allergic asthma. A unifying model reconciling these conflicting

39). By contrast, it has been demonstrated by the same group that AMs do not display any suppressive or tolerogenic function during the sensitization phase of lung immune responses (namely, in naive animals exposed for the first time to innocuous inhaled antigens; ref. 37). Our study provides clear evidence that IMs, unlike AMs, play a crucial role in preventing the development of aberrant immune responses against harmless inhaled antigens. It is therefore possible that IMs and AMs have complementary immunosuppressive effects – IMs being able to prevent sensitization to harmless aeroantigens and AMs being capable of dampening the effector phase of lung immune responses. Another interesting point is that the suppressive properties of AMs are abrogated in the presence of proinflammatory cytokines such as GM-CSF (26). Our results further show that AMs do not display

**Figure 7**

IMs prevent LPS-triggered Th2 responses to innocuous inhaled antigens. (A–G) Naive BALB/c mice were injected i.p. daily from day 1 to 3 with depleting anti-F4/80 or control isotype antibodies. (A–C) On day 2, mice received an i.t. injection of OVA_{LPS}. (A and B) On day 6, MLN cells were restimulated for 3 days with 25 $\mu\text{g/ml}$ OVA. The proliferation was measured (A), and culture supernatants were assayed for IL-4 and IL-5 by ELISA (B). (C) From day 11 to 14, mice were challenged intranasally with 25 μg OVA (grade V; Sigma-Aldrich) in 50 μl PBS. On day 15, BALF was subjected to differential cell counts. (D) On day 2, IM-depleted and control mice were injected i.t. with 100 μg FITC-OVA (rather than unlabeled OVA) and 10 ng LPS. On day 3, MLNs were analyzed by flow cytometry for the presence of OVA-loaded DCs (FITC⁺F4/80⁺CD11c⁺). Percentages (left) and total numbers (right) of migrating DCs are shown. (E) On day 4, the percentages of T (CD3 ϵ ⁺, CD4⁺, or CD8 α ⁺ cells) and B (CD19⁺ cells) cells in MLNs were measured by flow cytometry. (F and G) On day 4, B and T cells were isolated from MLNs and stimulated ex vivo with agonist anti-CD40 antibodies or anti-CD3 and anti-CD28 antibodies, respectively. Control cells were left unstimulated. B cell (F) and T cell (G) proliferation was measured as [^3H]thymidine incorporation during the last 16 hours of a 2-day culture. * $P < 0.05$ versus results obtained with isotype control antibodies (A–D).



any immunoregulatory activity following stimulation with low concentrations of LPS. Indeed, LPS-treated AMs were unable to alter migratory and immunostimulatory properties of LPS-stimulated DCs. As the respiratory mucosal surfaces, and therefore AMs, are constantly exposed to low LPS doses (5, 6, 9), our data provide an explanation for the fact that AMs are ineffective in preventing the development of pulmonary immune responses in naive mice exposed to low LPS doses and harmless inhaled antigens. It is also likely that unidentified factors are necessary to boost the immunomodulatory effects of these cells during the effector phase of lung inflammation. By contrast, our data unambiguously demonstrate that IMs are capable of preventing LPS-triggered Th2 priming through functional inhibition of lung DCs. Taken together, our results indicate that IMs are not merely the precursors of AMs as previously proposed by Blussé van Oud Alblas et al. (40), but constitute a phenotypically and functionally distinct macrophage population that contributes to the fine-tuning of respiratory immune responses. Determining their ontological relation with AMs will require additional investigation.

Atopic asthma is mainly characterized by bronchial symptoms. Thus, it may come as a surprise that IMs, which are located in the parenchyma, may prevent the disease. Even though the effector phase of atopic asthma results in a bronchial disease, the site of allergic sensitization in the lungs still has not been formally identified. What we know is that IMs are directly exposed to instilled allergens, as evidenced by their uptake of OVA-FITC *in vivo* (data not shown), and are in close contact with parenchymal DCs (Figure 1, D and F). It is also known that plasmacytoid DCs, which play a key role in preventing allergic sensitization in the airways, are located in the parenchyma (3), reinforcing the idea that parenchymal cells are key regulators of Th2 responses in the lung. All these considerations support the idea that IMs in the parenchyma inhibit allergic sensitization by resident DCs, consequently preventing the development of bronchial inflammation upon reexposure to the allergen. Whether IMs play a role during the effector phase of experimental asthma still needs to be investigated.

Our results showed that lung DCs were no longer able to produce high quantities of IL-12, a Th1 cytokine, in the presence of IMs. Reduced secretion of IL-12 by DCs usually results in skewing of the immune responses toward a Th2 phenotype (41, 42), which was not the case in our study. In fact, it is not surprising that activation of lung DCs in the presence of IMs did not result in a Th2 bias of lung immune responses, as IMs prevented not only IL-12 production by DCs but also their membrane maturation and their migration to the lymph nodes, where presentation of the antigen would occur. Nevertheless, the function of IL-12 in pulmonary disease is still controversial. Indeed, while some reports showed that administration of recombinant IL-12 ameliorates allergic airway inflammation (43), studies in IL-12-deficient mice showed that IL-12 deficiency does not alter the Th1/Th2 balance after secondary allergen exposure (44). Finally, a more recent report, in which neutralizing monoclonal antibodies against IL-12 were used, proposed an aggravating effect of IL-12 during allergen exposure (45). This shows how controversial the role of IL-12 remains and even suggests that inhibition of IL-12 production by lung DCs could be one of the mechanisms by which IMs prevent Th2 responses in the airways.

Our experiments using *Il10*^{-/-} IMs and neutralizing antibodies against IL-10 revealed an important role for this immunosuppressive cytokine in IM function. Indeed, DCs that were cocultured

with *Il10*^{-/-} IMs, unlike those that were cocultured with wild-type IMs, upregulated MHC II, CD80, and IL-12 expression in response to LPS, had the ability to migrate to MLNs, and induced strong primary and secondary Th2 responses to OVA. These findings are consistent with previous reports that IL-10 not only inhibits DC maturation but also impairs the trafficking of antigen-loaded DCs from tissues to the draining lymph nodes (46–48). It is interesting to note that DC function was not fully restored when *Il10*^{-/-} IMs were used, suggesting that other soluble factors secreted by IMs act in conjunction with IL-10 to impair DC maturation and migration. Experiments are currently underway to identify these factors. IMs were the only pulmonary APC population capable of secreting high quantities of IL-10 following LPS stimulation. AMs spontaneously produced low levels of IL-10 but failed to increase IL-10 secretion in response to LPS, a finding in agreement with previous literature (49). It has been reported that lung DCs from mice exposed to harmless inhaled antigens transiently produce IL-10 (1). These IL-10-secreting DCs are phenotypically mature and migrate to the MLNs, where they stimulate the development of IL-10-secreting, antigen-specific Tregs (1). Here, we show that LPS at concentrations comparable to those found in the environment is sufficient to abrogate IL-10 production by antigen-pulsed lung DCs, enabling them to promote Th2 cell differentiation rather than tolerance.

It has recently been shown that intestinal lamina propria macrophages play a crucial role in maintaining mucosal tolerance (50). Like IMs, lamina propria macrophages are positive for F4/80 and CD11b, but negative for CD11c, and secrete high amounts of IL-10 even after stimulation with LPS. In addition, these macrophages can counteract the ability of intestinal DCs to induce immune responses. However, although lamina propria macrophages are capable of inducing the differentiation of Foxp3⁺ Tregs, IMs did not induce any T cell response in our *in vivo* experiments. The results of Denning et al. (50), together with ours, show for the first time to our knowledge that the intestinal and respiratory tracts both contain regulatory macrophage subsets that protect against aberrant immune responses to nonpathogenic environmental antigens, even in the presence of proinflammatory stimuli. These regulatory macrophage populations, namely lamina propria macrophages and IMs, share many phenotypic and functional characteristics but differ in their ability to induce Tregs.

It has been shown that tissue macrophages may differentiate into regulatory macrophages after stimulation with various factors, including immune complexes, glucocorticoids, apoptotic cells, prostaglandins, IL-10, and adenosine (51–57). These “induced” regulatory macrophages produce high levels of IL-10 and are therefore able to suppress immune responses (52, 54, 57, 58). Induced regulatory macrophages require two distinct stimuli to be efficiently reprogrammed and to exert their immunomodulatory activities (54, 57, 58). The first signal (for example, immune complexes, prostaglandins, adenosine, or apoptotic cells) has little or no function on its own but primes for subsequent IL-10-dependent responses to the second signal, such as a TLR ligand. Interestingly, our results show that IMs, unlike induced regulatory macrophages, do not need to be reprogrammed to acquire regulatory functions but primarily act as regulatory cells. IMs should therefore be considered as “constitutive” or “natural” regulatory macrophages.

The respiratory tract has evolved to limit access of foreign antigens to the immune system with barriers such as the mucus layer and intercellular tight junctions. However, the respiratory mucosa is not totally impenetrable, and active mechanisms have devel-



oped that suppress unwanted immune responses against harmless inhaled antigens. In the absence of proinflammatory stimuli, plasmacytoid and IL-10-producing myeloid lung DCs stimulate the development of Tregs specific for airborne antigens and, therefore, induce immune tolerance (1, 3, 59). However, given that LPS is ubiquitous in the environment and that lung DCs lose their tolerogenic properties to become pro-Th2 APCs after stimulation with innocuous antigens combined with low LPS doses (60, 61), it seems reasonable to suggest that induction of Tregs by lung DCs is an infrequent event. If Treg development were the normal outcome of harmless antigen encounter, MLNs would be the place of incessant Treg proliferation, which is highly improbable in view of the small size of MLNs in uninfected and unsensitized mice and the low percentage of Tregs in these lymph nodes (~5%; our unpublished observations). Here, we propose a model in which IMs paralyze lung DCs upon LPS stimulation, thereby breaking the link between innate and adaptive immunity and allowing concomitant inhaled antigens to escape from Th2 responses. Supporting this model, IM-depleted mice, but not normal mice, developed overt asthmatic reactions in response to OVA combined with low LPS doses. In our model, protection against aberrant pulmonary immune responses is explained by immunological ignorance of harmless inhaled antigens rather than by induction of Tregs. This is of pathophysiological importance. Indeed, although most of the antigens that are inhaled along with LPS are innocuous, some of them are potentially pathogenic, and it is evident that tolerating these antigens could have disastrous immunopathological consequences. However, additional experiments will be required to formally determine whether IMs paralyze all populations of lung DCs or whether they act in conjunction with tolerogenic DCs such as IL-10-producing myeloid DCs or plasmacytoid DCs (1, 3) to abrogate the development of allergic airway inflammation.

A question remaining open is how IM function is disabled in situations leading to airway sensitization. It is known that i.t. administration of high LPS doses induces allergic sensitization in mice (11), suggesting that the immunosuppressive activity of IMs is overcome in these conditions. Further investigation will be needed to verify this hypothesis.

We further postulate that inhibition or dysfunction of IMs might contribute to the development of asthma in humans. It has been shown that infections by respiratory viruses such as the respiratory syncytial virus in early life represent an important risk factor for asthma (62, 63). As respiratory viruses can infect pulmonary macrophages (64), it is possible that infected IMs are no longer able to inhibit maturation and migration of lung DCs, thus favoring both the immune response to virus infection and Th2 sensitization to harmless inhaled antigens. In light of our findings, it will be interesting to determine whether the function of IMs in humans with atopic asthma is altered and whether therapeutic strategies aimed at enhancing the inhibitory properties of IMs could help control the disease.

Methods

Mice. Wild-type BALB/c and C57BL/6 mice were purchased from Harlan Nederland. *Il10*^{-/-} mice (C57BL/6 background) and OVA-specific, MHC II-restricted, TCR transgenic DO11.10 (H-2^d; BALB/c background), and OT-II (H-2^b; C57BL/6 background) mice were from The Jackson Laboratory. *Tlr4*^{-/-} mice (C57BL/6 background) were a gift from S. Florquin (University of Amsterdam, Amsterdam, The Netherlands). All mice were housed in a specific pathogen-free facility and used at 6–10 weeks of age.

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Liège.

Reagents and antibodies. The DO11.10 OVA peptide (chicken OVA peptide 323–339 ISQAVHAAHAEINEAGR) was purchased from Neosystem. LPS-free OVA (EndoGrade Ovalbumin; endotoxin concentration, <1 EU/mg) was from Profos. LPS from *Escherichia coli* (serotype 055:B5) was purchased from Sigma-Aldrich. CFSE, DAPI, and FITC-dextran and -OVA were from Invitrogen. Methacholine was purchased from Sigma-Aldrich. Recombinant murine GM-CSF was provided by K. Thielemans (Medical School of the Vrije Universiteit Brussel).

PE-conjugated anti-F4/80 (BM8), allophycocyanin-conjugated (APC-conjugated) anti-CD11c (N418) and anti-TCR V α 2 (B20.1), Pacific Blue-anti-CD4 (RM4-5), and biotinylated anti-CD19 (MB19-1) were from eBioscience. Biotinylated anti-MHC II (I-E^k) (clone 17-3-3), PE-anti-CD3 ϵ (clone 145-2C11), and FITC-anti-CD8 α (clone 53-6.7) were from BD Biosciences. FITC-conjugated anti-CD40 (3/23), anti-CD80 (RM80), and anti-CD86 (PO3) were from Serotec. Biotinylated anti-DO11.10 TCR (KJI-26) was from Caltag Laboratories. 2.4G2 Fc receptor antibodies were produced in house. APC- and FITC-streptavidin were from eBioscience. Neutralizing anti-IL-10 antibodies (JES5-2A5) were from eBioscience.

Flow cytometry. Staining reactions were performed at 4°C. Cells were incubated with 2.4G2 Fc receptor antibodies to reduce nonspecific binding.

Cell isolation. To obtain single-lung-cell suspensions, lungs were perfused with 20 ml PBS through the right ventricle, cut into small pieces, and digested for 1 hour at 37°C in 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNaseI (Roche) in HBSS. IMs, AMs, and lung DCs were sorted by flow cytometry (FACSARIA) based on their differential F4/80/CD11c expression. DO11.10 CD4⁺ T cells were isolated using magnetic bead purification (CD4⁺ T Cell Isolation Kit 130-090-860; Miltenyi Biotec). Isolated cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μ M β -mercaptoethanol, 50 μ g/ml streptomycin, and 50 IU/ml penicillin (all from Invitrogen).

BAL and cytology. The trachea was catheterized, and the lungs were lavaged with 1 ml of ice-cold Mg- and Ca-free PBS containing 0.6 mM EDTA. The lavage procedure in Figure 1B was repeated 10 times. Cell density in BALF was assessed by the use of a hemocytometer. Cell differentials were performed on cytospin preparations stained with Diff-Quick (Dade Behring).

Immunohistochemistry. Lungs from naive BALB/c mice were inflated with Tissue-Tek OCT compound (Sakura Finetek) through the trachea, removed from the pulmonary cavity, embedded in Tissue-Tek, and snap-frozen in liquid nitrogen. Tissues were stored at -80°C. Frozen sections of lung tissues (5 μ m) were fixed in ice-cold ethanol. Sections were rehydrated in phosphate-buffered saline for 5 minutes, followed by treatment for 30 minutes with blocking reagent (1% in PBS; BoehringerMannheim) to saturate the sites of nonspecific reactions. The slides were then incubated with biotinylated anti-F4/80 Abs (CI:A3-1; Abd Serotec) for 1 hour (10 μ g/ml in 0.5% PBS-blocking reagent). They were rinsed in buffer and treated with the avidin-biotin-complex-HRP (ABC-HRP) reagent for 30 minutes. After rinsing, the slides were stained with the HRP substrate NovaRed. Thereafter, the excess of biotin from the first antibodies was blocked using avidin-biotin blocking kit. Then, biotinylated anti-CD11c Abs (HL3; BD Biosciences) (10 μ g/ml in 0.5% PBS-blocking reagent) were applied for 1 hour. After rinsing, the sections were treated again with ABC-HRP, followed by development with Vector SG (Vector Laboratories). Slides were dehydrated with alcohol, cleared, and mounted with Vectamount (Vector Laboratories). In some experiments, the ABC-HRP reagent was replaced by ABC-alkaline phosphatase (ABC-AP) reagent. In this case, the slides were stained with the AP substrate Vector Red (Vector Laboratories). Levamisole was used to inactivate endogenous AP.



The same protocol was used when lung cryosections were stained for CD68 (FA-11 antibody; Serotec), except that 0.2% Tween-20 was added to the blocking reagent in order to permeabilize cell membranes.

Phagocytic activity. IMs, AMs, and lung DCs (1×10^6 cells/well) were cultured in the presence of 1 mg/ml FITC-labeled dextran (40,000 kDa; Molecular Probes, Invitrogen) for 45 minutes. As a control for nonspecific dextran attachment, 0.02% sodium azide was added. To determine phagocytic activity, the uptake of FITC-labeled dextran was measured by flow cytometry.

In vitro proliferation assay. Isolated IMs, AMs, and lung DCs (1×10^4 or 4×10^4 cells/well) were incubated with the DO11.10 OVA peptide (50 μ g/ml) and kept at 4°C for 90 minutes. Loaded APCs were then cocultured for 3 days with 2×10^5 DO11.10 CD4⁺ T cells. Proliferation of DO11.10 T cells was measured as [³H]thymidine incorporation during the last 16 hours of the 3-day culture.

Th2 sensitization induced by i.t. administration of LPS-stimulated, OVA-pulsed BMDCs. To generate BMDCs, bone marrow cells from naive mice were grown in medium containing 20 ng/ml recombinant murine GM-CSF (65). At day 8, the culture medium was replaced with medium devoid of GM-CSF. One hour later, BMDCs were pulsed with 100 μ g/ml LPS-free OVA and concomitantly stimulated with 10 ng/ml LPS (namely, an LPS dose analogous to those commonly found in extracts from household dust; ref. 14). In some experiments, BMDCs were only pulsed with LPS-free OVA. Control BMDCs were left unpulsed and unstimulated. At day 9, cells were collected, washed, and resuspended in PBS. Cells (10^6) were then injected i.t. into anesthetized naive recipients. Ten days after i.t. immunization, mice were challenged with OVA (1% wt/vol in PBS; grade III; Sigma-Aldrich) aerosol during a daily 30-minute challenge on 5 consecutive days. Twenty-four hours after the last challenge, AHR was measured, the mice were killed, and airway allergy was evaluated.

Cocultures. FACS-sorted IMs and/or AMs were added to BMDC cultures at day 8, 1 hour before stimulation with OVA_{LPS}. Cells were cocultured at a ratio of 1:1 (BMDCs/IMs), 1:2 (BMDCs/AMs), or 1:1:2 (BMDCs/IMs/AMs). These proportions were chosen based on our observation that AMs are 2 times more abundant than IMs and DCs in the lung (Figure 1A). At day 9, 1×10^6 IMs and/or 2×10^6 AMs were injected i.t. together with 1×10^6 BMDCs. In some experiments, IMs were cocultured with BMDCs across a semipermeable membrane (cell culture insert, 1.0- μ m pore size; BD Biosciences).

Lung histology. Lungs were fixed in 10% formalin, paraffin embedded, cut in 5- μ m sections, and stained with hematoxylin and eosin. Intracytoplasmic and luminal mucin was assessed by PAS stains.

Determination of serum levels of OVA-specific IgE. OVA-specific IgE levels were measured by ELISA. Briefly, 96-well plates (ELISA plate; Microolon; Greiner Bio One) were coated with rat anti-mouse IgE. After addition of serum samples, biotinylated OVA (203112; Calbiochem) was added to individual wells, and its binding was detected with peroxidase-conjugated streptavidin (43-4323; Invitrogen). A serum pool from OVA-sensitized animals was used as internal laboratory standard; 1 U was arbitrarily defined as 1:50 dilution of this pool.

Restimulation of MLN cells. MLN cells (2×10^5 cells in a 96-well plate) were cultured in Click's medium supplemented with 0.5% heat-inactivated mouse serum and additives, with or without OVA (50 μ g/ml; grade V; Sigma-Aldrich). The proliferation was measured as [³H]thymidine incorporation during the last 16 hours of a 3-day culture. Culture supernatants were assayed for IL-4, IL-5, IL-13, and IFN- γ by ELISA (Biosource).

Measurement of AHR. Mice were anesthetized by i.p. injection (200 μ l) of a mixture of ketamine (10 mg/ml; Merial) and xylazine (1 mg/ml; VMD). A tracheotomy was performed by insertion of a 20-gauge polyethylene catheter into the trachea. A ligature was performed around the catheter to avoid leaks and disconnections. Mice were ventilated with a flexiVent small animal ventilator (SCIREQ) at a frequency of 450 breaths per minute and

a tidal volume of 10 ml/kg. A positive end-expiratory pressure was set at 2 hectopascal. Measurement started after 2 minutes of mechanical ventilation. A sinusoidal 1-Hz oscillation was then applied to the tracheal tube and allowed a calculation of dynamic resistance, elastance, and compliance of the airway by multiple linear regressions. After measurement of baseline lung function, mice were exposed to a saline aerosol (PBS) followed by aerosols containing increasing doses (3, 6, 9, 12 g/l) of methacholine. Aerosols were generated via use of an ultrasonic nebulizer (SCIREQ) and delivered to the inspiratory line of the flexiVent using a bias flow of medical air according to the manufacturer's instructions. Each aerosol was delivered for 10 seconds and periods of measurement as described above were made at 1-minute intervals after each aerosol. Dynamic resistance was the main parameter measured during the challenge (66).

Assessment of primary T cell activation. On day -1, mice received an i.v. injection of CFSE-labeled DO11.10 cells (equivalent of 10×10^6 transgenic T cells). On day 0, mice were injected i.t. with either PBS-BMDCs, OVA_{LPS}-BMDCs, OVA_{LPS}-BMDCs/AMs, OVA_{LPS}-BMDCs/IMs, OVA_{LPS}-BMDCs_{IMmemb}, or OVA_{LPS}-BMDCs/IMs/AMs as described above. On day 3, T cell responses in MLNs were analyzed by observing CFSE division profiles of live KJ1-26⁺ CD4⁺ T cells. The division index was used to quantify T cell division. The division index is the average number of divisions that a cell (present in the starting population) has undergone. This index was calculated using FlowJo software (Tree Star). Production of IL-4 and IL-5 by OVA-restimulated MLN cells was measured as described above. In some experiments, IMs from IL-10-deficient mice were used. As *Il10*^{-/-} mice were generated in the C57BL/6 background, CFSE-labeled OT-II cells were used rather than DO11.10 cells. In this case, T cell responses in MLNs were analyzed by observing CFSE division profiles of live TCR V α 2⁺ CD4⁺ T cells.

CFSE labeling. Splenic and lymph node cells from DO11.10 mice (5×10^7 cells/ml) were incubated with CFSE (0.5 μ M in PBS) for 10 minutes at 37°C. Cells were washed in PBS containing 10% FCS and then in PBS and injected i.v.

Intracellular staining. For analysis of IL-12 production by DCs, FACS-sorted lung DCs were placed in fresh RPMI medium (see above) or in the culture supernatant of OVA_{LPS}-stimulated IMs or AMs (16-hour stimulation). Cells were then treated with OVA_{LPS} (100 μ g/ml LPS-free OVA and 10 ng/ml LPS) for 2 hours and incubated with Brefeldin A (GolgiPlug; BD Biosciences) for an additional 5 hours. Freshly isolated lung DCs were used as controls. Cells were fixed and permeabilized with BD Cytotfix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained intracellularly with an APC-conjugated anti-IL-12p40 antibody (C15.6; BD Biosciences). Isotype control (APC Rat IgG1) was from BD Biosciences. Flow cytometry was performed with a FACScanto (BD Biosciences).

For evaluation of CD68 expression by pulmonary APCs, FACS-sorted lung DCs, IMs, and AMs were fixed and permeabilized as described above and stained intracellularly with an FITC-labeled anti-CD68 antibody (FA-11; Serotec). Isotype control (FITC Rat IgG2a) was from BD Biosciences.

The Mouse Regulatory T Cell Staining Kit (eBioscience) was used to determine the percentage of Foxp3⁺ T cells among the CD4⁺KJ1-26⁺ DO11.10 T cell population.

Stimulation of OVA-specific CD4⁺ T cells by OVA_{LPS}-DCs, OVA_{LPS}-DCs/AMs, or OVA_{LPS}-DCs/IMs. FACS-sorted lung DCs (2×10^4 cells) from naive BALB/c mice were stimulated for 16 hours with OVA_{LPS} (100 μ g/ml LPS-free OVA and 10 ng/ml LPS) in the presence or absence of AMs (4×10^4 cells) or IMs (2×10^4 cells). APCs were then extensively washed, irradiated, and cocultured for 72 hours with freshly isolated DO11.10 CD4⁺ T cells (2×10^5 cells). Unpulsed DCs were used as controls. The proliferation of DO11.10 CD4⁺ T cells was measured as [³H]thymidine incorporation during the last 16 hours of the 72-hour culture. Culture supernatants were assayed for IL-4 and IL-5 by ELISA (Biosource).



Cell migration. Sixteen-hour-cultured OVA_{LPS}-DCs, OVA_{LPS}-DCs/AMs, or OVA_{LPS}-DCs/IMs were collected and labeled with CFSE (5 μM) for 10 minutes at 37°C. Cells were washed, resuspended in PBS, and instilled into the trachea of naive recipients (DCs: 1 × 10⁶ cells; IMs: 1 × 10⁶ cells; AMs: 2 × 10⁶ cells). Control mice received PBS. Twenty-four hours later, MLNs were collected, minced, and digested for 30 minutes at 37°C in 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNaseI (Roche) in HBSS. CFSE⁺F4/80⁺CD11c⁺, CFSE⁺F4/80⁺CD11c⁻, and CFSE⁻F4/80⁺CD11c⁻ cells were detected by flow cytometry.

In depletion experiments, *in vivo* migration of lung DCs was evaluated by injection of 100 μg FITC-labeled OVA and 10 ng LPS in the trachea of control and IM-depleted BALB/c mice. Twenty-four hours later, MLNs were collected and analyzed for the presence of FITC⁺ DCs (F4/80⁺CD11c⁺ cells).

Production of IL-10 and TGF-β by IMs, AMs, and lung DCs. FACS-sorted IMs, AMs, and lung DCs were stimulated or not with OVA_{LPS} for 16 hours. Culture supernatants were assayed for IL-10 and TGF-β by ELISA (eBioscience).

Depletion of IMs. For depletion of IMs, 250 μg of depleting anti-F4/80 (HB-198 hybridoma; ATCC) (31, 32) or control isotype antibodies (IgG2b; Serotec) were injected *i.p.* on 3 consecutive days, starting 1 day before *i.t.* administration of OVA (100 μg/mouse) and LPS (10 ng/mouse). On day 3, the depletion of F4/80⁺CD11c⁻ cells was assessed on total lung cells by flow cytometry.

Intratracheal instillation of depleting anti-F4/80 antibody. Anesthetized mice received one *i.t.* injection of 250 μg of depleting anti-F4/80 antibodies on 3 consecutive days.

Depletion of AMs. Liposomes encapsulated with clodronate (dichloromethylene diphosphonate, Cl₂MDP) or PBS were prepared as described previously (67). Clodronate was a gift of Roche Diagnostics GmbH. Liposomes (100 μl) were injected *i.t.* to each anesthetized naive recipient.

B cell stimulation. B cells were purified from MLNs using CD19 MicroBeads (Miltenyi) and then cultured (2 × 10⁵ cells in a 96-well plate) in RPMI supplemented with 10% heat-inactivated fetal calf serum and additives, with or without agonist CD40 antibodies (10 μg/ml; 1C10; eBiosciences). The proliferation was measured as [³H]thymidine incorporation during the last 16 hours of a 2-day culture.

T cell stimulation. T cells were purified from MLNs using the Pan T cell Isolation Kit (Miltenyi Biotec). They were then placed (2 × 10⁵ cells) into 96-well plates coated with anti-CD3 antibodies (10 μg/ml; 145-2C11; eBiosciences) and cultured with anti-CD28 antibodies (5 μg/ml; 37.51;

eBiosciences) in RPMI supplemented with 10% heat-inactivated fetal calf serum and additives. Controls were cultured in uncoated wells without anti-CD28 antibodies. The proliferation was measured as [³H]thymidine incorporation during the last 16 hours of a 2-day culture.

Statistics. Data are presented as mean ± SD. The differences between mean values were estimated using an ANOVA test followed by a Fisher's protected least standard deviation test. A *P* value less than 0.05 was considered significant. All experiments were repeated at least 3 times; *n* ≥ 6 in each experimental group.

Acknowledgments

We thank Kris Thielemans for providing recombinant murine GM-CSF; Sandrine Florquin for providing *Tlr4*^{-/-} mice; Sandra Ormense and the Cell Imaging and Flow Cytometry GIGA Technological Platform for FACS analysis; and Ilham Sbai, Cédric François, and Fabrice Jaspas for excellent technical and secretarial assistance. Clodronate (Cl₂MDP) was a gift of Roche Diagnostics GmbH. The Laboratory of Cellular and Molecular Physiology is supported by grants from the Fonds National de la Recherche Scientifique (FNRS; Belgium; Mandat d'Impulsion Scientifique), by the Fonds de la Recherche Scientifique Médicale (FRSM; Belgium), by the Belgian Programme on Interuniversity Attraction Poles (IUAP; FEDIMMUNE) initiated by the Belgian State (Belgian Science Policy), and by an Action de Recherche Concertée de la Communauté Française de Belgique. D. Bedoret and T. Marichal are research fellows, C. Desmet is a postdoctoral researcher, and M. Moser is a research director at the FNRS.

Received for publication May 1, 2009, and accepted in revised form September 9, 2009.

Address correspondence to: Fabrice Bureau, Laboratory of Cellular and Molecular Physiology, GIGA-Research, University of Liège, Avenue de l'Hôpital, Bâtiment B34, Sart-Tilman, B-4000 Liège, Belgium. Phone: 32-4-366-42-84; Fax: 32-4-366-42-85; E-mail: fabrice.bureau@ulg.ac.be.

Rodrigue Closset is deceased.

- Akbari, O., DeKruyff, R., and Umetsu, D. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* **2**:725–731.
- Akbari, O., et al. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat. Med.* **8**:1024–1032.
- de Heer, H., et al. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J. Exp. Med.* **200**:89–98.
- Michel, O., et al. 1996. Severity of asthma is related to endotoxin in house dust. *Am. J. Respir. Crit. Care Med.* **154**:1641–1646.
- Milton, D., Johnson, D., and Park, J. 1997. Environmental endotoxin measurement: interference and sources of variation in the Limulus assay of house dust. *Am. Ind. Hyg. Assoc. J.* **58**:861–867.
- Rizzo, M., et al. 1997. Endotoxin exposure and symptoms in asthmatic children. *Pediatr. Allergy Immunol.* **8**:121–126.
- Thorn, J., and Rylander, R. 1998. Airways inflammation and glucan in a rowhouse area. *Am. J. Respir. Crit. Care Med.* **157**:1798–1803.
- Douwes, J., et al. 1999. Fungal extracellular polysaccharides in house dust as a marker for exposure to fungi: relations with culturable fungi, reported home dampness, and respiratory symptoms. *J. Allergy Clin. Immunol.* **103**:494–500.
- Douwes, J., et al. 2000. (1→3)-beta-D-glucan and endotoxin in house dust and peak flow variability in children. *Am. J. Respir. Crit. Care Med.* **162**:1348–1354.
- Alexis, N., et al. 2005. Acute LPS inhalation in healthy volunteers induces dendritic cell maturation *in vivo*. *J. Allergy Clin. Immunol.* **115**:345–350.
- Eisenbarth, S., et al. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196**:1645–1651.
- Hammad, H., et al. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15**:410–416.
- Gereda, J., et al. 2000. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet.* **355**:1680–1683.
- Braun-Fahrlander, C., et al. 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N. Engl. J. Med.* **347**:869–877.
- Douwes, J., et al. 2006. Does early indoor microbial exposure reduce the risk of asthma? The Prevention and Incidence of Asthma and Mite Allergy birth cohort study. *J. Allergy Clin. Immunol.* **117**:1067–1073.
- Park, J., Gold, D., Spiegelman, D., Burge, H., and Milton, D. 2001. House dust endotoxin and wheeze in the first year of life. *Am. J. Respir. Crit. Care Med.* **163**:322–328.
- Alexis, N., Lay, J., Almond, M., and Peden, D. 2004. Inhalation of low-dose endotoxin favors local T(H)2 response and primes airway phagocytes *in vivo*. *J. Allergy Clin. Immunol.* **114**:1325–1331.
- Thorne, P., et al. 2005. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. *Am. J. Respir. Crit. Care Med.* **172**:1371–1377.
- Smit, L., et al. 2008. Exposure-response analysis of allergy and respiratory symptoms in endotoxin exposed adults. *Eur. Respir. J.* **31**:1241–1248.
- Lagranderie, M., et al. 2003. Dendritic cells recruited to the lung shortly after intranasal delivery of *Mycobacterium bovis* BCG drive the primary immune response towards a type 1 cytokine production. *Immunology.* **108**:352–364.
- Steinman, R., and Cohn, Z. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137**:1142–1162.
- Steinman, R., and Witmer, M. 1978. Lymphoid dendritic cells are potent stimulators of the pri-



- mary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5132–5136.
23. Murphy, K., Heimberger, A., and Loh, D. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCR α 0 thymocytes in vivo. *Science*. **250**:1720–1723.
24. Franke-Ullmann, G., et al. 1996. Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages in vitro. *J. Immunol.* **157**:3097–3104.
25. Lambrecht, B., et al. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest.* **106**:551–559.
26. Bilyk, N., and Holt, P. 1993. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **177**:1773–1777.
27. Iwasaki, A., and Medzhitov, R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**:987–995.
28. Buelens, C., et al. 1995. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur. J. Immunol.* **25**:2668–2672.
29. De Smedt, T., et al. 1997. Effect of interleukin-10 on dendritic cell maturation and function. *Eur. J. Immunol.* **27**:1229–1235.
30. Geissmann, F., et al. 1999. TGF- β 1 prevents the noncongenate maturation of human dendritic Langerhans cells. *J. Immunol.* **162**:4567–4575.
31. Austyn, J., and Gordon, S. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11**:805–815.
32. Tidball, J., and Wehling-Henricks, M. 2007. Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J. Physiol.* **578**:327–336.
33. Apte, R., Richter, J., Herndon, J., and Ferguson, T. 2006. Macrophages inhibit neovascularization in a murine model of age-related macular degeneration. *PLoS Med.* **3**:e310.
34. Constant, S., et al. 2002. Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. *J. Clin. Invest.* **110**:1441–1448.
35. Holt, P., Strickland, D., Wikström, M., and Jahnsen, F. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* **8**:142–152.
36. Thepen, T., Van Rooijen, N., and Kraal, G. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J. Exp. Med.* **170**:499–509.
37. Thepen, T., McMenamin, C., Oliver, J., Kraal, G., and Holt, P. 1991. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs. immunity. *Eur. J. Immunol.* **21**:2845–2850.
38. Thepen, T., McMenamin, C., Girn, B., Kraal, G., and Holt, P. 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. *Clin. Exp. Allergy*. **22**:1107–1114.
39. Holt, P., et al. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* **177**:397–407.
40. Blussé van Oud Alblas, A., van der Linden-Schreier, B., and van Furth, R. 1981. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intravenous administration of heat-killed bacillus Calmette-Guérin. *J. Exp. Med.* **154**:235–252.
41. Manetti, R., et al. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* **177**:1199–1204.
42. Kalinski, P., Hilkens, C.M., Snijdert, A., Snijdert, F.G., and Kapsenberg, M.L. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol.* **159**:28–35.
43. Schwarze, J., et al. 1998. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. *J. Allergy Clin. Immunol.* **102**:86–93.
44. Wang, S., et al. 2001. IL-12-dependent vascular cell adhesion molecule-1 expression contributes to airway eosinophilic inflammation in a mouse model of asthma-like reaction. *J. Immunol.* **166**:2741–2749.
45. Meyts, I., et al. 2006. IL-12 contributes to allergen-induced airway inflammation in experimental asthma. *J. Immunol.* **177**:6460–6470.
46. Demangel, C., Bertolino, P., and Britton, W. 2002. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur. J. Immunol.* **32**:994–1002.
47. Koch, F., et al. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* **184**:741–746.
48. Koya, T., et al. 2007. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J. Allergy Clin. Immunol.* **119**:1241–1250.
49. Salez, L., Singer, M., Balloy, V., Créminon, C., and Chignard, M. 2000. Lack of IL-10 synthesis by murine alveolar macrophages upon lipopolysaccharide exposure. Comparison with peritoneal macrophages. *J. Leukoc. Biol.* **67**:545–552.
50. Denning, T., Wang, Y., Patel, S., Williams, I., and Pulendran, B. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat. Immunol.* **8**:1086–1094.
51. Erwig, L., and Henson, P. 2007. Immunological consequences of apoptotic cell phagocytosis. *Am. J. Pathol.* **171**:2–8.
52. Martinez, F., Sica, A., Mantovani, A., and Locati, M. 2008. Macrophage activation and polarization. *Front. Biosci.* **13**:453–461.
53. Sternberg, E.M. 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat. Rev. Immunol.* **6**:318–328.
54. Gerber, J., and Mosser, D. 2001. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J. Immunol.* **166**:6861–6868.
55. Strassmann, G., et al. 1994. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J. Exp. Med.* **180**:2365–2370.
56. Haskó, G., Pacher, P., Deitch, E., and Vizi, E. 2007. Shaping of monocyte and macrophage function by adenosine receptors. *Pharmacol. Ther.* **113**:264–275.
57. Mosser, D.M., and Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**:958–969.
58. Edwards, J., Zhang, X., Frauwirth, K., and Mosser, D. 2006. Biochemical and functional characterization of three activated macrophage populations. *J. Leukoc. Biol.* **80**:1298–1307.
59. Stock, P., et al. 2004. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat. Immunol.* **5**:1149–1156.
60. van Rijt, L., et al. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* **201**:981–991.
61. Stumbles, P.A., et al. 1998. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J. Exp. Med.* **188**:2019–2031.
62. Stein, R., et al. 1999. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet.* **354**:541–545.
63. Sigurs, N., Bjarnason, R., Sigurbergsson, F., and Kjellman, B. 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am. J. Respir. Crit. Care Med.* **161**:1501–1507.
64. Panuska, J., et al. 1990. Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. *J. Clin. Invest.* **86**:113–119.
65. Inaba, K., et al. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176**:1693–1702.
66. Gueders, M.M., et al. 2008. A novel formulation of inhaled doxycycline reduces allergen-induced inflammation, hyperresponsiveness and remodeling by matrix metalloproteinases and cytokines modulation in a mouse model of asthma. *Biochem. Pharmacol.* **75**:514–526.
67. Van Rooijen, N., and Sanders, A. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods.* **174**:83–93.