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Inhibition of Th2 Adaptive Immune Responses and Pulmonary Inflammation by Leukocyte Ig-Like Receptor B4 on Dendritic Cells

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We previously established that the inhibitory receptor LILRB4 mitigates LPS-induced, neutrophil-dependent pathologic effector mechanisms in inflammation. We now report that LILRB4 on dendritic cells (DCs) counterregulates development of an adaptive Th2 immune response and ensuing inflammation in a model of allergic pulmonary inflammation, initiated by inhalation sensitization with OVA and LPS followed by airway challenge with OVA. We found that *Lilrb4*$^{-/-}$ mice had significantly exacerbated eosinophilic pulmonary inflammation, as assessed in bronchoalveolar lavage and lung tissue, as well as elevated levels of OVA-specific IgE and Th2 cytokines produced by OVA-restimulated lymph node cells. LILRB4 was preferentially expressed on MHC class II^{high}CD86^{high} OVA-bearing DCs in lung-draining lymph nodes after sensitization or challenge. Moreover, the lymph nodes of *Lilrb4*$^{-/-}$ mice had significantly more of these mature DCs after challenge with OVA, which was accompanied by significantly more IL-4–producing lymphocytes, compared with *Lilrb4*$^{+/+}$ mice. Sensitization of naive *Lilrb4*$^{+/+}$ mice by transfer of OVA-LPS-pulsed *Lilrb4*$^{-/-}$ bone marrow-derived DCs was sufficient to confer exacerbated allergic lung pathology upon challenge with OVA, compared with mice that received *Lilrb4*$^{+/+}$ bone marrow-derived DCs. Our findings establish that maturation and migration of pulmonary DCs to lymph nodes in response to Ag and an innate immune stimulus is associated with upregulated expression of LILRB4. In addition, this receptor attenuates the number of these mature DCs and attendant IL-4–producing lymphocytes in the lymph nodes, and accordingly, the ability of DCs to elicit pathologic Th2 pulmonary inflammation. ***The Journal of Immunology*****, 2010, 184: 000–000.**

The ITIM-bearing inhibitory receptor LILRB4 suppresses the activation of mast cells induced by passive administration of IgE and Ag or stem cell factor leading to a reduction in the ensuing anaphylactic reactions in mouse models (1, 2). In addition, LILRB4 diminishes neutrophil-dependent pathologic inflammation induced by LPS in the thrombohemorrhagic Shwartzman reaction and an effector phase model of immune complex arthritis induced by the combination of anti-type II collagen mAbs and LPS (3–5). Traditionally, LPS has been associated with innate immune responses to bacterial infections initiated by TLR4 signaling, which can lead to Th1 polarized adaptive immune responses driven by the production of IL-12 (6). The ability of Th1 polarization to prevent or suppress Th2 responses (7) led to the hypothesis that exposure to microbial components that foster Th1 polarization would reduce the incidence of allergic pulmonary disorders. Indeed, exposure of children to environments high in LPS early in life has been associated with a reduced risk for subsequent development of bronchial asthma (8–10). Conversely, environmental LPS levels have been directly correlated with the severity of asthma in older subjects (11, 12). In a number of animal models, exposure to LPS near the time of sensitization to experimental Ags introduced intraperitoneally with adjuvants reduces pulmonary inflammation and/or airway hyperresponsiveness (AHR) induced by Ag challenge in the airway (13–15). In contrast, inhalation of LPS before or during sensitization with inhaled OVA can result in augmented Ag-specific serum IgE levels and lung eosinophilia upon airway challenge with OVA (16, 17). The ability to detect a contribution for LPS in the development of the Th2 adaptive immune response and pulmonary inflammation may reflect the fact that sensitization with OVA alone via the airway is tolerogenic (18–21), and an innate immune "danger signal," such as LPS, is necessary to convert the tolerogenic response to a pathologic immune and inflammatory response.

Nevertheless, the contribution of LPS to models in which both sensitization and challenge occur through the airway is not restricted to inducing Th2 inflammation. An elegant study, in which the major variable was the dose of LPS administered during airway sensitization to OVA, established that the amount of LPS materially alters the nature of the pulmonary inflammation that ensues upon challenge with OVA (17). A relatively low dose of LPS (300 ng delivered in three intranasal instillations with a total of 300 μg of OVA) leads to iconic Th2 pulmonary inflammation upon OVA challenge, characterized by elevated serum levels of Ag-specific IgE and IgG1, the absence of serum IL-12, eosinophilia and goblet cell metaplasia in the lung, and preferential production of Th2 cytokines IL-5 and IL-13 by Ag-restimulated lymph node cells in a TLR4- and MyD88-dependent manner (17, 22). In contrast, the administration of 1000-fold more LPS during sensitization with the same dose of OVA leads to generation of OVA-specific serum IgG2a, serum IL-12, neutrophilic pulmonary inflammation, and

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Abbreviations used in this paper: AF-OVA, Alexa Fluor 647-labeled ovalbumin; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; MFI, mean fluorescence intensity.

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release of IFN-γ from Ag-restimulated lymph node cells, all of which are characteristic of Th1 responses (17). LPS in the low-dose protocol made an essential contribution to the migration of pulmonary dendritic cells (DCs) to the draining intrathoracic lymph nodes (17, 23) and likely to MyD88-dependent upregulation of MHC class II and CD86 on pulmonary DCs during sensitization, the latter favoring Th2 polarization in the absence of IL-12 (22, 23). Indeed, LPS also contributes importantly to the recruitment of Ag-specific Th2 cells to the lung in a similar model (24).

A better understanding of the mechanisms that regulate the ability of LPS to influence the development of allergic pulmonary inflammation would help explain the complex contributions of this innate and ubiquitous molecule to asthma. Given that LILRB4 counterregulates LPS-mediated inflammation in several neutrophil-dependent acute effector phase models (3–5), we considered the possibility that LILRB4 might also counterregulate the development of the LPS-dependent, adaptive Th2 response to inhaled OVA through expression on DCs (25). In our study, expression of LILRB4 was strikingly upregulated on DCs bearing OVA and high levels of MHC class II and CD86 in the lung-draining lymph nodes of mice after inhalation of OVA and low-dose LPS. In addition, after challenge with OVA, *Lilrb4*$^{-/-}$ mice had significantly exacerbated eosinophilic lung inflammation in the OVA/low-dose LPS model, as well as elevated levels of Ag-specific serum IgE and Th2 cytokines produced by OVA-restimulated intrathoracic lymph node cells. The greater pathologic Th2 response in *Lilrb4*$^{-/-}$ mice was preceded by the appearance of a significantly greater number of mature, OVA-bearing DCs in the lung-draining lymph nodes, as well as IL-4–producing lymphocytes likely accumulated there in response to the OVA-bearing DCs. Moreover, sensitization of *Lilrb4*$^{+/+}$ mice by transfer of *Lilrb4*$^{-/-}$ OVA/LPS-pulsed bone marrow-derived dendritic cells (BMDCs) to *Lilrb4*$^{+/+}$ mice was sufficient to reiterate upon Ag-challenge the exacerbated allergic lung pathology observed in *Lilrb4*$^{-/-}$ mice sensitized by inhalation of OVA-LPS. Therefore, LILRB4 on DCs can temper the course of a pathologic adaptive immune response in vivo and ensuing pulmonary Th2 inflammation.

Materials and Methods

Animals

Lilrb4$^{+/+}$ and *Lilrb4*$^{-/-}$ mice were generated on the BALB/c background as previously described (4). Mice were maintained in a specific pathogen-free barrier facility. Female mice, 6–12 wk old, were used for experiments. The use of mice for these studies was reviewed and approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

LPS depletion and measurement

For some experiments, Endotoxin Detoxi-gel (Pierce Biotechnology, Rockford, IL) was used according to the manufacturer's instructions to remove >99% of the contaminating LPS in the administered OVA (Grade V; Sigma-Aldrich, St. Louis, MO) solution. For other experiments, OVA was administered without prior detoxification. However, as determined with the limulus amebocyte assay (Cambrex, East Rutherford, NJ), in all cases <2 ng of contaminating LPS per mouse was delivered by the OVA solutions during sensitization and challenge.

Sensitization and challenge protocol

Mice were lightly anesthetized with isoflurane and then sensitized intranasally with 50 μl PBS alone or containing 100 μg LPS-depleted OVA and 100 ng of LPS (from *Escherichia coli* O55:B5; Sigma-Aldrich L4524) on days 0, 1, and 2 (17). On days 14, 15, 18, and 19, mice were lightly anesthetized with isoflurane and challenged intranasally with 25 μg OVA in 50 μl PBS. In some experiments, the challenges were delivered in 30 μl with comparable results. Mice were sacrificed for analyses on day 21 by i.p. injection of an overdose of pentobarbital.

Analysis of bronchoalveolar lavage

A 22-gauge plastic cannula was inserted into the upper third of the trachea and secured with cotton thread. Bronchoalveolar lavage (BAL) fluid was obtained by infusion and withdrawal of 0.7 ml of calcium- and magnesium-free HBSS containing 0.1 mM EDTA or Dulbecco's PBS containing 0.5 mM EDTA; this was repeated three times to achieve a total instillation of 2.1 ml. Cells were pelleted and washed by centrifugation, counted with a hemacytometer, and spun onto slides with a Shandon Cytospin (Thermo Scientific, Rockford, IL). The slides were stained with Diff-Quik (Dade Behring, Deerfield, IL), and differential cell counts were determined on the basis of cell morphology and staining characteristics by counting at least 200 cells per sample.

Lung mononuclear cell isolation

Lungs were perfused with 10 ml of ice-cold calcium- and magnesium-free HBSS through the right ventricle, removed intact, and chopped into 1-mm fragments with 18-mm scalpel blades. Lung fragments were incubated in 500 U/ml collagenase (CLS-IV; Worthington Biochemical, Lakewood, NJ) and 0.02 mg/ml DNAse I (Sigma-Aldrich) at 37°C for 60 min with agitation at 200 rpm. The resulting suspensions were strained through metal sieves to remove debris, and mononuclear cells (> 97% pure) were isolated from the interface of a Nycoprep (Axis-Shield, Oslo, Norway) gradient (300 × *g* for 20 min at 4°C). Cells were washed with PBS, counted, and subjected to flow cytometric analysis.

Flow cytometric analysis

Cells were resuspended in PBS containing 0.5% FBS or BSA and 0.05% sodium azide at 4°C and incubated with anti-FcγRII/III mAb (eBioscience, San Diego, CA) and mouse IgG (Sigma-Aldrich) for 20 min on ice to block FcγR. Cells were then incubated with saturating concentrations of fluorochrome-conjugated anti-CD4, CD8, CD11c, CD19, CD86, or MHC class II mAbs (eBioscience), or mAb B23.1 prepared as previously described (5), for 30 min at 4°C, washed by centrifugation, and analyzed on a BD Biosciences (San Jose, CA) FACSCanto flow cytometer, using FACSDiva acquisition software and FlowJo analysis software (Tree Star, Ashland, OR).

Isolation of intrathoracic lymph node cells for Ag restimulation and flow cytometry

For the Ag restimulation assay, cells were isolated from the parathymic, hilar, and paratracheal lymph nodes by mechanical dispersion through mesh and were cultured at 2×10^6 cells/ml with 0.1 mg/ml of LPS-depleted OVA for 72 h. The concentrations of cytokines in culture supernatants were measured with ELISA kits (eBioscience). For flow cytometric analysis of DCs, the residual tissue fragments on the mesh were added to the filtrate that was then digested with collagenase and DNAse I at 37°C for 20 min with agitation at 200 rpm. The reaction was stopped with an equal volume of PBS containing 2 mM EDTA, and cells were then washed by centrifugation in PBS containing 1 mM EDTA.

Measurements of anti-OVA Ig in sera

For quantification of anti-OVA IgE levels, anti-mouse IgE (2 μg/ml; clone R35-72; BD Biosciences) was incubated in 96-well flat bottom Maxisorp plates (Thermo Fisher, Pittsburgh, PA) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 and blocked with PBS containing 10% heat-inactivated FBS (Sigma-Aldrich). Serially-diluted sera samples were added, and the plates were incubated for 1 h at 37°C. Biotinylated OVA (2 μg/ml) was added to each well, and the plates were incubated for 1 h at 37°C. Plates were washed several times and incubated with HRP-conjugated streptavidin for 1 h at 37°C. The plates were washed several times, substrate solution (tetramethylbenzidine and hydrogen peroxide) was added, and the plates were incubated for 30 min at room temperature. The reactions were terminated by the addition of 1 M H_2SO_4, and the absorbances at 570 nm were subtracted from the absorbances at 450 nm. OVA-specific IgE concentrations in experimental samples were calculated by comparison with those of a serum containing high-titer anti-OVA IgE generated by i.p. injection and intranasal administration of OVA in BALB/c mice. The concentration of anti-OVA IgE in that serum was initially determined by comparison with a monoclonal anti-OVA IgE (26) provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA). Anti-OVA IgE levels in the BMDC transfer experiments were determined with an ELISA kit from MD Biosciences.

For quantification of OVA-specific IgG1, plates were coated with OVA (10 μg/ml), the serially-diluted sera were added, and the plates were incubated with biotinylated anti-mouse IgG1 (clone A85-1; BD Biosciences), and HRP-conjugated streptavidin. Anti-OVA mouse IgG1 mAb (clone OVA-14; Sigma-Aldrich) was used as the standard.

Analysis of Ag-bearing DCs in intrathoracic lymph nodes

In one set of experiments, mice received 100 μg Alexa Fluor 647-labeled OVA (AF-OVA) (Invitrogen, Carlsbad, CA) and 100 ng LPS intranasally on day 0; mice were analyzed on day 1. In other experiments, mice received 100 μg unlabeled OVA and 100 ng LPS intranasally on days 0, 1, and 2; some were analyzed on day 14, whereas others were challenged with 25 μg AF-OVA on day 14 and analyzed on day 15. In each type of experiment, some mice received unlabeled OVA rather than AF-OVA to serve as negative controls for defining AF-OVA positivity in flow cytometry. On the days of analysis, mice were euthanized, and their pulmonary and systemic circulation was perfused with 10 ml HBSS. Four draining intrathoracic lymph nodes (two right parathymic, one paratracheal, and one right hilar) were harvested per animal, pooled, and disaggregated to single-cell suspensions with the flat end of a tuberculin syringe on a 70-μm cell strainer (Costar, Cambridge, MA). The cells were centrifuged, resuspended in 3 ml of prewarmed RPMI 1640 containing 5% FBS, and digested with collagenase and DNAse as described above for lungs, except that the incubation time was 20 min. The digestion was stopped by the addition of 3 ml ice-cold PBS containing 2 mM EDTA. The cells were then washed with 5 ml PBS containing 1 mM EDTA and resuspended in 500 μl cold PBS containing 0.5% FBS or BSA and 0.05% sodium azide.

Quantification of IL-4–producing cells by ELISPOT assay

Mice were sensitized by intranasal instillation of 100 μg OVA and 100 ng LPS on days 0, 1, and 2. On day 14, mice were challenged by intranasal instillation of 25 μg OVA. On day 15, four intratracheal lymph nodes were isolated from each mouse as described above. Four serial 2-fold dilutions of cells, starting with 8×10^5 cells/ml, were cultured in 96-well sterile multiscreen tissue culture plates (Millipore, Bedford, MA) coated with capture Abs (eBioscience). Cells were stimulated with 50 ng/ml PMA and 1 μM ionomycin for 36 h. IL-4 spots were detected with an ELISPOT kit (eBioscience) according to the manufacturer's instructions and were counted using a dissecting microscope. The frequency of IL-4–producing cells was calculated and multiplied by the total number of lymph node cells to determine the total number of IL-4–producing cells.

Generation and adoptive transfer of BMDCs

BMDCs from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice were generated according to the method of Lutz et al. (27). Cells were flushed from the femurs of euthanized mice with 10 ml cold RPMI 1640 and filtered through a 70-μm cell strainer. The cells were then plated in extra deep 100 × 25-mm Petri dishes (Thermo Fisher) at 4×10^5 cells/ml in 10 ml RPMI 1640 supplemented with 10% FBS, 42.9 mM 2-mercaptoethanol, 10,000 U/ml Pen-Strep, 200 mM L-glutamine, MEM non-essential amino acids (R10 medium), and 20 ng/ml mouse recombinant GM-CSF (PeproTech, Rocky Hill, NJ). On day 3 of culture, 10 ml fresh R10 medium with 20 ng/ml GM-CSF was added to each dish. On days 6 and 8, half the volume of each dish was removed, centrifuged, resuspended in 10 ml/dish of fresh R10 medium with 20 ng/ml GM-CSF, and added back to the original dish. On day 9, nonadherent cells were harvested, washed with PBS at 4°C, and replated in 35 × 10 mm Petri dishes (Thermo Fisher) at 1×10^6 cells/ml in 3 ml/dish R10 medium with 20 ng/ml GM-CSF, 100 μg/ml OVA, and 100 ng/ml LPS. After overnight incubation, nonadherent cells were harvested, washed by centrifugation three times with PBS, and resuspended in PBS at 1×10^5 cells/50 μl. *Lilrb4*$^{+/+}$ BALB/c recipients were lightly anesthetized with isoflurane and received 1×10^5 *Lilrb4*$^{+/+}$ or *Lilrb4*$^{-/-}$ Ag-loaded BMDCs in 50 μl PBS intranasally. On days 14, 15, 18, and 19, mice were challenged with intranasal instillations of 25 μg OVA in 30 μl PBS or given PBS alone. Mice were euthanized and analyzed 48 h after the last instillation.

Lung histology

The left lungs of mice were fixed for at least 8 h in 4% paraformaldehyde and embedded in glycolmethacrylate, and 2.5 μm sections were cut, as described previously (28). Areas of inflammation and total areas in eight sections from each lung were quantified by digital image analysis to determine the percent inflamed area. Eosinophilia was revealed by staining sections with Congo red (28, 29). Other sections were stained with periodic acid-Schiff for enumeration of goblet cells in the bronchial epithelium of at least four independent bronchovascular bundles from each mouse. Only comparable, large caliber, preterminal bronchi (200–220 μm diameter) were examined, because minimal goblet cell metaplasia occurs in terminal bronchioles (30). The length of basal lamina of each bronchus was measured by Image J (National Institutes of Health image analysis software), and the data are expressed as the number of goblet cells per millimeter of basal lamina.

Statistical analyses

Statistical significance was determined using Student unpaired, two-tailed *t* test or the Mann-Whitney *U* test. $p < 0.05$ was considered to be significant.

Results

Effects of LILRB4 deficiency on Th2 pulmonary inflammation in an LPS-dependent model

To detect contributions of LILRB4 to pulmonary inflammation, we chose a model of airway sensitization and challenge to OVA that depends on the presence of relatively low levels of LPS during the sensitization phase to elicit Th2 inflammation in the lung (17). On days 0, 1, and 2, *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice received intranasal instillations of PBS alone or containing 100 μg LPS-depleted OVA and 100 ng highly purified LPS. On days 14, 15, 18, and 19, all mice received 25 μg LPS-depleted OVA intranasally, and mice were euthanized for analysis on day 21 (17). The infiltration of inflammatory cells into the lungs was assessed by analysis of cells in BAL. The BAL from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice that were sensitized with OVA-LPS and challenged with OVA (OVA-LPS/OVA mice) developed an eosinophilia that did not occur in the BAL fluid rom mice that received PBS during the sensitization phase (PBS/OVA mice) (Fig. 1). However, the number of eosinophils in the BAL of *Lilrb4*$^{-/-}$ OVA-LPS/OVA mice was significantly greater ($p = 0.006$) than in *Lilrb4*$^{+/+}$ mice. In addition, the number of BAL lymphocytes induced in OVA-LPS/OVA mice was also significantly greater ($p = 0.04$) in *Lilrb4*$^{-/-}$ mice compared with *Lilrb4*$^{+/+}$ mice, whereas there were no differences in the numbers of neutrophils or macrophages. These data indicate that the absence of LILRB4 leads to significantly exacerbated Th2 pulmonary inflammation in this model.

To quantify pulmonary inflammation in a second manner, the lungs of *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice at day 21 were perfused to remove intravascular cells, minced, and digested with collagenase and DNAse I; parenchymal mononuclear cells were isolated by density gradient centrifugation. Greater numbers of mononuclear cells were recovered from both *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ OVA-LPS/OVA mice compared with PBS/OVA mice (Fig. 2), demonstrating that the inflammation induced in this model was not limited to cells recovered with BAL (Fig. 1). Moreover, the number of lung mononuclear cells obtained from *Lilrb4*$^{-/-}$ mice was significantly greater than from *Lilrb4*$^{+/+}$ animals ($p = 0.0004$; Fig. 2). The mononuclear cells were phenotyped by flow cytometry to define their composition. The lung mononuclear cells from *Lilrb4*$^{-/-}$ mice had significantly more CD4$^+$ cells ($p = 0.02$), CD8$^+$ cells ($p = 0.04$), and CD19$^+$ cells ($p = 0.0002$; Fig. 2). Therefore, the greater number of lung mononuclear cells in *Lilrb4*$^{-/-}$ OVA-LPS/OVA mice at day 21 included both T and B lymphocytes. These changes primarily reflected the greater number of total lung mononuclear cells in *Lilrb4*$^{-/-}$ mice rather than selective increases in particular populations of lymphocytes. Therefore, as revealed by significantly increased cellularity in both BAL and parenchymal lung mononuclear cells, the absence of LILRB4 leads to exacerbated pathologic Th2 pulmonary inflammation.

Effects of LILRB4 deficiency on serum Ig levels and Ag-recall responses

Our previous studies identified counterregulatory effects of LIRB4 on mast cells and neutrophils in passive, effector phase models of inflammation (1–5). The augmented pulmonary inflammation induced by active immunization in the OVA-LPS model suggested that LILRB4 might contribute to regulating the development of

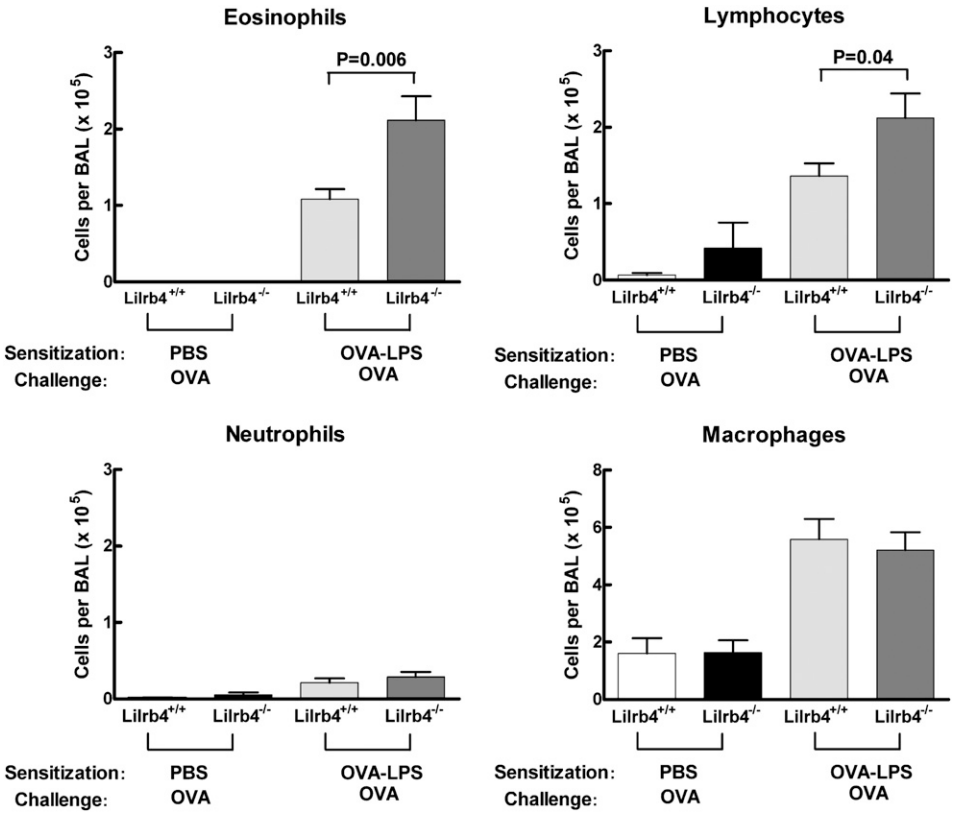

FIGURE 1. BAL cell numbers and differentials from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice received PBS alone or with 100 μg OVA and 100 ng LPS intranasally on days 0, 1, and 2. Mice were challenged with 25 μg LPS-depleted OVA intranasally on days 14, 15, 18, and 19. BAL was collected on day 21, cells in the BAL were counted, cytospin slides were prepared and stained with Diff-Quik, and leukocyte differential counts were determined for each mouse and multiplied by the number of recovered cells. Data are expressed as mean ± SEM; n = 4, 5, 12, and 12 for the four columns from left to right, respectively.

adaptive immune responses. We therefore determined whether the Ag-specific IgE response was elevated in the absence of LILRB4. Serum samples were isolated from mice at D21, and the levels of OVA-specific IgE were quantified with an ELISA. IgE was essentially undetectable in *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ PBS/OVA mice, but the levels were elevated in OVA-LPS/OVA mice (Fig. 3). Moreover, sera from *Lilrb4*$^{-/-}$ mice contained a significantly greater amount of Ag-specific IgE compared with sera from *Lilrb4*$^{+/+}$ animals (p = 0.04). In addition, there was a trend toward higher levels of OVA-specific IgG1, a Th2-associated Ig subclass in

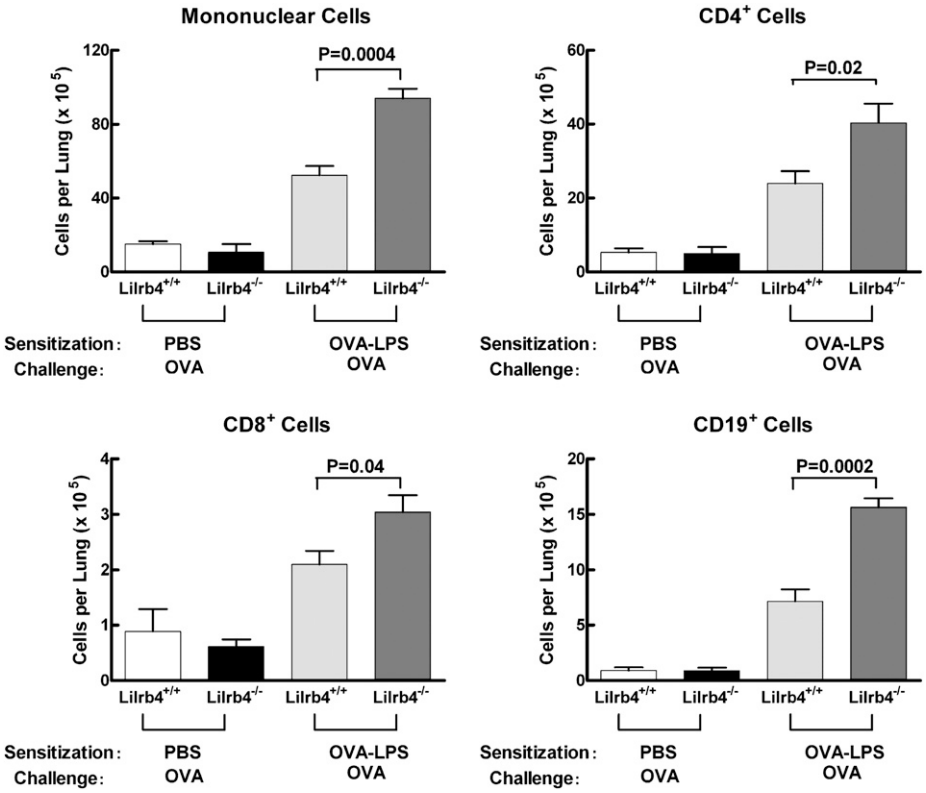

FIGURE 2. Cell numbers and surface phenotypes of parenchymal lung mononuclear cells from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Mice were sensitized and challenged as described in the Fig. 1 legend. Lungs were perfused, cut into fragments, and digested with collagenase and DNAse I. The mononuclear cells dispersed from each lung were isolated on Nycoprep gradients and counted. Fc receptors on the cells were blocked with mouse IgG and anti-FcγRIIB/III. The percentage of cells expressing CD4, CD8, or CD19 was determined by flow cytometry with directly fluorochrome-labeled mAbs, and the total number of cells expressing each receptor was calculated. Data are expressed as mean ± SEM; n = 2, 2, 5, and 5 for the four columns from left to right, respectively.

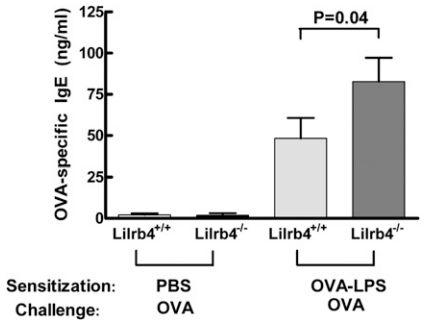

FIGURE 3. Serum IgE levels in *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Mice were sensitized and challenged as described in the Fig. 1 legend. Levels of anti-OVA IgE were measured by ELISA with an anti-mouse IgE mAb as the capture reagent, and biotinylated OVA/HRP-Streptavidin/TMB as the detecting reagents. Data are expressed as mean ± SEM; n = 8, 10, 16, and 16 for the four columns from left to right, respectively.

mice (31) in *Lilrb4*$^{-/-}$ versus *Lilrb4*$^{+/+}$ OVA-LPS/OVA mice (2.4 ± 0.3 and 1.6 ± 0.3 μg/ml; n = 16 and 17, respectively; p = 0.08). These data indicate that the absence of LILRB4 led to an enhanced Th2 polarized humoral response.

To examine a cellular adaptive immune response, we isolated intrathoracic lymph node cells from mice on day 21, restimulated the lymph node cells with OVA, and measured cytokine production 72 h later with ELISAs. The secretion of IL-4, IL-5, and IL-13 were all significantly greater in *Lilrb4*$^{-/-}$ cultures compared with *Lilrb4*$^{+/+}$ cultures (p = 0.004, 0.02, and 0.005, respectively; Fig. 4). In contrast, there was a trend toward less production of IFN-γ in the *Lilrb4*$^{-/-}$ cultures. These data indicate that there was an enhancement of the Th2-polarized T cell response in cells from *Lilrb4*$^{-/-}$ mice.

Expression of LILRB4 on intrathoracic lymph node DCs

Although the preceding data indicated that LILRB4 can negatively regulate a pulmonary adaptive immune response, we did not detect expression of LILRB4 on lung or lymph node T cells in this model (data not shown). We then considered the possibility that LILRB4 is expressed on DCs in the pulmonary system and might influence lymphocytes in lung-draining lymph nodes. To address this possibility, we instilled PBS alone or containing 100 μg AF-OVA and 100 ng LPS intranasally into *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Intrathoracic lymph node cells were isolated 18 h later as described above, and the binding of mAb B23.1 anti-LILRB4 (32) to CD11c$^+$ DCs was assessed with flow cytometry. In mice that received PBS alone, 17 ± 0.15% of the CD11c$^+$ cells expressed LILRB4, and the total number of CD11c$^+$LILRB4$^+$ cells was 0.5 × 10^4 ± 0 (n = 2); LILRB4 was not detected on cells from *Lilrb4*$^{-/-}$ mice (representative histograms are shown in Fig. 5*A*). In contrast, mice that received AF-OVA and LPS had a greater percentage (26 ± 1.8%; Fig. 5*A*) and total number (2.0 ± 0.3 × 10^4; n = 8) of CD11c$^+$ cells that expressed LILRB4 than mice that received PBS alone. Notably, whereas 70 ± 3.6% of CD11c$^+$AF-OVA$^+$ cells expressed LILRB4, a significantly lower 12 ± 0.7% of CD11c$^+$AF-OVA$^-$ cells expressed LILRB4 (representative histograms in Fig. 5*A*; compiled data in Fig. 5*B*). In addition, the number of LILRB4$^+$CD11c$^+$AF-OVA$^+$ cells in the lymph nodes was a significant 22-fold greater than the number of LILRB4$^+$ CD11c$^+$AF-OVA$^-$ cells. Moreover, the mean fluorescence intensity (MFI) value for LILRB4 on the OVA-bearing cells was a significant 6.4-fold greater than on the cells that did not have OVA (Fig. 5*B*). Furthermore, the MFI values for MHC class II and CD86 were a significant 5.7-fold and 6.6-fold greater, respectively, on CD11c$^+$AF-OVA$^+$ cells compared with CD11c$^+$AF-OVA$^-$ cells (Fig. 5*C*). Because the vast majority of OVA-bearing DCs in the intrathoracic lymph nodes of mice after intranasal instillation of OVA and LPS are derived from the airway (23), our data indicate that OVA-bearing DCs that had migrated from the lung to the draining lymph nodes and matured expressed LILRB4.

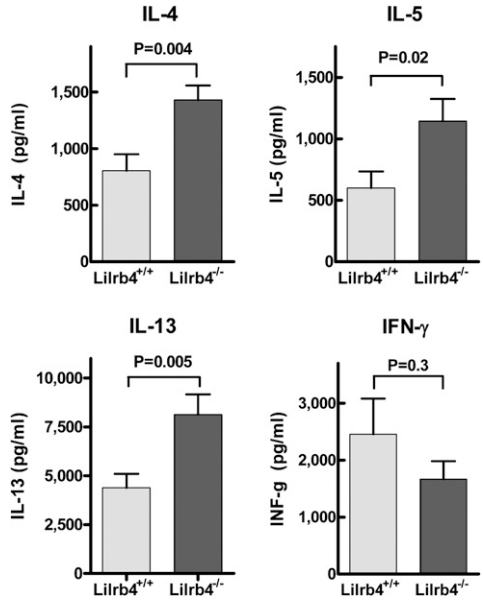

FIGURE 4. Secretion of cytokines by Ag-restimulated lymph node cells from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Mice were sensitized and challenged as described in the Fig. 1 legend. Intrathoracic lymph nodes were removed, cells were dispersed by mechanical disruption of the tissue, cells were cultured at 2 × 10^6 cell/ml with 0.1 mg/ml LPS-depleted OVA for 3 d, and mediators in the supernatants were measured with ELISAs. Data are expressed as mean ± SEM; n = 13 and 11 (IL-4), 12 and 9 (IL-5), 13 and 11 (IL-13), and 9 and 8 (IFN-γ) for each *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ pair, respectively.

To determine whether the absence of LILRB4 affected lymph node DCs 18 h after instillation of AF-OVA and LPS, we compared these cells in *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. There were no significant differences in the percentages or numbers of CD11c$^+$ cells, CD11c$^+$AF-OVA$^+$ cells, or in the expression levels of AF-OVA, MHC class II, and CD86 on CD11c$^+$ cells in the two strains of mice (n = 4–13). Nevertheless, the preferential expression of LILRB4 on OVA-bearing DCs that express high levels of MHC class II and CD86 in the draining lymph nodes of *Lilrb4*$^{+/+}$ mice raised the possibility that LILRB4 would influence the response of pulmonary DCs in sensitized mice to challenge with OVA. We therefore sensitized *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice by daily intranasal instillation of 100 μg OVA-LPS on days 0–2, and the intrathoracic lymph node cells of one group of mice were analyzed on day 14. Another group of sensitized mice was challenged with 25 μg AF-OVA on day 14, and their lymph node cells were analyzed on day 15. Before challenge, there were no differences in either the numbers of leukocytes or CD11c$^+$ cells in the lymph nodes of *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice (Fig. 6*A*). After the single challenge in *Lilrb4*$^{+/+}$ mice, there was no significant increase in either of these parameters, whereas there were significant 3- and 4-fold increases in the numbers of CD11c$^+$ cells and leukocytes, respectively, in the lymph nodes of *Lilrb4*$^{-/-}$ mice, and these numbers were significantly greater than those of challenged *Lilrb4*$^{+/+}$ mice (Fig. 6*A*). Moreover, there was a significantly greater percentage and number of CD11c$^+$ cells bearing AF-OVA in *Lilrb4*$^{-/-}$ mice, as well as a significantly greater MFI of AF-OVA in the CD11c$^+$ cells (Fig. 6*B*), indicative of greater Ag content. The AF-OVA$^+$ cells from *Lilrb4*$^{+/+}$ mice, when compared with AF-OVA$^-$ cells, expressed significantly greater levels of both MHC

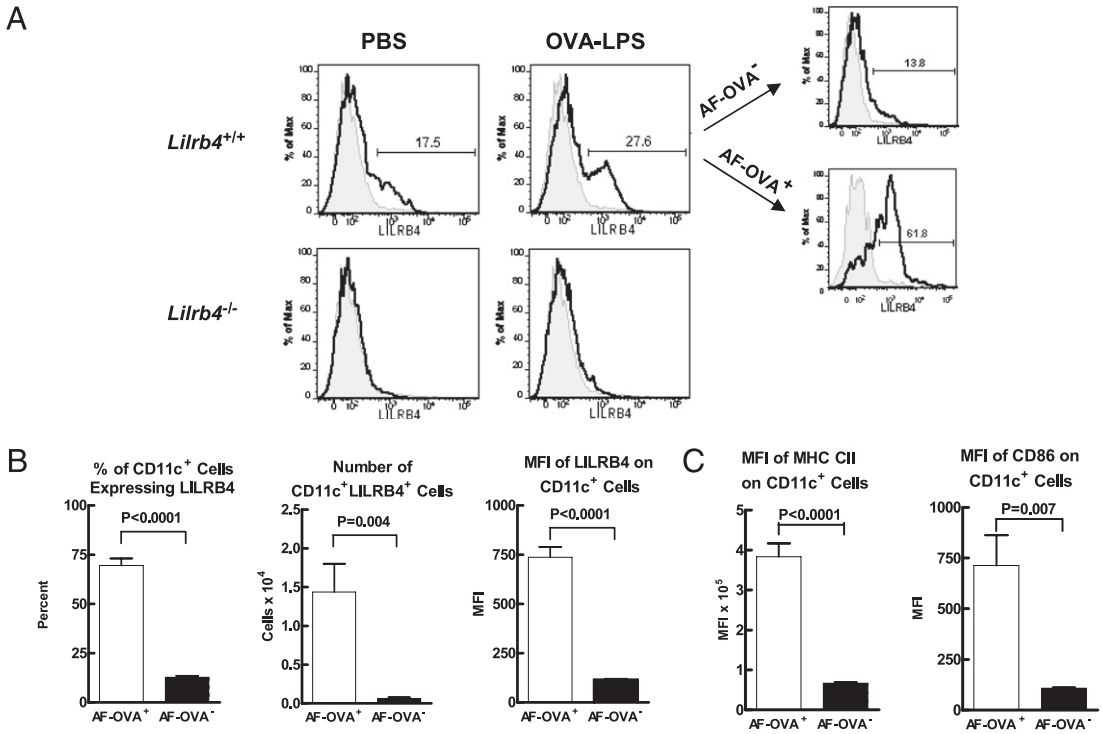

FIGURE 5. Expression of LILRB4 on intrathoracic lymph node DCs. PBS alone or containing 100 μg AF-OVA and 100 ng LPS was instilled intranasally into *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Four intrathoracic lymph nodes were removed from each mouse 18 h later. The cells were dispersed enzymatically from the tissue and counted, and FcγR on the cells was blocked as described in the Fig. 2 legend. The binding of anti-LILRB4 (bold histograms) and isotype control (light, shaded histograms) to CD11c$^+$ DCs was measured by flow cytometry. The cells in *Lilrb4*$^{+/+}$ mice that had received AF-OVA and LPS were further gated into AF-OVA$^+$ and AF-OVA$^-$ cells, and the percent, number, and MFI of LILRB4$^+$ cells were determined for the two cell populations. Representative flow cytometric histograms (*A*) and compiled data (*B*) are shown. The expression of MHC class II and CD86 on the AF-OVA$^+$ and AF-OVA$^-$ populations was determined in parallel (*C*). Data are expressed as mean ± SEM; $n = 6$ (*B*); $n = 4$ (*C*).

class II (MFI, 4095 ± 354 versus 1466 ± 19, respectively; $p < 0.0001$; $n = 7$) and CD86 (MFI, 1051 ± 85 versus 155 ± 4.9, respectively; $p < 0.0001$; $n = 7$). Although there were no differences in the MFI values of these cell populations in *Lilrb4*$^{-/-}$ mice (data not shown; $n = 7$), there were significantly more AF-OVA$^+$MHC class II^{high}CD86^{high} cells in the lymph nodes of *Lilrb4*$^{-/-}$ mice (Fig. 6*C*).

To determine whether the greater number of mature DCs bearing greater amounts of Ag in the lymph nodes of *Lilrb4*$^{-/-}$ mice affected the magnitude of the Th2 response in the lymph nodes, mice were sensitized daily with OVA-LPS three times, challenged on day 14 with unlabeled OVA, and the frequency of IL-4–producing cells in the lymph nodes was determined with an ELISPOT assay on day 15. In accordance with the greater number of mature, OVA-bearing DCs in the LNs of *Lilrb4*$^{-/-}$ mice, there was a significantly greater number of IL-4–producing cells in the LNs of these mice compared with *Lilrb4*$^{+/+}$ animals (Fig. 6*D*).

Effects of the absence of LIRB4 on DCs on Ag-induced Th2 pulmonary inflammation

Because LILRB4 is expressed on cell populations other than DCs that could influence the progression of pulmonary inflammation, we sought to determine whether the absence of LILRB4 on DCs alone is sufficient to cause exacerbated pulmonary inflammation in the OVA-LPS model. It has been reported that BMDCs pulsed with OVA or OVA-LPS transfer Ag sensitization to naive mice (33, 34), that BMDCs express LILRB4, and that the absence of this receptor on these culture-derived cells results in greater activation of T cells in vitro (25). We therefore cultured bone marrow cells from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice in GM-CSF to generate BMDCs (27). After 9 d of culture, the cells were cultured with medium alone or were pulsed with 100 μg/ml OVA and 100 ng/ml LPS for 18 h. As assessed by flow cytometry, *Lilrb4*$^{+/+}$ but not *Lilrb4*$^{-/-}$ CD11c$^+$ BMDCs expressed LILRB4 before and after Ag pulse (data not shown). BMDCs from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice were instilled intranasally (1×10^5 per mouse) into separate groups of *Lilrb4*$^{+/+}$ recipients. Thus, the sensitization step in these *Lilrb4*$^{+/+}$ mice was replaced with adoptive transfer of *Lilrb4*$^{+/+}$ or *Lilrb4*$^{-/-}$ DCs pulsed with OVA-LPS, and in mice that received *Lilrb4*$^{-/-}$ cells, only the OVA-LPS-pulsed DCs lacked expression of LILRB4. On days 14, 15, 18, and 19, mice were challenged with 25 μg OVA intranasally. As determined by analysis of BAL on day 21, *Lilrb4*$^{+/+}$ mice that had been sensitized with *Lilrb4*$^{-/-}$ DCs had significantly greater numbers of total leukocytes, eosinophils, and lymphocytes in their BAL compared with mice that received *Lilrb4*$^{+/+}$ DCs ($p = 0.01$, 0.002, and 0.03, respectively), but not neutrophils or macrophages (Fig. 7). In addition, there was more infiltration of inflammatory cells around the bronchovascular bundles in the lungs of *Lilrb4*$^{+/+}$ mice that had received *Lilrb4*$^{-/-}$ DCs compared with mice that had received *Lilrb4*$^{+/+}$ cells (Fig. 8*A*), and the percent of tissue that was inflamed was significantly greater ($p = 0.001$) as determined by quantitative digital image analysis of tissue sections stained with hematoxylin and eosin (Fig. 8*B*). Tissue eosinophilia in the infiltrates was markedly more pronounced in animals that received *Lilrb4*$^{-/-}$ DCs, as revealed in tissue sections stained with Congo Red (Fig. 8*C*); the extent of eosinophilia was so great in recipients of *Lilrb4*$^{-/-}$ DCs that it was not possible to quantify the cell numbers. Goblet cell metaplasia (Fig. 8*D*), as quantified by the number of goblet cells per length of bronchial epithelium stained with period acid-Schiff, was also significantly greater in recipients of *Lilrb4*$^{-/-}$ DCs compared with *Lilrb4*$^{+/+}$ DCs (Fig. 8*E*; $p = 0.03$). Recipients of

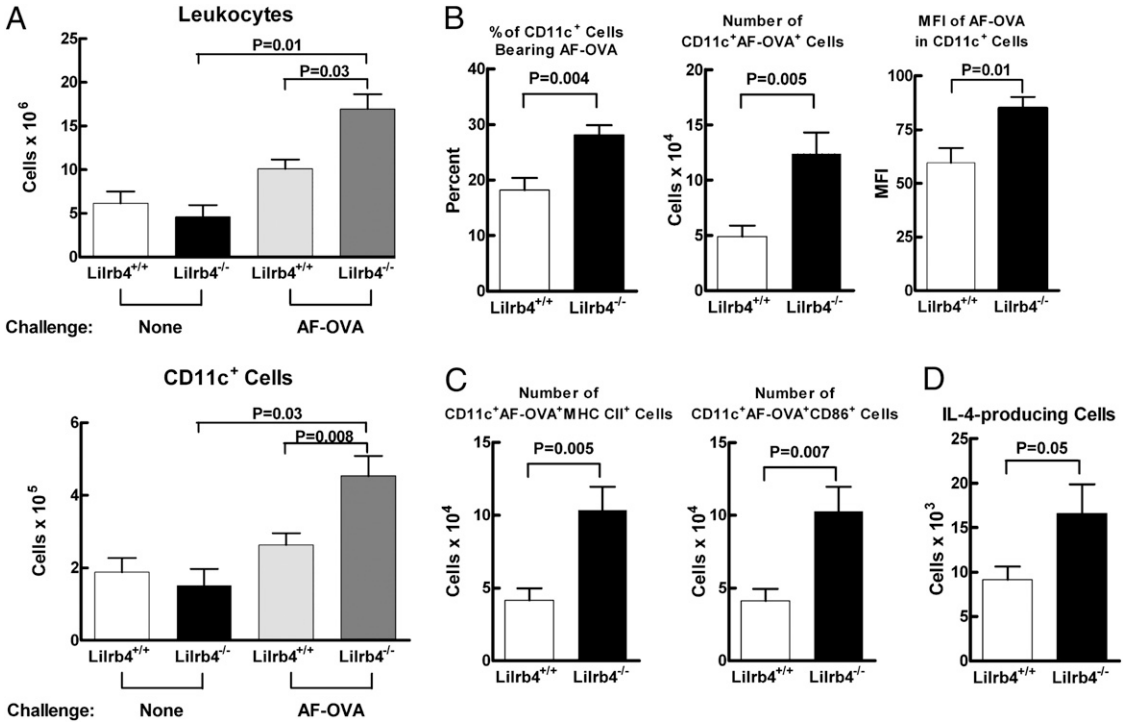

FIGURE 6. Intrathoracic lymph node cell DCs and IL-4 production after a single challenge of *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice. Mice were sensitized as described in the legend for Fig. 1. On day 14, intrathoracic lymph node cells were isolated from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice as described in the Fig. 5 legend, and the numbers of leukocytes (*A*, *upper panel*) and CD11c^{+} cells (*A*, *lower panel*) were determined by cell counting and flow cytometry. Another group of sensitized *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice received 25 μg AF-OVA intranasally on day 14, and lymph nodes cells were analyzed on day 15 as on day 14 with the addition of measurements of the percent, number, and MFI of AF-OVA^{+} cells (*B*) and the numbers of MHC class II^{+} and CD86^{+} cells (*C*) by flow cytometry. Lymph node cells were isolated on day 15 from an additional group of sensitized *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice that had been challenged with 25 μg unlabeled OVA on day 14, and the number of IL-4–producing cells was determined with an ELISPOT assay (*D*). Data are expressed mean ± SEM; n = 2, 2, 9, and 9 for the four columns from left to right (*A*); n = 7 (*B*, *C*); n = 8 (*D*).

Lilrb4$^{-/-}$ DCs also had 1.7-fold higher levels of serum OVA-specific IgE than mice that received *Lilrb4*$^{+/+}$ DCs (n = 10), an increment identical to that of *Lilrb4*$^{-/-}$ versus *Lilrb4*$^{+/+}$ OVA-LPS/OVA mice (Fig. 3). The combined data indicate that the absence of LILRB4 on DCs was sufficient to confer exacerbated Th2 pulmonary responses in this model.

Discussion

We have established that LILRB4 counterregulates the development of a Th2-polarized adaptive immune response and the ensuing allergic pulmonary inflammation induced by Ag sensitization in the presence of a relatively low concentration of LPS followed by Ag challenge in the airway. Moreover, we have found that LILRB4 is expressed on mature, Ag-bearing DCs in lung-draining intrathoracic lymph nodes during sensitization to Ag, and that *Lilrb4*$^{-/-}$ mice have a greater number of these DCs and IL-4–producing lymphocytes in their lymph nodes after a single challenge with Ag that initiates the pulmonary pathology that ensues after additional exposure to Ag. The absence of LILRB4 on DCs alone was sufficient to induce the exacerbated eosinophilic inflammation in this model, demonstrating that LILRB4 on DCs can contribute significantly to the counterregulatory process afforded by this ITIM-bearing receptor. Therefore, LILRB4 dampens not only IgE-induced mast cell activation that elicits immediate hypersensitivity reactions (1) and neutrophil-dependent pathologic inflammation induced by LPS (3) and the combination of IgG immune complexes and LPS (4, 5), but also plays an important role in regulating the amplification of an innate immune signal from DCs to the adaptive immune response of T cells.

Our studies confirmed that this model induces Th2 pulmonary inflammation in BALB/c wild-type mice as defined by the preferential infiltration of eosinophils rather than neutrophils into the lung as detected in BAL (Fig. 1). Moreover, we found that *Lilrb4*$^{-/-}$ mice had significantly greater numbers of both eosinophils and lymphocytes in the BAL compared with *Lilrb4*$^{+/+}$ mice (Fig. 1), demonstrating that the absence of LILRB4 can intensify the development of an immunization-induced Th2 response. Accordingly, the number of interstitial mononuclear cells isolated from the lungs of *Lilrb4*$^{-/-}$ mice was significantly greater than from *Lilrb4*$^{+/+}$ mice and consisted of significantly greater numbers of CD4^{+} and CD8^{+} T cells, as well as CD19^{+} B cells (Fig. 2). Hence, LILRB4 in wild-type mice suppressed the induced allergic pulmonary inflammation without altering its hallmark Th2 characteristics.

The inhibitory influence of LILRB4 on the development of the Th2-polarized adaptive immune response was revealed by the presence of significantly augmented levels of OVA-specific IgE in the serum of *Lilrb4*$^{-/-}$ mice compared with *Lilrb4*$^{+/+}$ mice (Fig. 3). We have previously reported that when *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice are immunized by i.p. injection of OVA adsorbed to alum, there are no differences in the levels of OVA-specific Igs, including IgE (1). Because Th2-polarized immune responses initiated by immunization with OVA/alum can develop in the absence of TLR4 (17) and MyD88 (22), our ability to detect more Ag-specific IgE in the sera of *Lilrb4*$^{-/-}$ mice sensitized with OVA-LPS in the absence of alum may reflect the TLR4-dependence of the response and its counterregulation by LILRB4 in vivo. Additional evidence that LILRB4 counterregulated the adaptive Th2 response to inhaled OVA-LPS is that upon rechallenge of intrathoracic lymph node cells with Ag, cells from *Lilrb4*$^{-/-}$ mice produced significantly more IL-4, IL-5, and IL-13, but not more IFN-γ, compared with *Lilrb4*$^{+/+}$ mice (Fig. 4). These findings indicate that LILRB4

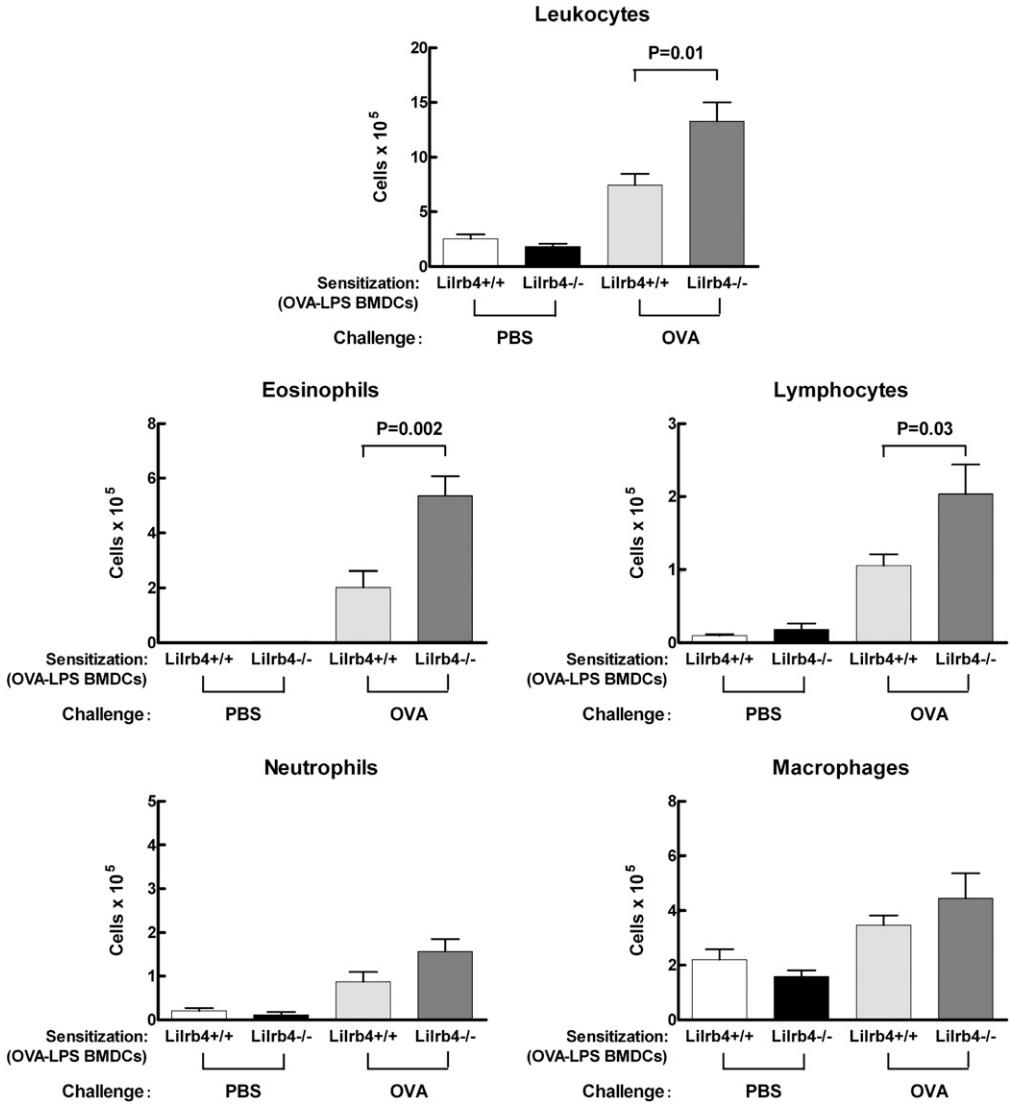

FIGURE 7. Effects of transferred *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ BMDCs on BAL cells in *Lilrb4*$^{+/+}$ mice. BMDCs from *Lilrb4*$^{+/+}$ or *Lilrb4*$^{-/-}$ mice were pulsed for 18 h with 100 μg/ml OVA and 100 ng/ml LPS, and 1×10^5 cells in 50 μl PBS were instilled intranasally into *Lilrb4*$^{+/+}$ mice. On days 14, 15, 18, and 19, mice were instilled intranasally with 30 μl PBS alone or containing 25 μg OVA, and the mice were euthanized on day 21. The numbers of the different cell populations in BAL fluid was determined as described in the Fig.1 legend.

counterregulates the development of a pathologic adaptive immune response initiated by an innate immune signal that renders an otherwise harmless and tolerizing molecule immunogenic when inhaled (17, 35).

A potential contribution for LILRB4 on DCs in this model was appreciated through the finding that a single intranasal instillation of OVA-LPS induced a substantial increase in the number of DCs expressing this molecule in the lung-draining intrathoracic lymph nodes of *Lilrb4*$^{+/+}$ mice 1 d later (Fig. 5). Moreover, there was a striking preferential expression of LILRB4 on OVA-bearing DCs that expressed high levels of MHC class II and CD86. The vast majority of OVA-bearing DCs in lung-draining lymph nodes 1 d after intranasal instillation of OVA are derived from the airway mucosa, and the number of these cells increases when LPS is administered with OVA, as does their expression of CD86 and ability to present Ag to T cells in vivo (23). Our data reveal that functionally mature pulmonary DCs that have the appropriate localization and capacity to present Ag to T cells preferentially express LILRB4, which is consistent with a counterregulatory role for this receptor on DCs in the development of allergic lung inflammation.

Although the absence of LILRB4 did not affect the number and/or phenotype of DCs in the intrathoracic lymph nodes of mice during sensitization to OVA-LPS, we considered the possibility that the receptor might influence the response of DCs in sensitized animals to subsequent inhalation of OVA. Airway challenge of Ag-sensitized animals results in increased numbers of airway DCs and migration of the cells to the lung-draining lymph nodes, particularly in the first 24 h after challenge (36–39). Similarly, there is a rapid increase in the number of DCs in the bronchial mucosa of individuals with allergic asthma in response to an inhaled allergen challenge (40). Pulmonary DCs that respond to Ag challenge in sensitized animals contribute importantly to the development and continuation of Th2 airway inflammation and AHR. Depletion of lung DCs at the time of Ag challenge substantially reduces the number of pulmonary T cells and their ability to generate Th2 cytokines in response to Ag; it also prevents the development of eosinophilic airway inflammation, goblet cell hyperplasia, and AHR (41, 42). Because of these key contributions of pulmonary DCs to the pathologic condition induced by inhalation challenge, we compared the DCs of OVA-LPS-sensitized *Lilrb4*$^{+/+}$ and

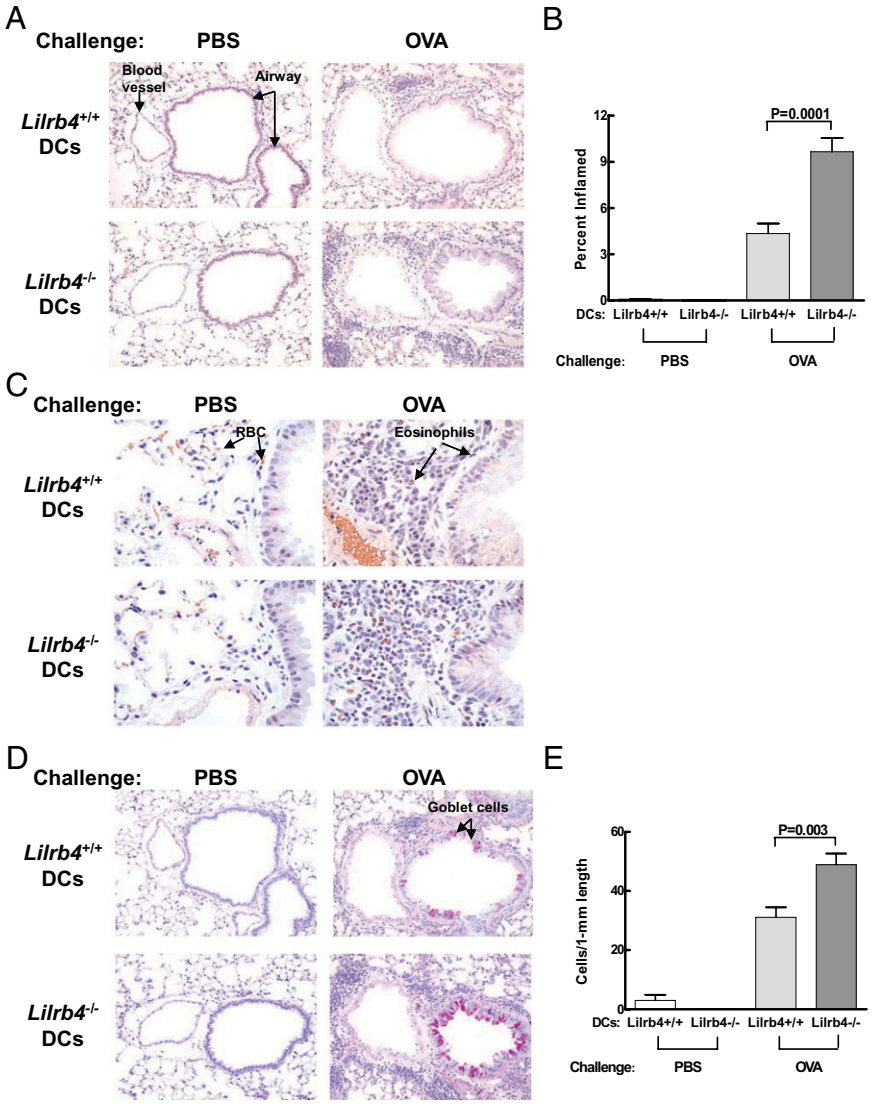

FIGURE 8. Effects of transferred *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ BMDCs on lung inflammation and goblet cell hyperplasia in *Lilrb4*$^{+/+}$ mice. Lungs from mice treated as described in the Fig. 7 legend were processed for histologic analysis, and tissue sections were stained with chloroacetate esterase to quantify inflammation (*A*, *B*), Congo Red to demonstrate tissue eosinophilia (*C*), and periodic acid-Schiff stain (*D*, *E*) to quantify goblet cells. Quantitative data in *B* and *E* are expressed as mean ± SEM; n = 5, 5, 10, and 10 for the four columns from left to right, respectively.

Lilrb4$^{-/-}$ mice 1 d after a single challenge with OVA. We found that a significantly greater percentage and number of OVA-bearing DCs expressing high levels of MHC class II and CD86 appeared in the intrathoracic lymph nodes of *Lilrb4*$^{-/-}$ mice compared with *Lilrb4*$^{+/+}$ mice (Fig. 6). In addition, the amount of OVA in the lymph node DCs of *Lilrb4*$^{-/-}$ mice was greater, which is notable because upregulation of Ag uptake by airway DCs is associated with the progression of allergic airway inflammation (43). We also found that the lymph nodes of challenged *Lilrb4*$^{-/-}$ mice had significantly more IL-4–producing cells (Fig. 6), which is consistent with the greater number of Ag-bearing mature DCs available to present Ag to memory T cells traversing the lymph nodes after the challenge. Overall, our findings in the lymph nodes of *Lilrb4*$^{-/-}$ mice are thus consonant with the exacerbated eosinophilic (Figs. 1, 2), Th2 polarized (Figs. 3, 4) inflammation that ensues with continued Ag challenge in these mice.

To address whether the expression of LILRB4 on DCs is sufficient to regulate pathologic Th2 responses, we pulsed BMDCs generated from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice with OVA in the presence of LPS. *Lilrb4*$^{+/+}$ mice were then sensitized by intranasal transfer of the DCs and subsequently challenged by intranasal instillation of OVA. In this adoptive transfer system, *Lilrb4*$^{+/+}$ mice that received *Lilrb4*$^{-/-}$ DCs lacked LILRB4 only on those DCs that would present Ag to T cells in the recipients. The transfer of *Lilrb4*$^{+/+}$ DCs led to a robust Th2 pulmonary response to Ag challenge, as measured by influx of eosinophils and lymphocytes into BAL (Fig. 7), as well as eosinophilic inflammation near bronchovascular bundles and goblet cell metaplasia in the airway epithelium (Fig. 8). All of these hallmark characteristics of allergic pulmonary inflammation were significantly greater in *Lilrb4*$^{+/+}$ mice that had received *Lilrb4*$^{-/-}$ Ag-pulsed DCs (Figs. 7, 8), demonstrating that the absence of LILRB4 on DCs alone was sufficient to induce the exacerbated response. It was recently reported that LILRB4 on mouse DCs can counterregulate their ability to activate T cells in vitro, and that the allogeneic graft-versus-host reaction is more severe in *Lilrb4*$^{-/-}$ recipients, in parallel with greater expression of CD86 on splenic DCs from *Lilrb4*$^{-/-}$ mice (25). Our in vivo DC transfer system directly establishes that LILRB4 on DCs can contribute importantly to negative regulation of a pathologic allergic airway response that is dependent on the generation of a Th2-polarized adaptive immune response (17).

The ability to discern an effect of LILRB4 on the adaptive immune response in models of allergic pulmonary inflammation may depend on the nature of the model and/or genetic contributions. For example, when *Lilrb4*$^{-/-}$ mice (on the C57BL/6 background) were sensitized by i.p. injection of ragweed extract and alum followed by intratracheal and intranasal challenges with ragweed, there were no significant differences between *Lilrb4*$^{+/+}$

and *Lilrb4*$^{-/-}$ mice in the levels of serum IgE and IgG1, the amounts of IL-5 or IL-13 secreted from Ag-restimulated splenocytes, and lung inflammation as assessed histologically, although the levels of IL-4 and IL-13 in the BAL were significantly greater in *Lilrb4*$^{-/-}$ mice (44). The inability to detect a difference in cytokine production by Ag-restimulated lymphocytes from *Lilrb4*$^{+/+}$ and *Lilrb4*$^{-/-}$ mice may have reflected the use of splenocytes rather than lymphocytes from the lung-draining intrathoracic lymph nodes, inasmuch as we detected significantly greater secretion of Th2 cytokines by Ag-rechallenged lymph node cells of *Lilrb4*$^{-/-}$ mice in the OVA-LPS model (Fig. 4). In addition, our ability to detect a contribution of LILRB4 to the negative regulation of lymphocyte Th2 cytokine production as well as serum IgE levels (Fig. 3) may reflect the fact that in our model, both the sensitizations and challenges were performed in the airway, without the use of i.p. injection of alum, which substantially alters the mechanism by which DCs initiate Th2 immune responses (45).

The ability of LILRB4 to negatively regulate DC biology is not limited to mouse cells and models. A human LILRB4 homolog is expressed on DCs (46, 47), is upregulated on populations that have been induced to exert negative regulatory functions on effector T cell activation, and contributes to the inhibitory process directly or via regulatory T cells (48–51). Human LILRB4 fosters allograft acceptance in a humanized mouse model (52), a finding consistent with the exacerbated rejection of allografts in *Lilrb4*$^{-/-}$ mice (25). It was recently reported that the extracellular domains of mouse and human LILRB4 bind collagen (53), thus identifying another similarity between the human and mouse receptors. Furthermore, LPS upregulates LILRB4 expression on human DCs (54, 55) suggesting that, as we have found in the mouse, this innate immune signal induces a counterregulatory response via increased expression of LILRB4.

In summary, we have established that the immune response to inhaled Ag includes an increase in mature, Ag-bearing DCs expressing LILRB4 in lung-draining lymph nodes. In the absence of LILRB4, there are increased numbers of these cells and cells producing the key Th2 polarizing cytokine IL-4. Concomitantly, a significant exacerbation of the pathologic adaptive immune response and ensuing pulmonary inflammation occur in *Lilrb4*$^{-/-}$ mice, an effect that can be reiterated when LILRB4 is absent only on DCs. Given the fundamental roles that DCs play in the initiation and perpetuation of allergic airway disease, and the effects of LPS in modulating responses to inhaled Ag in both mice and humans, our findings establish that LILRB4 on DCs can play an important role in counterregulating those processes that are key determinants in the development of allergic airway disease.

Disclosures

The authors have no financial conflicts of interest.

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