

Fc γ receptor-mediated antigen uptake by lung DC contributes to allergic airway hyper-responsiveness and inflammation

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During asthma, lung DC capture and process antigens to initiate and maintain allergic Th2 cell responses to inhaled allergens. The aim of the study was to investigate whether allergen-specific IgG, generated during sensitization, can potentiate the acute airway inflammation through Fc γ receptor (Fc γ R)-mediated antigen uptake and enhance antigen presentation resulting in augmented T-cell proliferation. We examined the impact of antigen presentation and T-cell stimulation on allergic airway hyperresponsiveness and inflammation using transgenic and gene-deficient mice. Both airway inflammation and eosinophilia in bronchoalveolar lavage fluid were markedly reduced in sensitized and challenged Fc γ R-deficient mice. Lung DC of WT, but not Fc γ R-deficient mice, induced increased antigen-specific CD4⁺ T-cell proliferation when pulsed with anti-OVA IgG immune complexes. Intranasal application of anti-OVA IgG immune complexes resulted in enhanced airway inflammation, eosinophilia and Th2 cytokine release, mediated through enhanced antigen-specific T-cell proliferation *in vivo*. Finally, antigen-specific IgG in the serum of sensitized mice led to a significant increase of antigen-specific CD4⁺ T-cell proliferation induced by WT, but not Fc γ R-deficient, lung DC. We conclude that Fc γ R-mediated enhanced antigen presentation and T-cell stimulation by lung DC has a significant impact on inflammatory responses following allergen challenge in asthma.

Key words: Antigen presentation/processing • Asthma • DC • Fc γ receptor • Inflammation

Introduction

Asthma is a chronic inflammatory disease of the lungs characterized by recurrent episodes of increased airway inflammation, enhanced mucus production and constriction of the airways [1]. Studies of asthma using animal models have shown that Th2 cells

play a predominant role in disease pathogenesis. Th2 cytokines produced by activated CD4⁺ T cells, such as IL4, IL-5 and IL-13, exacerbate the severity of the disease [2–4]. DC, comprised of phenotypically and functionally distinct subsets [5, 6], are generally held responsible for initiating and maintaining allergic Th2-responses to inhaled allergens in asthma [7]. Forming a network in the upper layers of the epithelium and lamina propria of the airways, DC remain in an immature state that is specialized for internalizing foreign antigens. Upon antigen internalization and recognition, DC mature, migrate to the draining LN, process

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and load the antigen into the MHC, and present these MHC–peptide complexes to initiate a polarized T-lymphocyte response. In mice, at least five conventional CD11c^{high} DC populations are consistently found in lymphoid tissue. The spleen contains three of these: CD8⁺CD4[−], CD8[−]CD4⁺ and CD8[−]CD4[−] DC. LN contain two additional subsets that are absent in the spleen: CD4[−]CD8[−]CD11b⁺ DC, thought to have immigrated from the interstitial tissue, and CD205⁺Langerin⁺ Langerhans cells, only found in skin draining LN.

Antigen presentation and IC-mediated maturation of DC is regulated by IgG Fc receptors (FcγR). In the mouse, there are three classes of activating FcγR: the high affinity receptor FcγRI, the low affinity receptor FcγRII and the recently described FcγRIV [8]. These FcγR form hetero-oligomeric complexes with the same FcR γ-chain, which contains an ITAM sequence required for cell activation and cell surface expression. Most FcγR-triggered responses are balanced by the signaling of inhibitory ITIM-bearing FcγRII. Via the simultaneous triggering of activating and inhibitory signaling pathways, FcγR control a wide array of cellular responses, including phagocytosis, antibody-dependent cell-mediated cytotoxicity and the release of inflammatory mediators, which ultimately lead to the amplification of normal and pathological immune reactions *in vivo* [8–11].

FcγR are expressed on many inflammatory cell types involved in allergic airway inflammation. It is, therefore, likely that FcγR, as well as polymorphisms in genes encoding FcγR, play a pivotal role in allergic airway disease [12]. Allergen-specific IgG is present in the serum of allergic individuals and sensitized mice [13], and a specific role has been postulated for FcγRIII signaling in the regulation of optimal Th2-cell differentiation in allergy. This augmented Th2 differentiation was found to be independent of FcR-mediated antigen uptake and processing [14]. Others [13] suggested that expression of FcγRI is important during the sensitization phase of the development of allergic airway inflammation and airway hyperresponsiveness. In our study, we used a mouse model of experimental asthma to verify the impact of FcγR on antigen uptake and presentation by DC. We hypothesized that activating FcγR control the strength and characteristics of airway hyperresponsiveness and inflammation, and sought to demonstrate that IC can potentiate acute airway inflammation after sensitization, mediated by augmented T-cell proliferation after challenge.

Results

Altered airway inflammation in FcγR-deficient mice

To compare the inflammatory response to inhaled antigens in B6 and FcγR-deficient mice, the animals were sensitized with OVA + alum as described and challenged with OVA by inhalation. On day 3 after challenge, B6 mice revealed the characteristic perivascular and peribronchiolar infiltrate of mononuclear cells, whereas the allergic airway inflammation was reduced in FcγR-

deficient mice (Fig. 1A). We observed significant eosinophilia in the BALF of B6 mice, which was virtually absent in FcγR-deficient (Fig. 1B). Control experiments of sensitized but non-challenged mice confirmed absence of eosinophilia and neutrophils in the BALF of all mice (data not shown). Both B6 as well as FcγR-deficient mice mounted a strong OVA-specific IgE response after sensitization, which resulted in equivalent mean OVA-specific IgE levels in both groups. No OVA-specific IgE responses were detectable in non-sensitized mice (data not shown).

In order to confirm the constitutive FcγR expression on murine splenic and lung DC, cells from B6 mice were enriched by density gradient centrifugation and cell sorting of CD11c⁺MHC class II⁺ cells (Fig. 2A). Cells obtained by this procedure have previously been confirmed as DC by their morphology and capacity to stimulate naïve T cells [15] and contain the recently described CD103⁺CD11b[−] and CD103[−]CD11b⁺ subpopulations (Fig. 2). Both surface expression measurement as well as real-time analysis of lung-derived CD11c⁺MHC class II⁺ DC confirmed the presence of various FcγR and revealed high RNA-levels of FcγRII and readily detectable mRNA of FcγRI and FcγRIII (Fig. 2). Similarly, all splenic DC subpopulations showed expression of the FcγR tested, with CD4[−]CD8[−] DC having slightly lower expression of FcγRI (Fig. 2A). Pulmonary macrophages expressed all tested FcγR on their surface (Fig. 2E).

Impact of IgG-IC on MHC class II antigen presentation by spleen-derived and lung DC

We hypothesized that increased antigen uptake by DC through FcγR could potentially lead to increased MHC class II-mediated T-cell proliferation, thereby facilitating allergic airway inflammation. To compare whether OVA and anti-OVA IgG immune complexes (OVA-IC) influences antigen presentation by DC subsets *in vitro*, OVA and anti-OVA IgG were mixed at increasing ratios (from 4:1 to 1:4) and IC-formation confirmed using gel electrophoresis and mass spectrometry (data not shown). A ratio of 1:4 (OVA:anti-OVA IgG) led to readily detectable OVA-IC. Hence, we used 25 µg/mL OVA or the same amount of OVA in immune-complexed form (OVA:anti-OVA IgG, 1:4) to pulse sorted spleen-derived DC subsets. The cells were then co-cultured with CFSE-labeled OT-II cells and antigen presentation was assessed by measuring T-cell proliferation, visualized as a progressive dilution of the CFSE fluorescent marker. CD4⁺CD8[−] DC and CD8[−]CD4[−] DC, but not CD8⁺CD4[−] DC, led to significantly increased T-cell proliferation when pulsed with OVA-IC, as compared to OVA alone. This effect was completely abrogated when DC deficient for FcR γ-chain were used indicating the specificity of this effect (Fig. 3A and B). Similarly, experiments using low-endotoxin OVA (EndoGrade™ OVA) in combination with anti-OVA IgG revealed significantly augmented T-cell stimulation by splenic DC. Alternatively, splenic DC from TLR4-deficient mice likewise led to a highly significant increase in T-cell proliferation when pulsed with OVA-IC as compared to OVA alone, suggesting no considerable contribution of LPS-contamination of OVA (data not shown). In order to better define the relevance of our observation

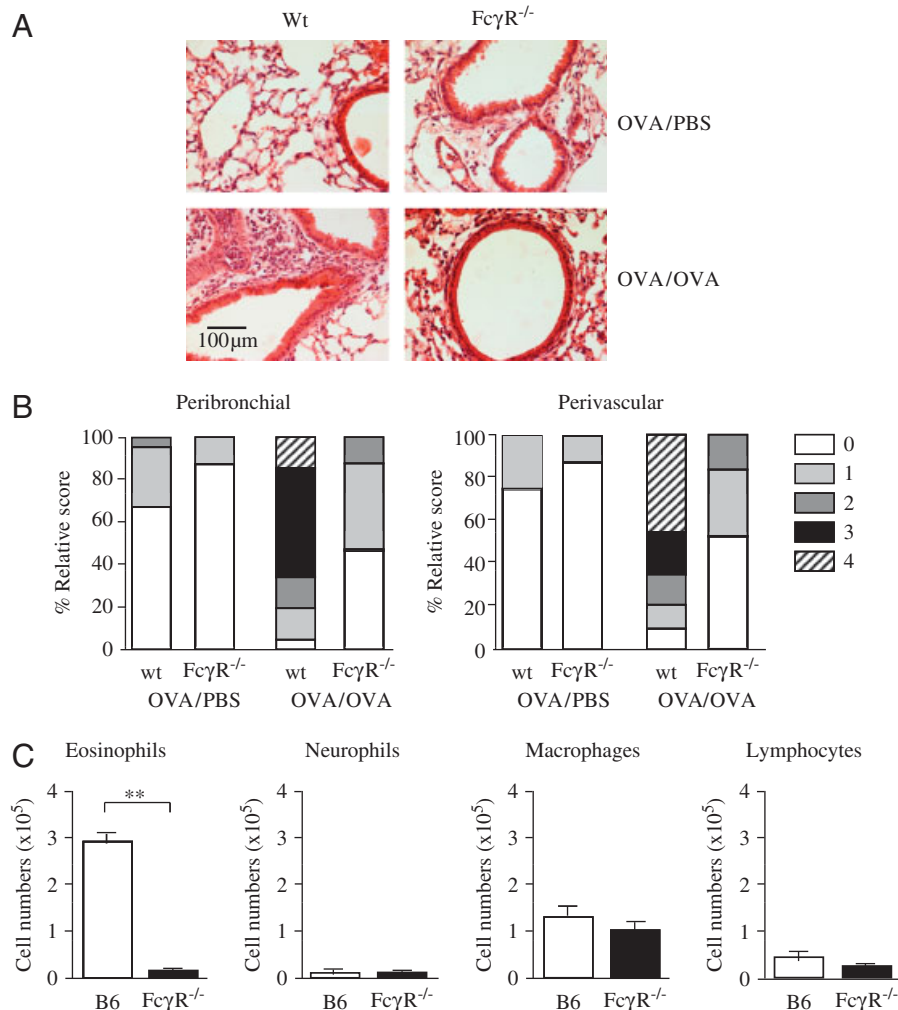


Figure 1. Lung histology and characterization of BALF obtained from FcγR-deficient and B6 mice. (A) Histological examinations of lung tissues from B6 (left panel) and FcγR^{-/-} mice (not expressing FcγRI, FcγRIII, FcγRIV and FcεRI) (right panel), sensitized with OVA and challenged with PBS (OVA/PBS, control) or OVA (OVA/OVA). Micrographs (original magnification × 10) depict lung sections stained with hematoxylin/eosin. Histology was examined using a peribronchial and perivascular inflammatory score ranging from 0 (normal) to 4 (severe inflammation) (bottom panels). (B) Eosinophil, neutrophil, macrophage and lymphocyte cell counts of BALF obtained from OVA sensitized and challenged (OVA/OVA) B6 (open bars) and FcγR^{-/-} (closed bars) mice. Data show mean ± SEM pooled from three independent experiments with 4–12 mice/group. ***p* < 0.001.

for allergic airway hyperresponsiveness, we then purified CD11c⁺MHCII⁺ DC from the lungs of the respective mice, pulsed the cells with OVA or IC and then used them to stimulate CFSE-labeled OT-II cells. Again, T-cell proliferation doubled when using lung DC from B6 mice, in a manner similar to splenic DC, but no such effect was observed when FcγR-deficient lung DC were used (Fig. 3A). Exposure of sorted CD11c⁺MHCII⁺ lung DC to OVA-IC led to IL-6 and TNF-α secretion (Fig. 3C) and up-regulation of the co-stimulatory molecule CD86 on BM-derived DC (BMDC) (Fig. 3D).

Enhanced airway inflammation and eosinophilia through IgG-IC

In order to better assess the role of OVA-IC on T-cell proliferation in allergic airway inflammation, we made use of an adoptive

transfer model, as described earlier [16]. Th2-biased OVA-specific DO11.10 cells were transferred into BALB/c mice, and these mice were challenged i.n. with either OVA or OVA-IC. Twenty-four hours after the last challenge, the mice that had received OVA-IC not only had significantly increased total cell counts in the BALF, as compared to PBS or anti-OVA IgG treated mice, but these mice also presented with significantly increased total cell numbers, as compared to OVA challenged mice (Fig. 4A). These differences resulted mainly from increased eosinophil counts, as mice challenged with OVA-IC had more than three times higher eosinophil counts in the BALF, as compared to OVA challenged mice. Eosinophilia was negligible in PBS and anti-OVA IgG-treated mice (Fig. 4B). Importantly, control animals not receiving Th2-biased DO11.10 cells but challenged three times with OVA-IC showed no peribronchial/perivascular inflammation and their BALF of was devoid of eosinophils (data not shown), suggesting that no other FcγR-expressing inflammatory cells

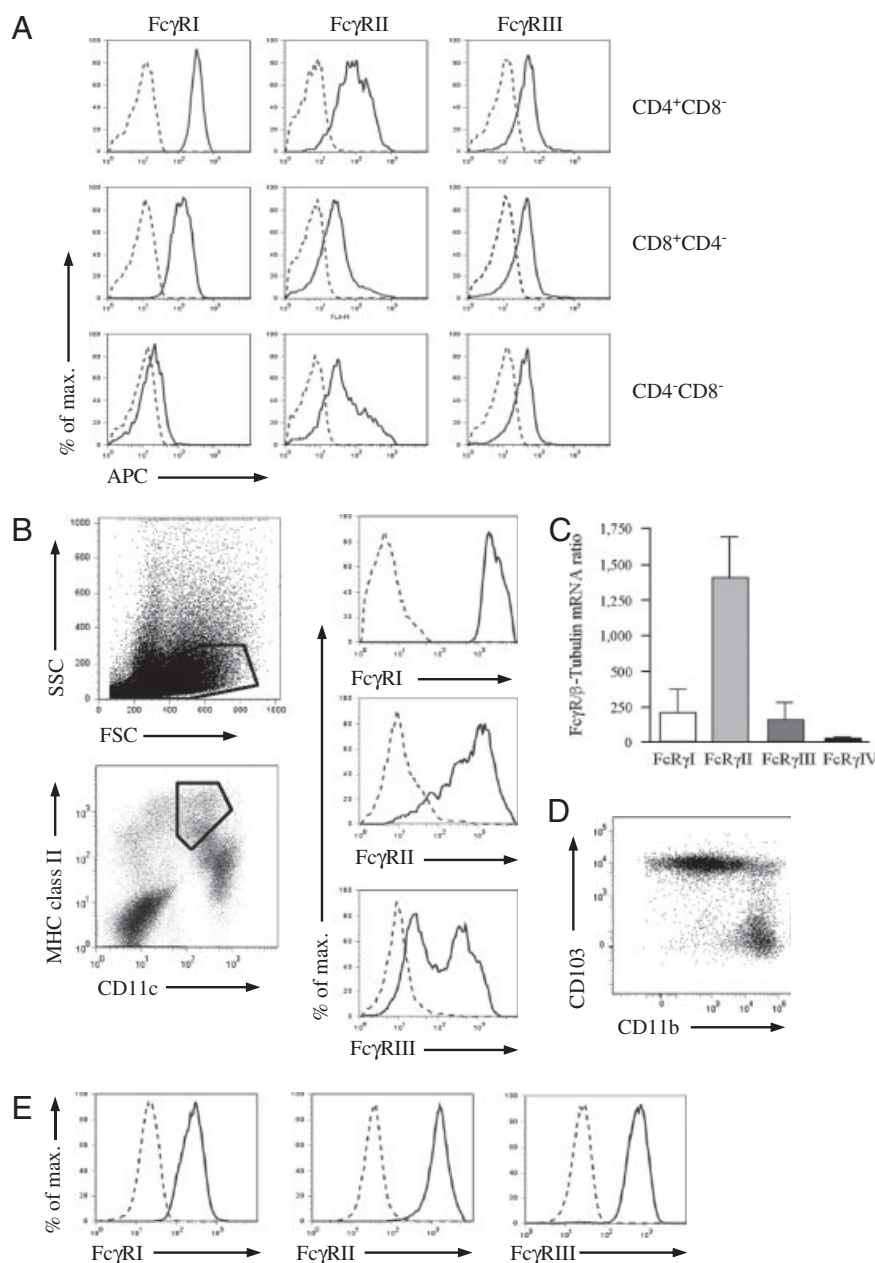


Figure 2. Phenotyping, cell sorting and analysis of Fc γ R expression on lung DC. (A) Surface expression of Fc γ R-I-III (black line) and isotype control antibody (dotted line) on splenic DC subpopulations as indicated. (B) Surface expression of Fc γ R-I-III (black line) or isotype control antibody (dotted line) on lung DC. (C) Total RNA was isolated from these DC and analyzed by quantitative Taqman PCR. The gene expression for each gene is indicated relative to β -tubulin control. Data show mean+SEM pooled from three independent experiments. (D) Lung DC (as gated in B on CD11c⁺ MHC class II^{high} cells) comprise at least two phenotypically distinct lung DC populations (CD11c⁺ MHC class II⁺ CD11b⁺ CD103⁻ and CD11c⁺ MHC class II⁺ CD11b⁻ CD103⁺). (E) Surface expression of Fc γ R-I-III (black line) and isotype control antibody (dotted lines) on pulmonary macrophages.

independently (e.g. macrophages) caused eosinophilia and inflammation.

In line with the cellular data, lung function confirmed the severe airway hypersensitivity reaction in mice treated with OVA (Fig. 4C). Because provocation was terminated for ethical reasons once the animals had reached an ED₂₀₀RL, the lung function did not quantify a further impairment when mice were challenged with OVA-IC. However, the mice treated with OVA-IC

revealed a markedly augmented perivascular and peribronchiolar infiltrate of mononuclear cells, thereby providing evidence for more severe pulmonary inflammation (Fig. 4D–F). Taken together, these data suggest that allergen-specific IgG-IC can contribute to enhanced eosinophilia, increased airway inflammation and resulting airway hyperresponsiveness when administered i.n. in a Th2 T-cell-dependent murine asthma model.

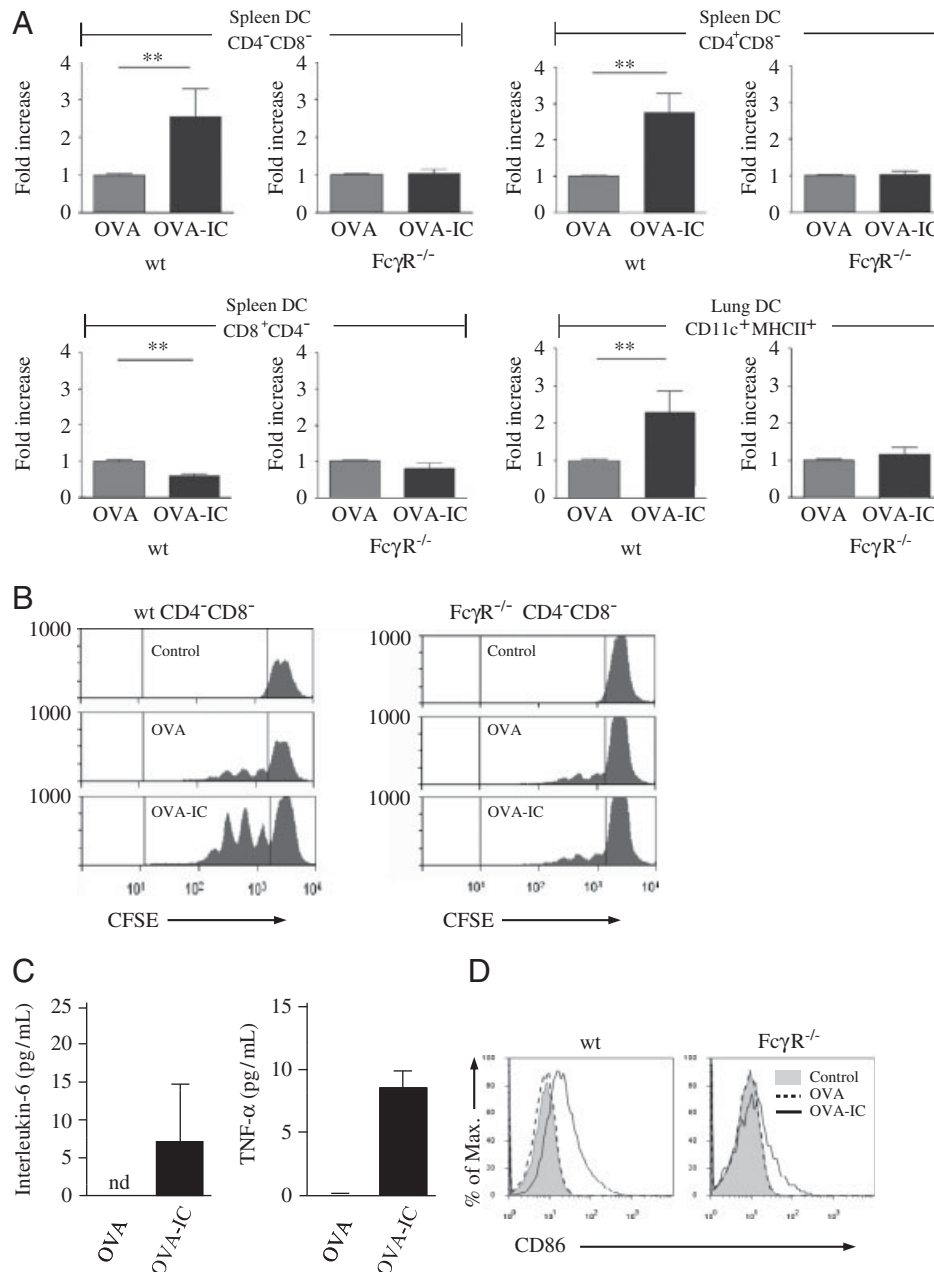


Figure 3. Antigen presentation and T-cell stimulation of anti-OVA IgG-IC by spleen- and lung-derived DC. (A) Spleen-derived DC subpopulations and lung DC as noted (1×10^4) were purified from B6 or FcγR^{-/-} mice (>95% pure) and pulsed with OVA (25 μg/mL) or OVA-IC (1:4) containing the same amount of OVA. Cells were washed and used to stimulate 5×10^4 CFSE labeled OT-II cells. Proliferation of OT-II cells after DC treatment with OVA-IC (black bars) was quantified in duplicate by measuring the numbers of CD4⁺Vα2⁺CFSE^{low} cells after 60 h. Data are depicted as the fold increase above proliferation of OVA only treated DC (grey bars). Data show mean + SEM pooled from three independent experiments with duplicate measurements. (B) Representative CFSE-proliferation profile resulting from co-culture of spleen CD8⁺CD4⁺ DC and OT-II cells. (C) Lung DC were purified, pulsed for 45 min as indicated and cytokines in the supernatant assessed after 16 h of culture. (D) BMDC were pulsed with OVA or OVA-IC for 2 days and surface expression of CD86 determined by FACS analysis. * $p < 0.05$; ** $p < 0.001$.

Increased airway inflammation is mediated by augmented T-cell proliferation

Next, we wished to better define whether or not the increased airway inflammation was a result of enhanced antigen presentation and T-cell proliferation. Therefore, we allowed IC-formation to occur *in vivo* and examined the resulting T-cell stimulation by

DC from lung-draining LN. BALB/c mice were treated i.n. with PBS or anti-OVA IgG (anti-OVA and OVA-IC groups), followed by inhalation of 1% OVA aerosol for 20 min (OVA and OVA-IC groups) on two consecutive days. Twelve hours after the last challenge, lung-draining LN were removed, and DC were isolated and co-cultured with CFSE-labeled DO11.10. As shown in Fig. 5A and B, DC isolated from mice that had received anti-OVA IgG i.n.

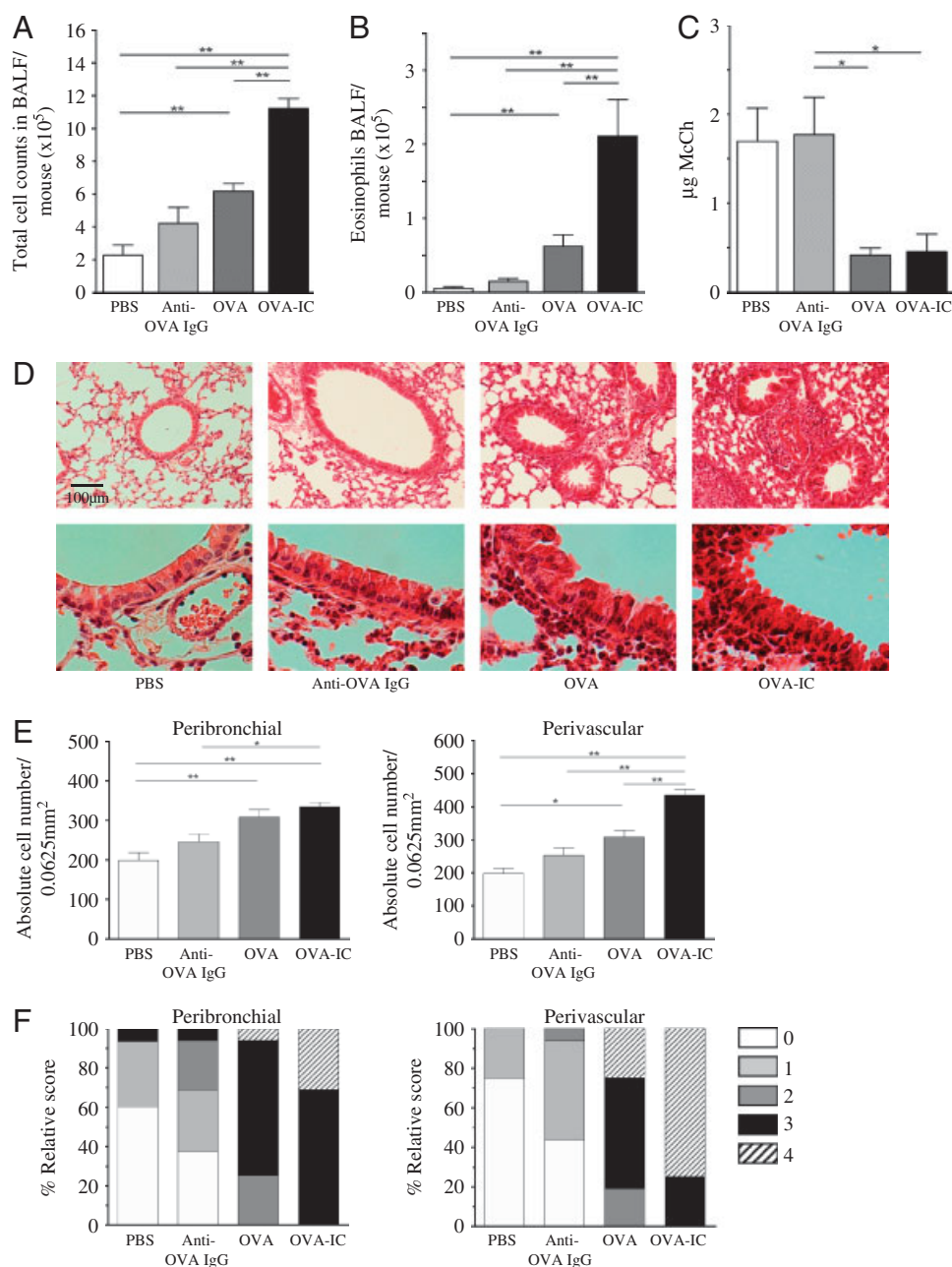


Figure 4. Enhanced airway inflammation by OVA-IC in an asthma model employing adoptive transfer of Th2-biased T lymphocytes. BALB/c mice were adoptively transferred with $3\text{--}4 \times 10^6$ *in vitro*-stimulated DO11.10 cells, followed by intranasal administration of PBS, anti-OVA, OVA (25 μ g/mL) or OVA-IC (1:4 mixture of 25 μ g OVA and anti-OVA) on three consecutive days. Twenty-four hours after the last challenge, lung function and BALF were analyzed, and lungs were fixed in formalin. Data show mean $\pm$ SEM pooled from two independent experiments. (A) Mean total cell counts/mouse in the BALF. (B) Mean eosinophil counts/mouse in BALF. (C) Effects of OVA or OVA-IC challenge on pulmonary reaction to MCh inhalation. The degree of bronchoconstriction is expressed as concentration of MCh that renders the lung resistance to approximately 200% of the basic value ($\text{ED}_{200\text{RL}}$). (D) Micrographs (magnification $\times 10$ (top row) or $\times 40$ (bottom row)) of lung sections stained with H&E. (E) Peribronchial and perivascular cell counts of inflammatory cells and (F) inflammatory score ranging from 0 (normal) to 4 (severe inflammation). * $p < 0.05$; ** $p < 0.001$.

followed by inhalative challenge with OVA led to a highly significant and at least 100% increase in antigen-specific T-cell stimulation, as compared to DC from mice that were challenged with OVA alone. These data suggest that allergen-specific IgG-IC formation *in vivo* following allergen inhalation can result in enhanced T-cell proliferation induced by DC in lung-draining LN.

Serum IgG of sensitized mice enhances the allergen-specific T-cell proliferation induced by lung DC

After considering the above data, we reasoned IC-formation occurs in the lungs during allergen exposure, thereby affecting lung DC-mediated T-cell stimulation. Thus, we aimed to more closely replicate the *in vivo* situation of antigen presentation

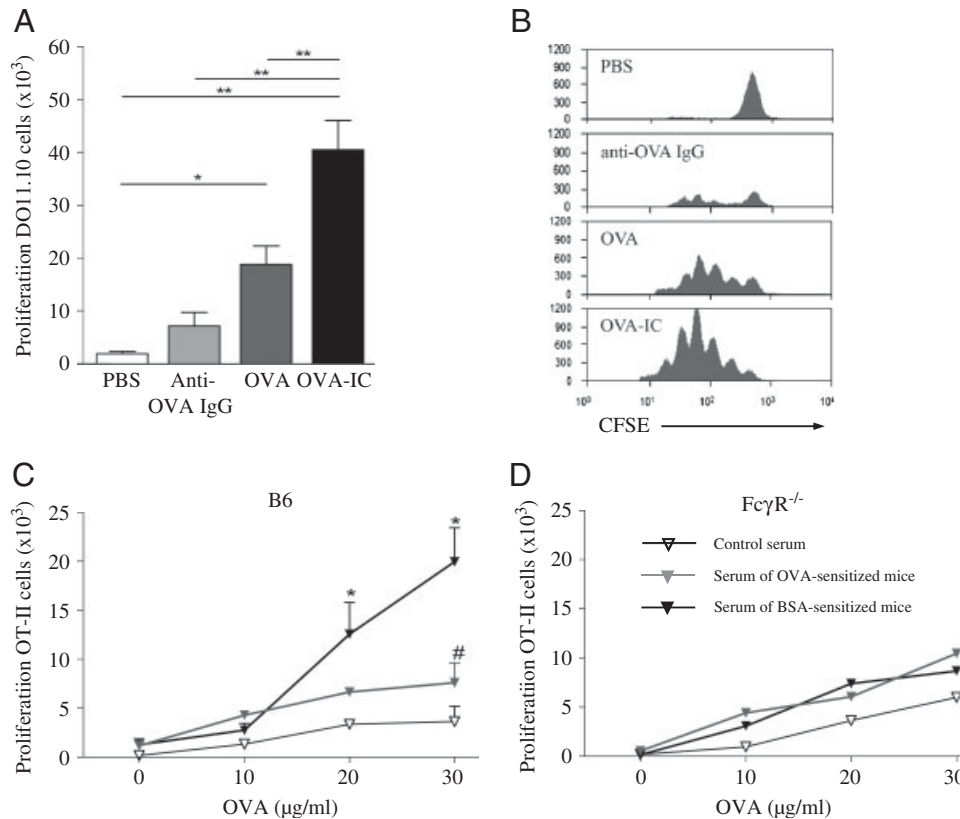


Figure 5. Enhanced airway inflammation by OVA-IC is mediated by augmented T-cell proliferation. (A) BALB/c mice were treated i.n. with PBS or 100 µg anti-OVA (anti-OVA and OVA-IC groups), followed by inhalation of 1% OVA aerosol (OVA and OVA-IC groups) or PBS (PBS and anti-OVA groups) for 20 min on two consecutive days. Twelve hours after the last challenge, mediastinal LN were removed, and DC isolated (>95% pure) and co-cultured with CFSE-labeled DO11.10 cells for 60 h. Proliferation assay was performed by flow cytometry and quantified by duplicate measurements of CD4⁺Vα2⁺CFSE^{low} cells. Data show mean+SEM pooled from two independent experiments with 2–6 mice/group. **p*<0.05; ***p*<0.001. (B) Representative T-cell proliferation in PBS, anti-OVA, OVA or OVA-IC treated mice. CD11c⁺ MHC class II⁺ cells were isolated from lungs of (C) B6 or (D) FcγR^{-/-} mice and incubated with graded OVA doses as indicated, or without OVA, or serum from B6 (control serum), OVA-, or BSA-sensitized B6 mice. After 1 h, DC were washed and then co-cultured with CFSE-labeled OT-II cells for 60 h. Proliferation assays were performed in duplicate by flow cytometry. Data show mean+SEM from three independent experiments. **p*<0.05 for comparison of the OVA-sensitized serum group and the control serum group; #*p*<0.05 for the comparison of the OVA-sensitized serum group and the BSA-sensitized serum group.

during allergic lung hypersensitivity. The purified lung DC obtained from B6 mice were given serum containing either anti-OVA IgG (obtained from OVA+Alum sensitized mice) or anti-BSA IgG (obtained from BSA+Alum sensitized mice) together with increasing OVA concentrations. The resulting antigen-specific T-cell stimulation was determined using CFSE-labeled OT-II cells after 60 h of culture. As depicted in Fig. 5C, serum of OVA+Alum sensitized mice yielded a significant three- to fourfold increased antigen-specific T-cell proliferation induced by lung DC, as compared to serum of BSA- or non-sensitized mice. To further prove the specificity of this observation, lung DC from FcγR-deficient mice were used as a control, revealing no increase in T-cell proliferation even at the highest OVA concentration tested and exposure to serum of OVA+Alum sensitized mice (Fig. 5D). These data strongly suggest that anti-OVA IgG-IC formation through increased DC-mediated antigen-specific T-cell proliferation is able to contribute to allergic airway hyperresponsiveness.

Discussion

Our study provides experimental evidence that allergen-specific IgG, generated during sensitization, can lead to IC formation upon antigen challenge and result in enhanced FcγR-mediated antigen presentation. This augmented antigen presentation and Th2 T-cell proliferation, possibly in concert with enhanced DC activation [17, 18], promotes the manifestation of pulmonary allergic hypersensitivity reaction during the effector phase. These findings expand significantly upon previous reports on the role of FcγR and allergen-specific IgG in allergic asthma [13, 14] in that we now show a novel mechanism and impact of FcγR during the airway challenge phase. Previous reports suggested a specific role for FcγRIII signaling in the regulation of optimal Th2 cell differentiation in allergy during sensitization, regulated by IL-10 production from the DC. Moreover, Kitamura *et al.* [13] demonstrated that expression of FcγR, most likely FcγRI, on DC is important during the sensitization phase for the development of allergic airway inflammation. Other studies

indirectly suggested that activating Fc γ R could contribute to inflammation through the activation of Syk, a downstream kinase by which Fc γ R are known to augment antigen presentation [17, 19, 20].

The reduced eosinophilia in FcR γ -chain deficient mice, which do not express Fc γ RI, Fc γ RIII, Fc γ RIV and Fc ϵ RI, corroborates a previous report [13] and could be a result of effects other than antigen presentation. Signaling via Fc γ RIII on mast cells has been demonstrated to induce the release of soluble mediators that have a role in the regulation of Th2 differentiation. In addition, reduced levels of IL-5 and eotaxin in BALF of Fc γ R^{-/-} mice [13] might account for the absence of eosinophilia. Finally, FcR γ -chain-deficient mice are devoid of Fc ϵ RI and therefore any Fc ϵ RI-mediated effects of OVA-specific IgE during the sensitization or challenge phase, either due to mast-cell activation or altered DC function, is absent in these mice. Although the sensitization/challenge model that we used does not require B cells or antibodies, including allergen-specific IgE, Fc ϵ RI, or mast cells [21, 22], it remains possible that *in vivo* Fc ϵ RI facilitated enhanced antigen-uptake or activation of pulmonary DC indirectly through mast-cell activation [23, 24].

In contrast to previous studies [13, 14, 17] that employed BMDC and sensitization of Fc γ R-deficient mice, we aimed to specifically delineate the contribution of Fc γ R on lung DC during the challenge phase of the murine asthma model. We first confirmed the expression of Fc γ R expression on lung DC and compared their function to spleen-derived DC subpopulations, as the importance of considering the phenotypic, functional and anatomical differences of various DC subsets has been supported by several studies [25]. Thus, our studies focus on DC populations obtained from lymphoid organs in addition to pulmonary DC to study the function of Fc γ R. This revealed that lung DC and splenic CD8⁻ DC gave rise to increased CD4⁺ T lymphocyte stimulation when DC acquired antigen as immune complexes via Fc γ RI, Fc γ RIII or Fc γ RIV. This effect was absent when CD8⁺ DC or FcR γ -chain deficient DC were used. These observations would be consistent with the view that contamination of OVA with endotoxins was not responsible for these alterations. Additional results support this interpretation. First, DC of TLR4-deficient mice led to increased T-cell proliferation after exposure to OVA-IC as compared to OVA alone. Second, serum of sensitized mice, which contained anti-OVA IgG, caused increased T-cell proliferation when given together with OVA to WT lung DC. This effect was antigen-specific, as serum of BSA-sensitized mice did not cause this outcome, and Fc γ R-dependent, given that FcR γ -deficient DC did not result in increased T-cell proliferation.

Several observations support the impact of Fc γ R on DC during the effector phase of pulmonary hypersensitivity. First, we adoptively transferred Th2-biased antigen-specific CD4⁺T lymphocytes [4] into antigen-naïve mice, thereby restricting the induction of pulmonary hypersensitivity mainly to the DC–T-cell interaction. Second, pulmonary exposure of mice to OVA-IC dramatically increased eosinophilia in the BALF and cellular

infiltration in the lungs, an effect that was not observed in naive mice and thus not induced non-specifically. Third, the increased pulmonary immune reaction induced by OVA-IC was paralleled by a highly significant increase in proliferation of antigen-specific T cells, both *in vitro* as well as *in vivo*. Finally, in order to overcome the possibility of artificially high antigen and antibody concentrations used in the adoptive transfer experiments, we were able to show that antigen-specific IgG in the serum of sensitized mice contributed to IC formation and significantly augmented T-cell proliferation induced by lung DC. The specificity of this observation was underlined by control experiments, in which the use of serum from mice sensitized against a different antigen did not result in increased T-cell activation when FcR γ -chain deficient DC were used. This largely excludes the possibility that circulating inflammatory factors, such as amyloid P or C reactive protein [26], in sensitized mice could account for the results. Furthermore, it confirms that Fc γ RI, Fc γ RIII or Fc γ RIV are required for augmented antigen presentation and also that lung DC lacking these receptors are devoid of constitutively defective processing or presentation via MHC class II. Considering that OVA-specific IgG₁ is generated during sensitization [13] and given the fact that IgG₁ binding by activating Fc γ R is exclusively dependent on Fc γ RIII [27–29], with no contribution of Fc γ RI and Fc γ RIV [11, 30], we speculate that Fc γ RIII is the major mediator of these effects. Further studies using targeted knock down of Fc γ R on DC after sensitization or Th2 cell transfer might help to further delineate the function and contribution of these effects to pulmonary hypersensitivity.

We assumed that under physiological conditions the formation of immune complexes would have a preferential impact on activation and antigen presentation of lung DC [17, 18] and thus examined DC outside secondary lymphoid organs. Further studies are required in order to identify specific lung DC subsets that might be involved in this process [28] and to investigate whether other Fc-receptor expressing cells contribute to this effect. It seems likely that migratory and resident LN DC populations could be regulated through IgG in a similar way. This would have important implications, not only for the priming of antigen-specific allergic T-cell responses and the re-challenge of existing T-cell populations, but presumably also on the collateral priming to inhaled antigens [4]. In this respect, the DC activation and cytokine production following engagement of activatory Fc γ R could be of further relevance. It is important to note, however, that allergen-specific IgG can also alleviate the strength of a pulmonary hypersensitivity reaction, presumable by modulating macrophage function through Fc γ R [31].

In summary, we conclude that not only IgE but also IgG and Fc γ R play an important role during the manifestation of allergen-induced airway hyperresponsiveness and inflammation. In addition to their function during sensitization against allergens, Fc γ R-mediated enhanced antigen presentation and T-cell stimulation by lung DC appears to have a significant impact on inflammatory responses during the airway challenge phase. These data support therapeutic strategies that target Fc γ R for the treatment of asthma.

Materials and methods

Animals

Eight- to ten-wk-old female C57BL/6 (B6) mice, male/female BALB/c mice (Charles River, Sulzfeld, Germany), DO11.10 mice, TLR4-deficient (both Jackson Laboratory, Bar Harbor, ME, USA), OT-II mice (from Dr. William Heath, Melbourne), and Fc γ R-deficient B6 mice, purchased from Taconic (Germantown, NY, USA), were used throughout the study. Fc γ R-deficient mice lack the γ -chain subunit of the Fc γ RIII and Fc ϵ RI receptors. The deleted γ -chain is also associated with Fc γ RI. The deleted γ -chain subunit is essential for receptor assembly, signal transduction and cell surface expression of Fc γ RIII and Fc ϵ RI molecules [32]. Mice were fed with OVA-free laboratory food and tap water *ad libitum*, and kept in a regular 12 h dark/light cycle at a temperature of $21 \pm 2^\circ\text{C}$. All experimental procedures were performed according to a protocol approved by the appropriate governmental authority and ethics committees.

Sensitization and allergen challenge of mice

The mice were sensitized with OVA (10 μg , Grade VI, Sigma, Deisenhofen, Germany) or PBS absorbed to aluminium hydroxide (1.5 mg, Pierce Biotechnology, Rockford, IL, USA) by i.p. injection on days 1, 14 and 21. On days 28 and 29, all mice were challenged with 1% OVA dissolved in PBS for 20 min. Allergen exposition was performed by dispersing of the relevant agent using a jet nebulizer, LC Star, 2.8 μm mass median aerodynamic diameter (Pari, Starnberg, Germany) in a closed plexiglass box, in which mice could move freely. To generate antigen-specific Th2-biased DO11.10 cells, T cells were obtained from LN and enriched and co-cultured with purified DC from BALB/c mice pulsed with OVA_{323–339} peptide (Biosyntan, Berlin, Germany) in complete medium containing IL-4, IL-2, and anti-IFN- γ . Five days later, Th2-biased DO11.10 cells were quantified and $3\text{--}4 \times 10^6$ were adoptively transferred i.v. into BALB/c recipients [4]. On three consecutive days, mice were challenged i.n. with PBS, 100 μg rabbit anti-OVA IgG (MP Biomedicals Germany, Heidelberg, Germany) (control groups), 25 μg OVA, or OVA-IC (made by mixing a 1:4 ratio of 25 μg OVA and anti-OVA IgG). Twenty-four hours after the last challenge, lung function was analyzed and mice were dissected.

Cell differentiation of the BALF

Total cell counts in BALF were scored using a Neubauer chamber (Brand, Wertheim, Germany). Leukocyte subsets (eosinophils, neutrophils, macrophages or lymphocytes) were counted in BALF using cytopspins (centrifuged preparations) stained with Diff-Quik (Medion Diagnostics, Düringen, Germany). A total of 400 cells were counted in each sample.

Histology

Twenty-four hours after the last airway challenge, lungs were fixed with 4% formalin and embedded in paraffin. The paraffin blocks were cut into 4 μm slices and stained with hematoxylin/eosin (Merck, Darmstadt, Germany). From each mouse lung, six sections (containing hilar structures and periphery) of the right and left lung were evaluated. Microphotographs were performed using a Nikon Eclipse 50i microscope with a Nikon Digital Sight DS-U1 Camera. Peribronchial and perivascular inflammation of four mice from each group were analyzed and scored as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one-cell layer deep; 3, a ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells of more than four-cells deep.

T-PCR analysis of Fc γ R expression on pulmonary DC. Purified lung DC were taken up in TriZol[®] Reagent (Invitrogen, Karlsruhe, Germany), total RNA was isolated from frozen samples with a chlorophorm-propanol-ethanol extraction procedure and cDNA synthesis was carried out *via* reverse transcriptase (Qiagen, Hilden, Germany). Quantitative real-time RT-PCR analysis was performed with an iCycler[®] (Biorad, Munich, Germany) and QuantiTect SYBR[®] Green PCR kit (Qiagen) in order to determine the levels of Fc γ RI-IV mRNA, normalized to tubulin and using published Fc γ RI-III primers [33, 34]. For detection of Fc γ RIV transcripts, the following Fc γ RIV-specific primers were used: sense, 5'-CAGAGGGCTCATTGGACA-3'; antisense, 5'-GTGATTTGATGCCACGGT-3'. The PCR condition was 95°C , 15 min one cycle, followed by 94°C , 15 s, 52.5°C , 30 s and 72°C , 30 s for 40 cycles for all primer pairs.

DC isolation and flow cytometric analysis

DC were isolated from mouse spleen or lungs as previously described [35–37]. In brief, the organs were cut into small fragments, digested with collagenase and DNase I (Sigma) and enriched by gradient centrifugation using Nycodenz reagents (Axis-Shield, Oslo, Norway) with a density of 1.073 for lung DC and 1.077 for splenic DC. DC were then enriched by negative depletion using magnetic separation and an antibody cocktail containing anti-Gr1, anti-B220, anti-erythrocytes, anti-CD19 and anti-CD3. To prevent DC maturation during the isolation protocol, the procedure was carried out on ice, with the exception of the initial 20 min digestion with collagenase/DNase, which was performed at room temperature. This protocol excluded B220⁺ “plasmacytoid DC” from the DC preparation [38]. DC were labeled with CD11c (HL3, FITC or PE), CD4 (GK1.5, FITC or PE), and CD8 (53-6.7, APC) monoclonal antibodies (all BD Biosciences, Heidelberg, Germany). Lung DC were stained for CD11c and MHC class II (2G9), CD11b (M1-70), CD103 (M290) (all BD Pharmingen, Germany), CD16 (275005, IgG2a, Alexa 647), CD32 (K9 361, IgG2b, Alexa 647), CD64 (290322, IgG2a, plus goat-anti-rat APC, Invitrogen) (all R&D Systems, Germany) or isotype control antibodies. Analytical and preparative fluorescent-activated cell sorting was done on a

FACSAria (BD Biosciences, San Jose, CA, USA), or a Mo-Flo (Cytomation, Fort Collins, CO, USA) instrument and sorts were usually 95–98% pure. Gating strategy for analysis and sort of lung DC and lung macrophages ($CD11c^+MHC\ class\ II^{low}$) is shown in Fig. 2B. For spleen-derived DC, dead cells were excluded by DAPI or PI-staining, and $CD11c^+$ cells were gated and analyzed for CD4 and CD8 expression. BMDC were generated by flushing out the BM from tibia and fibula of B6 mice. Red blood cells were lysed, cells were filtered through a 70-micron nylon mesh, counted and plated at a concentration of 1×10^6 cells/mL of complete IMDM (Biochrom AG, Germany) supplemented with 20 ng/mL rGM-CSF. Medium was changed on days 3 and 5 and usually 7-day-old cultures were used for experiments. For cytokine measurement, sorted lung DC or BMDC were pulsed with OVA or OVA-IC (see below) for 45 min or 48 h, respectively. Then supernatant was harvested and IL-6 and TNF- α determined using a commercial available Cytometric Bead Array (BD Biosciences, Germany) according to the manufacturer's instructions.

T-cell isolation and in vitro T-cell proliferation assay

For stimulation of OT-II or DO11.10 cells, single cell suspensions from the LN were treated with monoclonal antibodies against MAC-1, F4/80, erythroid cells, Gr-1, MHC class II and CD8 α . The antibody-coated cells were incubated with anti-rat IgG-coupled magnetic beads (Biomag[®] Quiagen, Germany) following the manufacturer's protocols. Finally, enriched T cells were labeled with CFSE as described elsewhere [6]. For the experiments using soluble OVA (Grade VI, Sigma or EndoGrade OVA, endotoxin conc. <1 EU/mg, Hyglos, Germany) or OVA-IC, DC were plated in U-bottom 96-well plates (1×10^4 cells/well in RPMI 1640 supplemented with 10% FBS and 25 mM HEPES) with 25 μ g/mL OVA or OVA-IC (made by mixing a 1:4 ratio of 25 μ g/mL OVA and anti-OVA IgG) for 45 min at 37°C in complete medium. A Limulus Amebocyte Lysate revealed that OVA grade VI (Sigma) had a non-significant endotoxin content of <0.1 EU at a concentration of 100 μ g/mL, that the EndoGrade[™] OVA (100 μ g/mL) was endotoxin free with no signal in the Limulus Amebocyte Lysate assay, and that the anti-OVA IgG at a concentration used in our experiments had an endotoxin level of 0.24 EU. The DC were washed three times and co-cultured in 200 μ L of complete medium containing 5×10^4 CFSE-labeled OT-II or DO11.10 cells. For experiments using OVA in combination with serum from sensitized (OVA or BSA) or non-sensitized mice, 1×10^4 lung DC were incubated with OVA (25 μ g/mL) and serum (100 μ L) for 1 h at 37°C. Afterwards, OVA and serum were removed by washing the cells with PBS and DC were used to stimulate 5×10^4 CFSE-labeled OT-II cells in 200 μ L of complete medium. For proliferation analysis after 60 h of culture, OT-II or DO11.10 cells were stained with CD4-APC (BD Biosciences, Germany), and samples were analyzed by flow cytometry. The total number of dividing ($CD4^+PI^-CFSE^{low}$) cells was determined in duplicate. For some experiments with OVA-IC, the data are presented as fold increase above T-cell proliferation obtained

with OVA-pulsed DC in order to normalize for variability among experiments.

Measurement of pulmonary function

Twenty-four hours after the last allergen challenge, airway hyperresponsiveness to inhaled methacholine (MCh) was assessed. Invasive but repetitive technique was performed to measure lung function in orotracheally intubated mice, using a body plethysmograph (HSE-Harvard Apparatus, March-Hugstetten, Germany) and an inhalation unit, which has been designed specifically for this mouse model [39]. Respiratory flow and esophageal pressure were measured and lung resistance was calculated using Notocord HEM software. Airway hyperresponsiveness was tested by provocation with increasing doses of MCh aerosol and according to ethics approval provocation was terminated once an animal had reached the ED₂₀₀ or above. Dried aerosols were generated by a computer-controlled aerosol generator system (Bronchy III+feedback dose control system, Fraunhofer Institute, Hannover, Germany).

Statistical analyses

All values are expressed as mean+SEM. Statistical analysis was performed using one-way ANOVA (Bonferroni *post hoc* test) or Mann-Whitney *U*-test using PRISM 4 (GraphPad, La Jolla, CA, USA). A *p*-value <0.05 was considered as statistically significant.

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Abbreviations: BMDC: BM-derived DC · FcγR: Fcγ receptor · MCh: methacholine · OVA-IC: anti-OVA IgG immune complexes

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