Signaling through FcγRIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation

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Although inhibitory Fcγ receptors have been demonstrated to promote mucosal tolerance, the role of activating Fcγ receptors in modulating T helper type (Th)2-dependent inflammatory responses characteristic of asthma and allergies remains unclear. Here, we demonstrate that signaling via activating Fcγ receptors in conjunction with Toll-like receptor 4 stimulation modulated cytokine production from bone marrow–derived dendritic cells (DCs) and augmented their ability to promote Th2 responses. Ligation of the low affinity receptor FcγRIII was specifically required for the enhanced Th2 responses, as FcγRIII$^{-/-}$ DCs failed to augment Th2-mediated airway inflammation in vivo or induce Th2 differentiation in vitro. Further, FcγRIII$^{-/-}$ mice had impaired Th2 cytokine production and exhibited reduced airway inflammation, whereas no defect was found in FcγRI$^{-/-}$ mice. The augmentation of Th2 immunity was regulated by interleukin 10 production from the DCs but was distinct and independent of the well-established role of FcγRIII in augmenting antigen presentation. Thus, our studies reveal a novel and specific role for FcγRIII signaling in the regulation of Th cell responses and suggest that in addition to immunoglobulin (Ig)E, antigen-specific IgG also contributes to the pathogenesis of Th2-mediated diseases such as asthma and allergies.

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Abbreviations used: B6, C57BL/6; BAL, bronchoalveolar lavage; BMDC, bone marrow–derived DC; i.t., intratracheally; MCP, monocyte chemoattractant protein; OVA-IC, OVA immune complex; PAS, periodic acid schiff; PI3K, phosphatitylinositol-3 kinase; Rrs, respiratory system resistance; SEA, soluble egg antigen; TLR, Toll-like receptor.

Asthma is a chronic disease characterized by increased airway inflammation, enhanced mucus production, and constriction of the airways (1). The adaptive immune response, in particular Th2 cells, plays a major role in the pathogenesis of airway inflammation. Th2 cytokines such as IL-4, IL-5, and IL-13 produced by activated CD4$^+$ T cells exacerbate the severity of the disease (2). Additionally, an important contribution of B cells in the pathogenesis of asthma is underlined by the central role for IgE in the activation and degranulation of mast cells (3).

Allergen-specific IgG is also present in the serum of allergic individuals; however, its exact role in asthma and allergy remains controversial (4–8). Allergen hyposensitization therapy involves "tolerizing" atopic individuals by long-term exposure to allergen. The success of this therapy has been correlated to increased serum IgG$_4$ levels (9). Antigen-specific IgG has been thought to exert its suppressive effects through different pathways that include competing with IgE for allergen epitopes and activation of inhibitory Fcγ receptors (10). In sharp contrast, a recent study by the Multicentre Allergy Study Group has demonstrated that the presence of antigen-specific IgG, in addition to antigen-specific IgE, increased the incidence of asthma symptoms in children (11). One possible explanation for these contradictory results may be the nature of the IgG isotype produced in each individual, as well as the preferential ligation of certain IgG isotypes to activating versus inhibitory Fcγ receptors.

Activating Fcγ receptors possess immunoreceptor tyrosine-based activation motifs, whereas inhibitory Fcγ receptors possess immunoreceptor tyrosine-based inhibition motifs in their intracellular domain. The activating receptors FcγRI (CD64) and FcγRIII (CD16) signal through a common signaling partner FcRγ (FcεRIγ), which contains an immunoreceptor tyrosine-based

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activation motif and is also associated with the high affinity receptor for IgE. A novel member of the Fcγ receptor family, FcγRIV, has been recently described (12). FcγRIV also associates with the FcRγ signaling chain, suggesting its role as an activating Fcγ receptor (12). FcγRIIb (CD32b) is the sole member of the inhibitory Fcγ receptor family in mice and does not associate with FcRγ. It has been demonstrated that ligation of the inhibitory FcγRIIb can suppress Fcε receptor signaling and ameliorate airway disease (13). FcγRIIb has also been shown to promote tolerance to inhaled antigens (14). Thus, the role of inhibitory Fcγ receptors in suppressing Th2-mediated inflammatory responses is well documented; however, the role of activating Fcγ receptors remains unclear.

Activating Fcγ receptors have both overlapping and nonredundant roles. Signaling via both FcγRI and FcγRIII on macrophages has been demonstrated to enhance phagocytosis of opsonized RBCs and antigen presentation to T cells (15, 16). However, evidence of nonredundant roles is provided by studies that demonstrate that both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice exhibit marked defects in their ability to mount Arthus reactions and have reduced susceptibility to passive anaphylaxis (16–18). In addition, the development of proteoglycan-induced arthritis was found to require FcγRIII but not FcγRI expression (19). The inability of the individual Fcγ receptors to compensate for each other may be explained by selective affinity of each receptor for a subtype of IgG antibodies. FcγRI binds IgG_{2a} antibodies with ~100-fold higher affinity than FcγRIII (12, 20, 21), and FcγRI is a high affinity receptor for monomeric IgG antibodies, whereas FcγRIII is thought to be a low affinity receptor that is engaged efficiently by IgG_1–immune complexes (15, 18).

In this study, we investigated whether activating Fcγ receptors regulate Th2-dependent inflammatory responses. We demonstrate that signaling via FcγRIII on DCs plays a key role in the development of optimal Th2-dependent airway inflammation. This augmentation of Th2 immunity by FcγRIII ligation is distinct from its role in antigen presentation and mediated by IL-10 production by the priming DCs. Our data suggest a novel role for FcγRIII on DCs in augmenting in vivo Th2 responses and the exacerbation of airway inflammation.

RESULTS

Ligation of Fcγ receptors modulates Toll-like receptor (TLR)-4–mediated cytokine production from DCs

DCs are thought to play a critical role in the regulation of Th cell differentiation (22) and for the development of airway inflammation (23–25). As DCs have been shown to express all Fcγ receptors (12, 26–28), we investigated the contribution of Fcγ receptor ligation in TLR-4–induced stimulation of DCs. Immune complexes were produced by incubation of OVA with anti-OVA sera (OVA immune complexes [OVA-ICs]) and used to ligate Fcγ receptors. To control for potential nonspecific effects from the antisera, an aliquot of antisera in which IgG had been depleted was incubated with OVA (OVA-IgG^{depl}). 22.1 EU/mg endotoxin present in the OVA served as the TLR-4 stimulus. Upon Fc receptor ligation, TLR-4–induced IL-10 production by the bone marrow–derived DCs (BMDCs) was significantly up-regulated, whereas IL-12 production was suppressed (Fig. 1 A). The ability of Fcγ receptors to modulate BMDC cytokine production was TLR-4 dependent, as OVA-ICs failed to modulate cytokine production from TLR-4$^{-/-}$ DCs. Polymixin B–mediated depletion of LPS from the OVA also abolished the ability of OVA-ICs to influence DC cytokine production (unpublished data). Further, Fcγ receptor ligation also augmented production of the chemokine monocyte chemoattractant protein (MCP)-1, which has been shown to promote Th2 differentiation (29).

To physically dissociate the TLR-4 stimulus from Fcγ receptor ligation, we sought to cross-link Fcγ receptors with an immobilized antibody specific for FcγRIIb/RIII (2.4G2) (30, 31). BMDCs were stimulated with 10 ng/ml LPS with varying concentrations of 2.4G2. As found with OVA-IC stimulation, ligation of Fcγ receptors on BMDCs with 2.4G2 resulted in a dose-dependent increase in IL-10 and MCP-1 production, and a decrease in IL-12 production (Fig. 1 B). Collectively, these data suggest that Fcγ receptor ligation modulates TLR-induced DC functions and augments cytokines known to promote a pro-Th2 milieu.

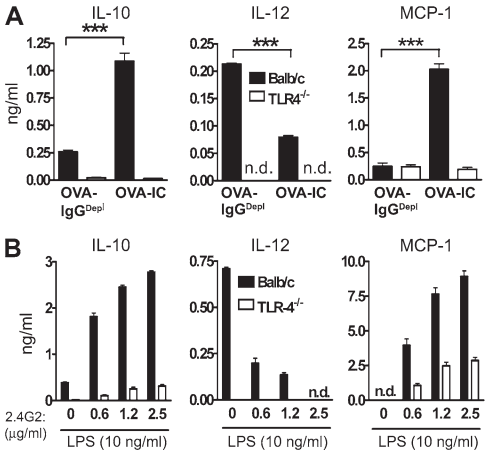

Figure 1. Ligation of Fcγ receptors modulates TLR-4–mediated cytokine production from DCs. (A) BMDCs generated from BALB/c (filled bars) or C.C3-Tlr4^{Lps-d} (TLR-4$^{-/-}$) (open bars) mice were loaded with immune complexes produced by incubation of OVA with anti-OVA sera (OVA-IC) or OVA mixed with an aliquot of antisera in which IgG had been depleted (OVA-IgG^{depl}) to account for potential nonspecific effects from the antisera (***, $P < 0.001$; n.d., not detected). (B) BALB/c (filled bars) or TLR-4$^{-/-}$ (open bars) BMDCs were cultured on plates coated with the indicated concentration of anti-FcγRIIb/RIII (2.4G2) in the presence of 10 ng/ml LPS. After 24 h of culture, cytokine production from the BMDCs was estimated. Fcγ receptor–mediated modulation of cytokine production was significantly reduced in the TLR-4$^{-/-}$ BMDCs as compared with BALB/c BMDCs ($P < 0.005$ by two-way ANOVA analysis). The data shown in A are representative of three independent experiments, and those presented in B are representative of two independent experiments.

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Ligation of Fcγ receptors on the priming DC augments airway inflammation

To determine if modulation of DC function upon Fcγ receptor ligation augments the ability of the DCs to induce Th2-mediated airway inflammation, we used DCs stimulated with a control rat IgG or 2.4G2 to sensitize naive mice. BMDCs were treated with 2.4G2 or a control antibody in the presence of OVA. After 24 h, the BMDCs were harvested and instilled intratracheally (i.t.) into naive recipients that were subsequently challenged with OVA on days 7, 8, and 9 (32). The mice were killed 24 h after the last challenge, and the extent of airway inflammation was assessed by determining the cellular composition in the bronchoalveolar lavage (BAL) fluid. Mice sensitized with 2.4G2-treated DCs exhibited increased numbers of eosinophils as well as $CD4^+$ T cells compared with mice sensitized with control DCs (Fig. 2 A). Analysis of cytokine production from the $CD4^+$ T cells in the BAL (Fig. 2 B) and the lungs (Fig. 2 C) revealed an increase in the percentage of IL-4^+ and IL-5^+ cells, indicating an increase in Th2 effector responses. These data demonstrate that signaling via Fcγ receptors on the priming DCs is sufficient to augment in vivo Th2 responses.

Modulation of cytokine production by 2.4G2 requires expression of activating Fcγ receptors on the DCs

The monoclonal antibody 2.4G2 that we used in the previous experiments can ligate both the activating Fcγ receptor FcγRIII as well as the inhibitory receptor FcγRIIb (27). To dissect whether the modulation of DC function was due to activating or inhibitory Fcγ receptors, we generated BMDCs from FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice as well as from FcRγ$^{-/-}$mice that lack all activating Fc receptors. Interestingly, modulation of IL-10 and IL-12 production upon 2.4G2 stimulation was dependent on expression of FcRγ chain and FcγRIII, but deletion of FcγRIIb actually enhanced the changes induced by the activating Fcγ receptors (Fig. 3 A). These data suggest that the modulation of cytokine production is dependent on signaling through the activating Fcγ receptors and that this signaling can be counteracted by the inhibitory FcγRIIb.

Ligation of activating Fcγ receptors has been shown to activate various signaling pathways including ERK, p38, and phosphatitylinositol-3 kinase (PI3K) (33). To ascertain the contribution of these signaling pathways in augmenting IL-10 production from the DCs, we stimulated BMDCs with LPS and 2.4G2 in the presence of selective inhibitors of p38 (SB202190), ERK (U0126), and PI3K (Ly294002) (Fig. 3 B). The PI3K inhibitor blocked 2.4G2-induced IL-10 production in a dose-dependent manner. In contrast, inhibition of ERK and p38 did not result in a significant reduction of IL-10 production. Collectively, these data suggest that signaling via activating Fcγ receptors leads to a PI3K-dependent modulation of DC cytokine production.

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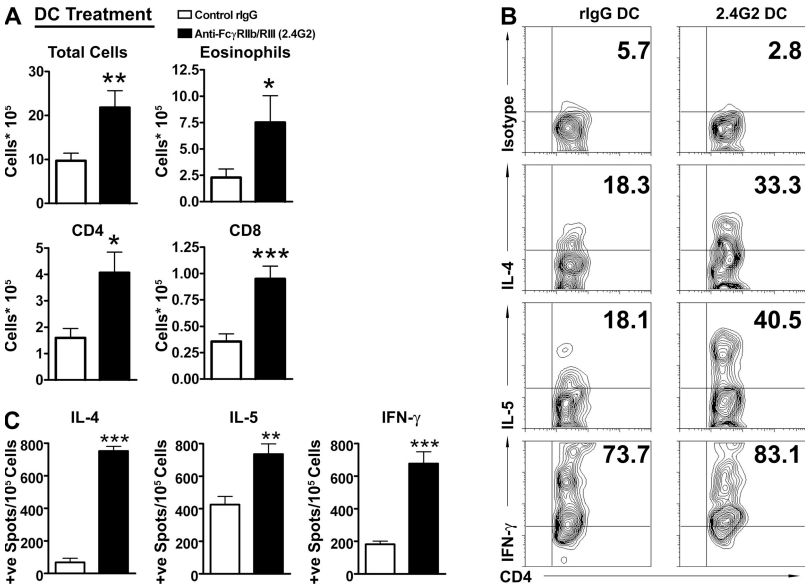

Figure 2. Ligation of Fcγ receptors on the priming DCs augments Th2-mediated airway inflammation and cytokine production in vivo. BMDCs generated from B6 mice were cultured on a nonspecific control rat IgG (open bars) or anti-FcγRIIb/RIII (2.4G2) (filled bars) –coated plates along with OVA. After 24 h, these DCs were harvested and instilled i.t. into naive B6 recipient mice. After sensitization, the recipient mice were challenged with soluble OVA i.t. daily for 3 d starting on day 7, and BAL was performed 24 h after last challenge. (A) Airway inflammation was assessed by determining the cellular composition of the BAL fluid (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The error bars indicate variation between individual mice. (B) For each group, the BAL cells were pooled and cytokine production of the $CD4^+$ T cells was determined by intracellular staining. (C) Pooled lung cells were restimulated in vitro with OVA for 48 h, and cytokine production was assessed by ELISPOT analysis (**, $P < 0.01$; ***, $P < 0.001$). The error bars indicate the SEM between replicate stimulations in different wells. The data represented in this figure were obtained from three independent experiments, and 9–10 mice per group were analyzed.

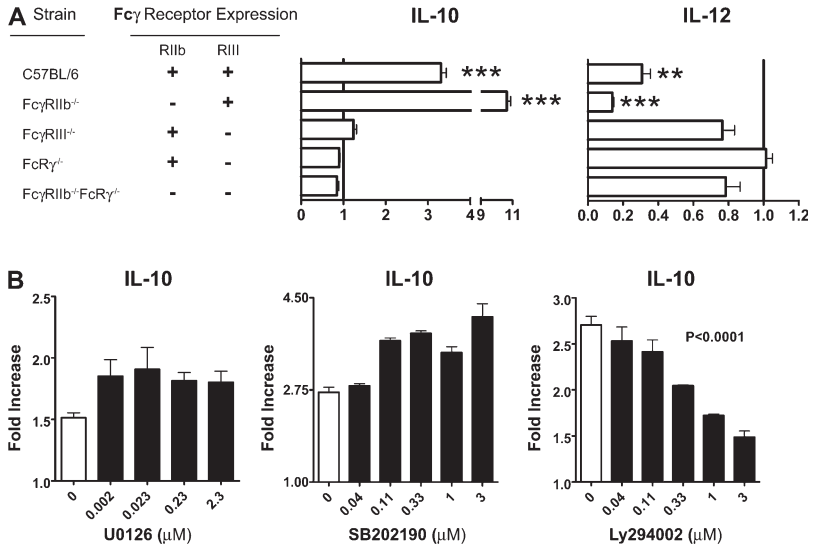

Figure 3. Modulation of cytokine production by 2.4G2 requires expression of activating Fcγ receptors on the DCs. (A) BMDCs generated from B6 or Fcγ receptor–deficient mice were stimulated with 10 ng/ml LPS along with a control IgG or anti-FcγRIIb/RIII (2.4G2). The amount of cytokines present in culture supernatants was determined by ELISA. The amount of cytokine produced upon 2.4G2 treatment was normalized to the amount produced upon treatment with control IgG (**, $P < 0.01$; ***, $P < 0.001$). (B) The fold increase in IL-10 production from DCs stimulated with 2.4G2 in the presence of the indicated concentrations of ERK inhibitor (U0126), p38 inhibitor (SB202190), and PI3K inhibitor (Ly294002) was determined. The data shown in this figure are representative of two independent experiments. The error bars indicate variation between replicate stimulations in different wells ($P < 0.0001$ by one-way ANOVA analysis).

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Signaling via FcγRIII on the priming DCs is required for immune complex–mediated up-regulation of airway inflammation

As FcγRIII is the sole activating Fcγ receptor engaged by 2.4G2, the data from the previous experiments suggested that FcγRIII signaling could play an important role in the augmentation of Th2-mediated airway inflammation. To investigate whether FcγRIII is required for Fcγ receptor–mediated augmentation of Th2-dependent airway inflammation or can be compensated for by other activating Fcγ receptors, OVA-ICs were used to ligate Fcγ receptors on FcγRIII$^{-/-}$ BMDCs.

BMDCs generated from C57BL/6 (B6) and FcγRIII$^{-/-}$ were cultured with either OVA-IC or control OVA-IgG^{depl} and instilled i.t. to sensitize naive B6 recipients. The recipients were challenged as in Fig. 2, and the severity of airway inflammation was determined on day 10. BAL eosinophilia was significantly increased in mice that were sensitized with B6 DCs loaded with OVA-IC as compared with mice sensitized with B6 DCs loaded with OVA-IgG^{Depl} (Fig. 4 A). However, FcγRIII$^{-/-}$ DCs loaded with OVA-IC failed to augment eosinophilia in recipient mice. Similarly, further evaluation of Th2 responses in the LNs revealed an augmentation of IL-4, IL-5, and IL-13 production by T cells from recipients that were sensitized with OVA-IC–loaded B6 DCs. The production of the pleiotropic cytokine IL-10 was also coordinately up-regulated along with the classical Th2 cytokines from T cells stimulated with OVA-IC–loaded B6 DCs. However, OVA-IC–loaded FcγRIII$^{-/-}$ DCs failed to augment production of all bonafide Th2 cytokines: IL-4, IL-5, and IL-13, as well as IL-10 (Fig. 4 B). The amount of IFN-γ produced by the T cells was relatively low, and no significant changes were observed in IFN-γ production in any of the experimental groups.

In this experimental system, only the priming DCs had a defect in FcγRIII expression, whereas the expression of FcγRIII on all other cells was unaltered. Thus, these data suggest an important role for FcγRIII signaling on the priming DCs for the stimulatory effects of OVA-IC treatment on Th2 responses in vivo. Furthermore, these data suggest a specific requirement of FcγRIII expression on DCs for the augmentation of Th2-mediated airway inflammation, as other Fcγ receptors could not compensate for the deficiency of FcγRIII.

Deletion of FcγRIII on DCs does not diminish their ability to process or present antigens to CD4$^+$ T cells

One possible mechanism by which FcγRIII deficiency on BMDCs leads to the reduction of Th2 responses could be decreased uptake and processing of immune complexes and diminished antigen presentation to CD4$^+$ T cells. To investigate this possibility, we first analyzed the ability of B6 and FcγRIII$^{-/-}$ BMDCs to internalize and process immune-complexed antigens. To quantitate the ability of Fcγ receptors to enhance uptake and processing of internalized antigens, we used DQ-OVA, a self-quenching conjugate that fluoresces only upon proteolysis (34). Increased fluorescence in this case would be representative of internalization and processing of antigens, and not just due to increased surface binding of immune-complexed antigens. B6 and FcγRIII$^{-/-}$ DCs were

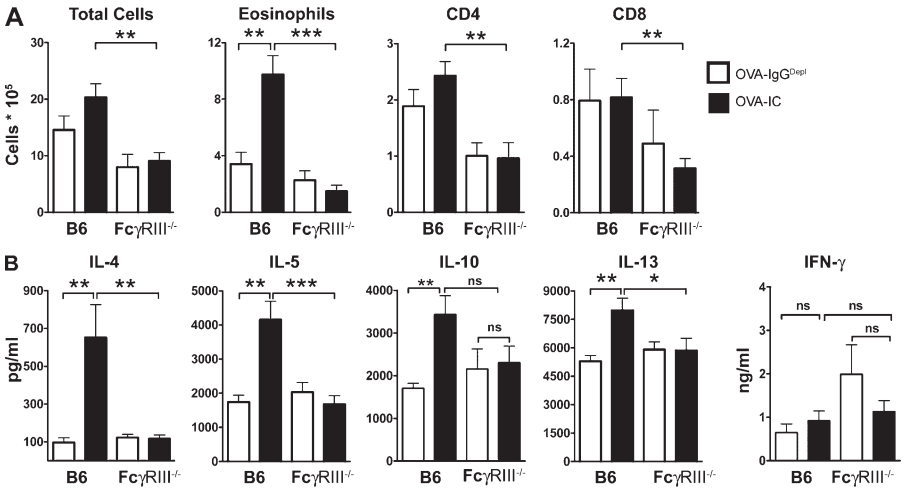

Figure 4. Immune complexes up-regulate Th2 responses by cross-linking FcγRIII on DCs. BMDCs generated from B6 or FcγRIII$^{-/-}$ mice were loaded with immune complexes produced by incubation of OVA with anti-OVA sera (OVA-IC; filled bars) or OVA mixed with an aliquot of antisera in which IgG had been depleted (OVA-IgG^{depl}; open bars). These DCs were instilled i.t. into naive B6 recipient mice challenged and analyzed as in Fig. 2. (A) Airway inflammation was assessed by determining the cellular composition of the BAL fluid (**, $P < 0.01$; ***, $P < 0.001$). (B) LN cells were restimulated in vitro with OVA for 48 h, and the amount of cytokines in culture supernatants was determined by ELISA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The data represented in this figure were obtained from two independent experiments. At least seven mice per group were analyzed.

incubated with DQ-OVA or DQ-OVA-IC for 15 min at 37°C to allow for uptake. After extensive washing to remove any un-internalized OVA, the cells were incubated for the indicated times at 37°C and amount of processing was quantitated as an increase in FITC fluorescence. As early as 5 min, both B6 and FcγRIII$^{-/-}$ BMDCs exhibited an enhanced ability to internalize and process DQ-OVA-IC as compared with uncomplexed DQ-OVA (Fig. 5 A). At later time points, similar to B6 DCs, FcγRIII$^{-/-}$ DCs loaded with DQ-OVA-IC exhibited an accelerated rate of processing compared with FcγRIII$^{-/-}$ DCs loaded with uncomplexed DQ-OVA. Thus, neither the ability of the DCs to take up antigen nor the ability of the DCs to augment processing of immune complexes was diminished in the absence of FcγRIII expression.

To determine whether FcγRIII$^{-/-}$ DCs had an impairment in stimulating T cell proliferation that was distinct from their ability to internalize and process antigens, OVA-pulsed B6 and FcγRIII$^{-/-}$ DCs were used to stimulate proliferation of TCR transgenic T cells from the OVA-specific OTII mice. Pulsing DCs with immune complexes dramatically increased proliferation of the responding T cells (Fig. 5 B). However, FcγRIII$^{-/-}$ and B6 DCs were equally efficient at augmenting T cell proliferation when loaded with OVA-IC. These data demonstrate that FcγRIII$^{-/-}$ DCs exhibit no defect in the ability to internalize, process, or present antigens and can induce expansion of T cells equivalent to that induced by B6 DCs.

Augmentation of Th2 differentiation requires FcγRIII expression but is independent of increased antigen presentation by the DCs

As no appreciable defect was found in the ability of FcγRIII$^{-/-}$ DCs to drive T cell expansion in vitro, we investigated whether FcγRIII$^{-/-}$ DCs had any defect in their ability to prime Th2 differentiation. OTII T cells stimulated with B6 DCs loaded with OVA-IC produced augmented levels of Th2 cytokines IL-4, IL-10, and IL-13 as compared with DCs loaded with OVA alone (Fig. 6 A). However, FcγRIII$^{-/-}$ DCs loaded with OVA-IC failed to augment Th2 cytokine production but were able to up-regulate IFN-γ production from the T cells similar to that induced by B6 DCs loaded with OVA-IC. These data argue that FcγRIII$^{-/-}$ DCs do not have a global impairment in responding to immune complexes but nevertheless fail to augment Th2 cytokine production in these cultures.

Finally, to investigate if the increase in antigen processing was required for augmented Th2 differentiation by DCs upon Fcγ receptor signaling, we eliminated the need for antigen uptake and processing. Control- or 2.4G2-treated DCs were loaded with the agonist $OVA_{323–339}$ peptide that can directly bind to MHC class II molecules present on the cell surface and hence does not require antigen processing. These DCs were used to stimulate TCR transgenic T cells, and cytokine production from the T cells was assessed. 2.4G2-treated DCs augmented Th2 cytokine production from the T cells as compared with control-treated DCs, even when T cell proliferation induced by these DCs was made independent of antigen uptake and processing (Fig. 6 B). Again, this effect was dependent on activating Fcγ receptors because 2.4G2 treatment failed to augment the ability of FcγRIII$^{-/-}$ DCs to induce Th2 differentiation (Fig. 6 C). Collectively, these data demonstrate that increased antigen presentation by the DCs upon Fcγ receptor ligation is not required for the increase in Th2 differentiation and argue for a distinct role for FcγRIII signaling in regulating DC-directed Th differentiation.

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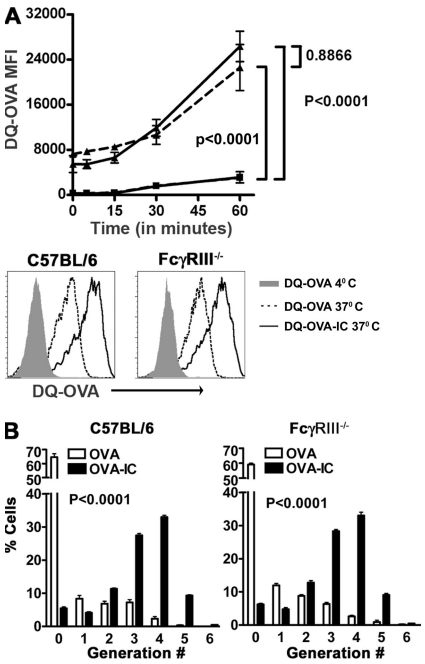

Figure 5. BMDCs from FcγRIII$^{-/-}$ mice do not have a defect in antigen uptake, processing, or presentation. (A) To measure antigen uptake and processing, DQ-OVA (■) or DQ OVA-ICs (▲) were loaded onto BMDCs generated from B6 (solid lines) or FcγRIII$^{-/-}$ (dashed lines) mice. The cells were incubated at 37°C, and the amount of OVA processed into peptides was measured at the indicated time points as an increase in FITC fluorescence. Error bars represent variation observed between independent experiments. Statistical data presented were obtained using a two-way ANOVA analysis. The bottom panel depicts a representative histogram of the data obtained at 60 min. The shaded histograms represent baseline 60 min. (B) CFSE-dyed OTII TCR transgenic T cells were cultured for 96 h with B6 or FcγRIII$^{-/-}$ DCs loaded with OVA (open bars) or OVA-IC (filled bars). The extent of proliferation was assessed as a dilution of the CFSE dye ($P < 0.0001$ by two-way ANOVA analysis).

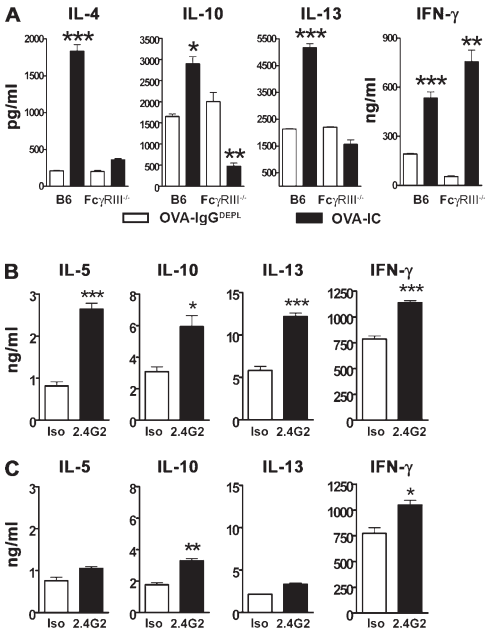

Figure 6. T cells exhibit reduced Th2 differentiation upon stimulation with FcγRIII$^{-/-}$ BMDCs. (A) OTII T cells were cultured with OVA-IgG^{Depl} (open bars) or OVA-IC (filled bars) –loaded B6 or FcγRIII$^{-/-}$ DCs for 7 d and restimulated with anti-CD3 for 48 h. The amount of cytokines in culture supernatants was determined by ELISA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). OTII TCR transgenic T cells were stimulated for 7 d with (B) B6 BMDCs or (C) FcγRIII$^{-/-}$ DCs that were left untreated (Iso) or treated with anti-FcRIIb/RIII (2.4G2) and loaded with the agonist OVA$_{(323-339)}$ peptide. T cells were subsequently restimulated for 48 h as in A, and the amount of cytokine production was determined by ELISA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The data shown are representative of two independent experiments. The error bars indicate SEM between replicate stimulations in different wells.

IL-10 production from the DCs plays a key role in Fcγ receptor–mediated augmentation of Th2 differentiation

Although the mechanism by which DCs augment Th2 responses remains controversial, it has been suggested that modulation of DC cytokine production influences the differentiation status of responding T cells (35, 36). Several studies have demonstrated an important role of DC cytokines IL-10 and MCP-1 in the augmentation of Th2 responses (29, 37–39). We demonstrated in Figs. 1 and 3 that ligation of activating Fcγ receptors on DCs led to the potent up-regulation of IL-10 and MCP-1. To elucidate the contribution of these cytokines in the modulation of DC function upon Fcγ receptor engagement, we stimulated B6, IL-10$^{-/-}$, and MCP-1$^{-/-}$ BMDCs with control OVA or OVA-IC and analyzed cytokine production from the DCs after 24 h of stimulation.

In agreement with previous reports (37, 40), IL-10$^{-/-}$ DCs exhibited increased levels of IL-12 production as compared with B6 DCs when stimulated with OVA (Fig. 7 A). However, ligation of Fcγ receptors with OVA-IC significantly reduced the amount of IL-12 production from IL-10$^{-/-}$ DCs. These data suggest that in addition to the autocrine suppression of IL-12 production induced by IL-10, Fcγ receptor ligation leads to distinct signaling events that can suppress IL-12 production in an IL-10–independent manner. Upon stimulation with OVA-ICs, MCP-1$^{-/-}$ DCs showed augmentation of IL-10 production and a suppression of IL-12 production similar to that induced by OVA-IC–treated B6 DCs (unpublished data).

To determine if production of these cytokines by DCs directly influenced Fcγ receptor–mediated augmentation of Th2 differentiation, we assessed the ability of OVA-IC–loaded IL-10$^{-/-}$ or MCP-1$^{-/-}$ DCs to induce Th2 differentiation of OTII T cells. Differentiation of OTII T cells with IL-10$^{-/-}$ DCs loaded with OVA-IC failed to induce augmented T cell production of IL-4, yet induced increased levels of IFN-γ (Fig. 7 B). Unexpectedly, OTII T cells stimulated with OVA-IC–loaded MCP-1$^{-/-}$ DCs exhibited no defects in their ability to enhance Th2 differentiation (Fig. 7 C). Collectively, our data suggest a central role for IL-10 in augmenting the ability of DCs to direct Th2 differentiation upon Fcγ receptor ligation, whereas MCP-1 production from the DCs was found to be dispensable.

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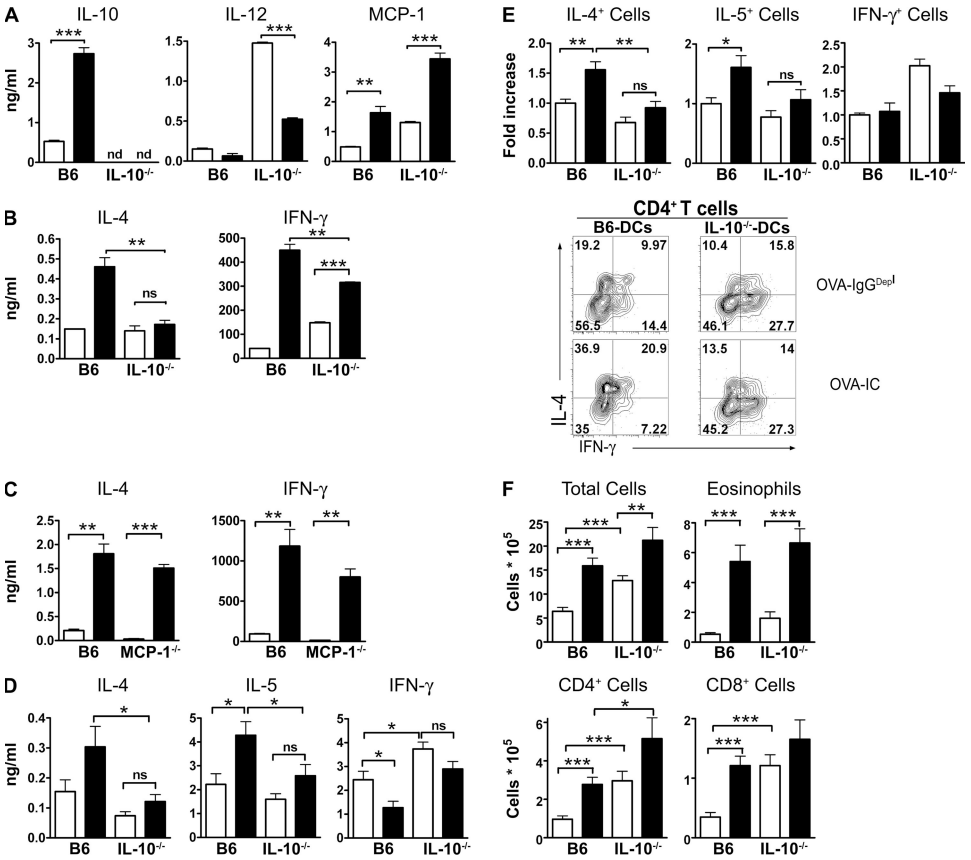

Figure 7. IL-10 production from DCs plays a central role in the up-regulation of Th2 differentiation by immune complexes. (A) BMDCs generated from B6 or IL-$10^{-/-}$ mice were loaded with immune complexes (filled bars) or control OVA-IgG^{Depl} (open bars). After 24 h of culture, cytokine production from the BMDCs was measured by ELISA. Th2 differentiation status of OTII T cells stimulated with (B) IL-$10^{-/-}$ or (C) MCP-$1^{-/-}$ DCs treated with immune complexes was assessed by analyzing cytokine production from the T cells as described in Fig. 6 A (**, $P < 0.01$; ***, $P < 0.001$). (D) Control OVA-IgG^{Depl} (open bars) or OVA-IC (filled bars) –loaded B6 or IL-$10^{-/-}$ DCs were instilled into naive B6 recipients that were subsequently challenged as described in Fig. 2. Cytokine production from cells in the draining LNs was determined by ELISA. (E) Quantitative analysis (top) and a representative flow cytometry plot (bottom) of cytokine production from cells in the BAL as determined by intracellular staining. Data in the top panel are normalized to the amounts produced by recipients sensitized with OVA-IgG^{Depl}–loaded B6 DCs. (F) Analysis of cellular composition of the BAL fluid. These data are representative of three independent experiments. The error bars indicate SEM between (A–C) replicate stimulations in different wells or (D–F) variation between individual mice. At least eight mice per group were analyzed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.d., not detected).

We next evaluated if IL-10 production from the priming DCs influenced in vivo Fcγ receptor–mediated augmentation of Th2 differentiation and airway inflammation. BM-DCs generated from B6 or IL-$10^{-/-}$ mice loaded with either OVA-IC or OVA-IgG^{Depl} were used to prime naive B6 recipients as described in Fig. 4. The recipients were subsequently challenged by i.t. administration of soluble OVA, and the differentiation status of T cells in the draining LNs was assessed by determining cytokine production by the cells upon restimulation with antigen. Similar to the results obtained from in vitro experiments, Fcγ receptor ligation of IL-$10^{-/-}$ DCs failed to augment production of Th2 cytokines IL-4 and IL-5 from cells in the draining LNs (Fig. 7 D). Priming with IL-$10^{-/-}$ DCs, as compared with priming with B6 DCs, led to an augmentation of IFN-γ production from draining LNs cells, regardless of whether the DCs were loaded with OVA-IC or OVA-IgG^{Depl}. A similar defect in Th2 cytokine production was found in $CD4^+$ T cells in the BAL fluid of mice sensitized with OVA-IC–loaded IL-$10^{-/-}$ as determined by intracellular cytokine analysis (Fig. 7 E). Collectively, these data argue for a central role of DC-derived IL-10 in Fcγ receptor–mediated augmentation of Th2 differentiation both in vitro and in vivo.

To determine if the failure to augment Th2 differentiation upon Fcγ receptor ligation of IL-$10^{-/-}$ DCs would result in a corresponding failure to augment airway inflammation, we assessed the extent of airway inflammation in the recipient mice described above. Contrary to our expectation, Fcγ receptor ligation of IL-$10^{-/-}$ DCs augmented their ability to induce eosinophilia (Fig. 7 F). Furthermore, there was a substantial and significant increase in T cells in the BAL fluid of mice sensitized with IL-$10^{-/-}$ DCs whether or not Fcγ receptors were ligated. These data suggest that even though Fcγ receptor–induced IL-10 contributes to the regulation of

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CD4+ T cell differentiation, IL-10 may not play a role in the augmentation of eosinophilia.

Development of airway inflammation and airway hyperresponsiveness is impaired in FcγRIII-deficient mice

Our data demonstrate that ligation of Fcγ receptors on DCs augments their ability to regulate Th2 responses in vitro and in vivo. We thus hypothesized that mice deficient in activating Fcγ receptors would have impaired Th2-dependent inflammatory responses. To investigate the role of activating Fcγ receptors in the development of Th2-mediated airway disease, we assessed the severity of airway inflammation in FcRγ$^{-/-}$, FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and control B6 mice that were sensitized and challenged with noninfectious *Schistosoma mansoni* antigens. This model of sensitization and challenge with *S. mansoni* antigens is well established and previously reported by our lab as well as others (41–44). B6 mice sensitized with *S. mansoni* eggs and challenged with soluble egg antigen (SEA) develop a robust airway inflammation consisting of 70–75% eosinophils, 10–15% T cells, and 10–15% macrophages. Furthermore, on the B6 background, this model of sensitization and challenge induces a log increase in airway eosinophilia compared with the OVA model of sensitization and challenge. We have previously established that eosinophilia in our model is totally dependent on both sensitization and challenge (42, 44). In the absence of either the sensitization or the challenge, the composition of the small number of BAL cells is predominantly macrophages and

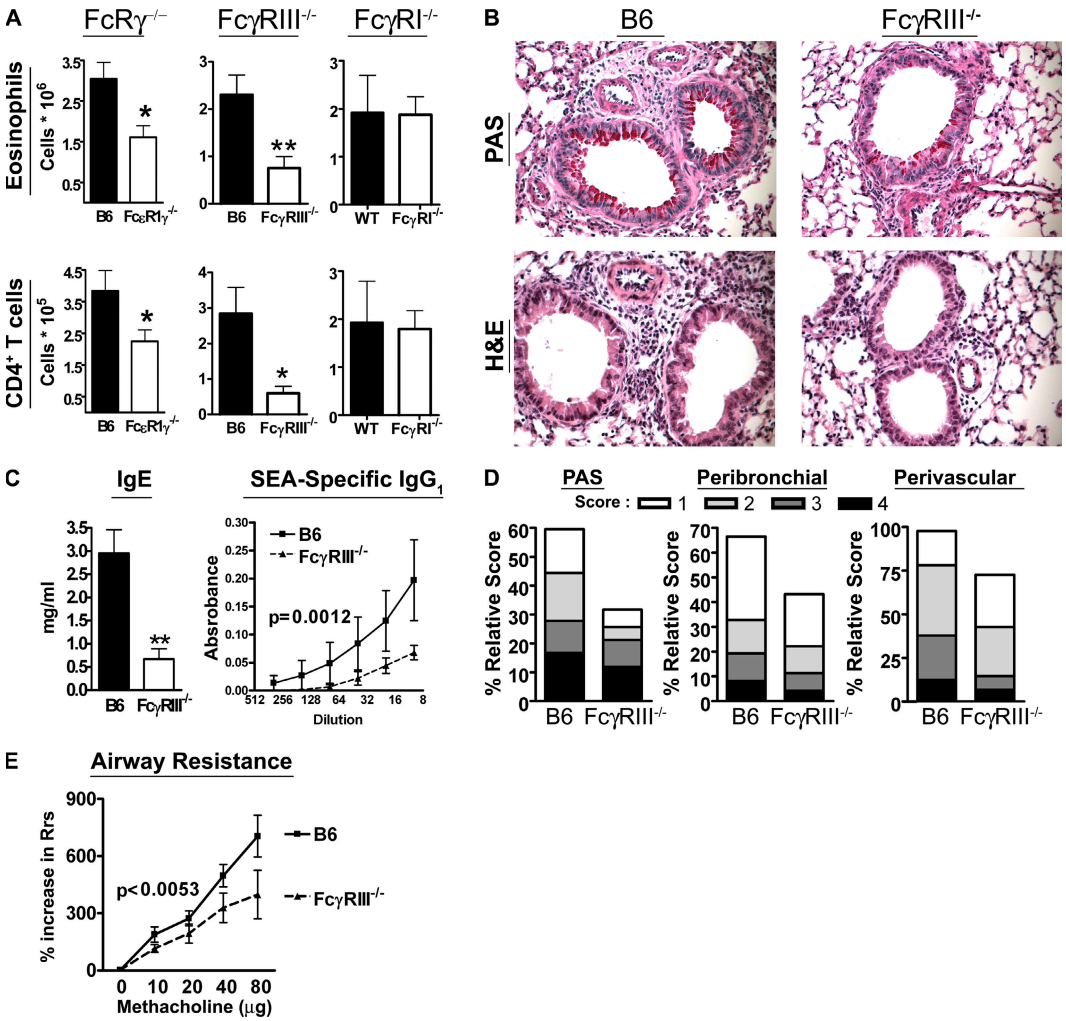

Figure 8. FcγRIII$^{-/-}$ mice exhibit a decrease in Th2-mediated airway inflammation and airway hyperresponsiveness. (A) FcRγ$^{-/-}$ ($n = 9$), FcγRIII$^{-/-}$ ($n = 8$), FcγRI$^{-/-}$ ($n = 8$), and B6 mice were sensitized with i.p. injection of inactivated *S. mansoni* eggs on day 0. These mice were challenged with i.t. administration of SEA on day 7. On day 11, the mice were killed and the cellular composition of the BAL fluid was examined by flow cytometry (*, $P < 0.05$; **, $P < 0.01$). (B) Lung tissues from sensitized and challenged B6 and FcγRIII$^{-/-}$ mice were fixed in 4% formalin and embedded in paraffin. The sections were stained with PAS or hematoxylin and eosin as noted. (C) Amount of IgE and SEA-specific IgG$_1$ in the sera of sensitized and challenged mice was estimated by ELISA (**, $P < 0.01$; $P = 0.0012$ obtained by two-way ANOVA analysis). (D) Goblet cell metaplasia, perivascular, and peribronchial inflammation were scored as described in Materials and methods. Four mice per group were analyzed. (E) Rrs to methacholine was measured at day 4 after challenge ($P < 0.0053$ by two-way ANOVA analysis). Data are representative of three independent experiments.

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indistinguishable from the BAL of mice without sensitization and challenge.

In agreement with our hypothesis, mice lacking all activating Fcγ receptors (FcRγ$^{-/-}$) exhibited a decrease in airway inflammation as represented by the decrease in eosinophils and CD4^{+} cells in the BAL fluid as compared with control B6 mice (Fig. 8 A). Deletion of the FcγRI receptor had no effect on the Th2-mediated inflammatory response; however, there was a significant decrease in the numbers of eosinophils and CD4^{+} T cells in the BAL fluid from FcγRIII$^{-/-}$ mice as compared with the control B6 mice. Thus, despite sharing the common FcRγ chain, the defect was specific to the FcγRIII receptor.

In addition to increased BAL eosinophilia, goblet cell metaplasia and mucus production are also indicative of the degree of inflammation. Histological examination of lungs showed that there was a decrease in mucus production as well as in the influx of inflammatory cells in sensitized and challenged FcγRIII$^{-/-}$ mice as compared with sensitized and challenged B6 mice (Fig. 8, B and D). Furthermore, serum IgE levels and antigen-specific IgG_1 levels were reduced in FcγRIII$^{-/-}$ mice, suggesting a reduction in the capacity of these mice to mount Th2 responses to *S. mansoni* antigens (Fig. 8 C). These data support our conclusion that expression of FcγRIII is required for optimal induction of Th2-mediated airway inflammation.

Airway hyperresponsiveness is a hallmark of asthma and is often associated with increased airway inflammation (45, 46). To investigate if the reduction in airway inflammation in FcγRIII$^{-/-}$ mice also leads to a reduction in airway hyperresponsiveness, we measured respiratory system resistance (Rrs) changes in response to methacholine. Sensitized and challenged FcγRIII$^{-/-}$ mice exhibited a reduction in Rrs to graded doses of methacholine as compared with similarly treated B6 mice (Fig. 8 E). Collectively, these data demonstrate that the sensitized and challenged FcγRIII$^{-/-}$ mice develop reduced Th2-dependent inflammatory responses and show a reduction in many of the major pathological features associated with human asthma.

FcγRIII$^{-/-}$ mice exhibit reduced in vivo Th2 differentiation and cytokine production

To determine if the decrease in airway inflammation in FcγRIII$^{-/-}$ mice was due to reduction in Th2 differentiation, we determined the cytokine production profile of effector cells in sensitized and challenged mice. Intracellular staining of CD4^{+} T cells obtained from the lungs of FcγRIII$^{-/-}$ mice demonstrated a defect in the ability to produce the hallmark Th2 cytokines IL-4 and IL-5, but exhibited no defect in IFN-γ production (representative contour plots are shown in Fig. 9 A, and the mean and SEM of five individual mice per experimental group are shown in Fig. 9 B). Similarly, upon in vitro restimulation with antigen, there was a specific reduction in IL-4, IL-5, and IL-13 secretion by T cells from the draining lymph nodes, whereas IFN-γ production was unaffected (Fig. 9 C). No defect was seen in Th2 cytokine production from the lymph node cells of FcγRI$^{-/-}$ mice upon in vitro restimulation (not depicted), demonstrating that there was a specific defect in Th2 effector functions in FcγRIII$^{-/-}$ mice, but not in FcγRI$^{-/-}$ mice. Thus, in addition

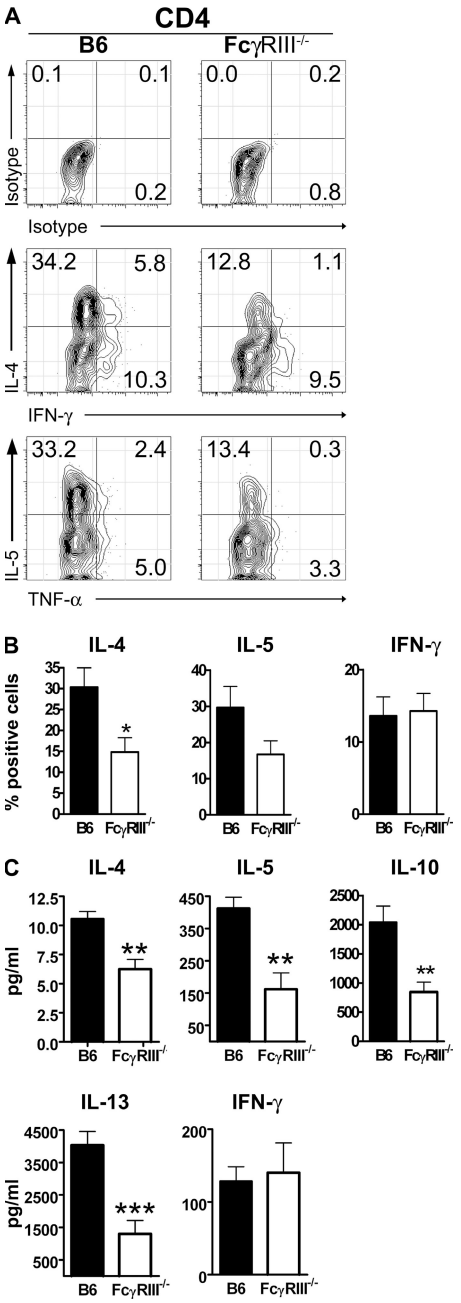

Figure 9. In vivo Th2 differentiation and cytokine production are impaired in FcγRIII$^{-/-}$ mice. B6 and FcγRIII$^{-/-}$ mice were sensitized and challenged with S. *mansoni* as described in Fig. 8. (A) Cytokine production of the effector cells in the lungs was determined by intracellular staining as described in Materials and methods. (B) Quantitative analysis of data presented in A (*, $P < 0.05$). (C) LN cells were restimulated in vitro with SEA for 48 h, and the amount of cytokines in culture supernatants was determined by ELISA (**, $P < 0.01$; ***, $P < 0.001$). The data represented in this figure were obtained from two independent experiments.

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to the well-established role of IgE–Fcε receptor interaction in the exacerbation of allergic hyperresponsiveness, ligation of the activating FcγRIII receptor can lead to augmented Th2 responses and the pathogenesis of airway disease.

DISCUSSION

This study establishes a role of FcγRIII in the regulation of DC function that is distinct from its role in augmenting antigen uptake and presentation. We find that signaling through FcγRIII on BMDCs promotes Th2 differentiation and leads to augmented Th2 effector responses in vivo. These data extend upon previous findings by now demonstrating a specific role of FcγRIII on DCs for optimal Th2 responses, and by demonstrating that this signal is independent of the role of FcγRIII in antigen presentation (47). Previous studies have suggested that activating Fcγ receptors could contribute to the severity of airway inflammation through the activation of Syk, a downstream kinase by which Fcγ receptors are known to augment antigen presentation (48–50). However, we demonstrate that signaling through FcγRIII augments DC-driven Th2 differentiation, even when the DCs are loaded with an agonist peptide, eliminating the need for antigen uptake and processing. Further, we demonstrated that in the absence of FcγRIII, immune complexes augment antigen presentation and induce augmented T cell proliferation without enhancing Th2 differentiation. These data uncouple the role of FcγRIII in regulating Th2 differentiation from its well-accepted role in augmenting antigen uptake and presentation to T cells (27, 28, 49, 51–53), and reveal a novel signaling function of the activating FcγRIII receptor.

We demonstrate that optimal Th2 responses and airway inflammation are dependent on FcγRIII but not FcγRI expression. Th2-dependent airway inflammation was induced by immunization with inactivated *S. mansoni* eggs, which induce potent Th2 immunity accompanied by production of IgG_1 antibodies (54). This IgG subclass preferentially stimulates FcγRIII as compared with FcγRI (55), and thus antigen-specific IgG1 may account for the selective requirement of FcγRIII in augmenting Th2 responses. Whether FcγRI signaling has a contributing role in the augmentation of Th2-mediated responses would need to be tested in experimental models where other IgG isotypes predominate.

An alternative and nonmutually exclusive explanation could be that FcγRI and FcγRIII have unique cellular expression profiles. In mice, FcγRIII is expressed on a variety of cell types that have been demonstrated to play a role in allergic disease, including DCs, macrophages, eosinophils (56), and mast cells (57). In contrast, FcγRI is primarily expressed on monocytes, macrophages, and DCs (58). Signaling via FcγRIII on mast cells has been demonstrated to induce mast cell degranulation and release of soluble mediators that have a role in the regulation of Th2 differentiation (59). Although FcγRI cannot compensate for the requirement of FcγRIII, a contribution of FcγRI cannot be ruled out. Thus, multiple mechanisms might contribute to the Th2 defect observed in FcγRIII$^{-/-}$ mice sensitized and challenged with *S. mansoni* antigens.

Although our data suggest a role for activating Fcγ receptors on DCs in augmenting airway inflammation, it has been proposed that ligation of Fcγ receptors on macrophages limits the severity of airway inflammation (60). Macrophages and DCs play diametrically opposite roles in the regulation of airway inflammation. Studies have demonstrated a suppressive role for macrophages in airway inflammation, whereas myeloid DCs are found to be required for the induction and exacerbation of airway inflammation (23, 25, 32, 61). Thus, it is possible that Fcγ receptors may have distinct functions in macrophages and DCs. The difference in Fcγ receptor function on these two cell types is highlighted by the observations that FcγRI, but not FcγRIII, has an important role in augmenting IL-10 production from the macrophages (62). In contrast to macrophages, our analysis of DCs from individual Fcγ receptor–deficient mice has substantiated a role for FcγRIII in augmenting LPS-induced IL-10 production independent of any other Fcγ receptor.

In our study, we demonstrate a central role of DC-derived IL-10 production in the augmentation of Th2 differentiation induced upon Fcγ receptor ligation both in vitro and in vivo. Nevertheless, the augmentation of eosinophilia induced by Fcγ receptor ligation on the priming DCs was found to be independent of IL-10 production from the DCs. Our data are supported by previous studies that demonstrate that IL-10$^{-/-}$ mice do not have a reduction in eosinophilia associated with mouse models of airway inflammation, even though IL-5 production is severely attenuated in IL-10$^{-/-}$ mice (63, 64). One possible explanation that could account for this disassociation of eosinophilia from Th2 differentiation may be that increased expansion of T cells observed in the absence of IL-10 may compensate for the reduction in Th2 cytokine production. Thus, even though IL-5 production was reduced on a per-cell basis (Fig. 7, D and E), the increased expansion of T cells observed in the BAL fluid of mice sensitized with IL-10$^{-/-}$ DCs (Fig. 7 F) may result in equivalent levels of IL-5 in the airways and lead to the increase in eosinophilia. A second possible explanation could be that Fcγ receptor ligation on DCs may regulate Th2 differentiation and augmentation of eosinophilic inflammation through distinct mechanisms. In either case, our data establish a clear requirement for FcγRIII ligation on priming DCs for both the induction of optimal Th2 responses as well as the development of airway inflammation.

Although our data indicate a clear role for DC-derived IL-10 in the induction of Th2 differentiation, other studies have reported that DC-derived IL-10 can induce the development of regulatory T cells that can potently suppress Th2-dependent inflammatory responses (65, 66). One possible explanation for these apparently contradictory roles of IL-10 could be the context in which IL-10 is produced by the DCs. The reports that have suggested a regulatory role for IL-10 characterized the role of DC-derived IL-10 in a model of tolerance induction (66). In this system, soluble OVA, in the absence of any TLR stimuli or other adjuvants, was instilled i.t. to induce tolerance. In contrast, all our experimental

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protocols use the TLR-4 agonist LPS, which is a strong stimulator of proinflammatory cytokines and a potent activator of DC functions, in conjunction with Fcγ receptor ligation. These DCs are exposed to a "danger" signal and are primed to activate the adaptive immune response. Thus, in the presence of additional proinflammatory mediators, IL-10 may serve to modulate the T helper response toward a Th2 bias rather than a tolerogenic response. This hypothesis is also supported by studies that report that ligation of certain proinflammatory stimuli, such as the TLR-2 agonist zymosan and Pam-3-Cys, lead to augmented IL-10 production by the DCs, which in turn leads to increased Th2 responses in vitro and in vivo (67, 68).

Although our data clearly demonstrate a positive contribution of DC IL-10 production in the augmentation of Th2 differentiation upon FcγRIII engagement, the mechanism by which IL-10 production from the DCs leads to the increased Th2 differentiation remains unclear. One mechanism could be that FcγRIII-mediated increase in DC IL-10 production could directly modulate T cell function. As both T cells and DCs express IL-10 receptors, another likely mechanism could be that IL-10 modulates DC function in an autocrine manner, which in turn affects T cell differentiation. In fact, suppression of IL-12 production due to the autocrine effects of IL-10 has been suggested to play a role in increased Th2 differentiation induced upon treatment of DCs with PGE_2 and steroids (69, 70). However, our results with IL-$10^{-/-}$ DCs argue for an alternate role of IL-10 in the augmentation of Th2 differentiation induced upon FcγRIII ligation on DCs. We report that the downmodulation of IL-12 production upon FcγRIII engagement does not require IL-10 production from the DCs. IL-$10^{-/-}$ DCs potently suppressed IL-12 production upon Fcγ receptor engagement (Fig. 7 A). However, IL-$10^{-/-}$ DCs failed to induce Th2 differentiation upon Fcγ receptor ligation. These data clearly dissociate the ability of Fcγ receptors to suppress DC IL-12 production from their ability to promote Th2 differentiation and lead us to conclude an alternate and important role of IL-10 production in this process. In support, previous reports have also demonstrated that IL-12 absence or suppression is not sufficient for the induction of Th2 responses by DCs (37, 71).

The role of IL-10 production from the T cells remains unclear. Our data is in concordance with the model proposed by Umetsu et al. (72) that suggests that Th2 cells and IL-10–expressing regulatory T cells may have a common precursor and diverge further along their developmental pathway. Whereas Th2 cells express Th2 cytokines such as IL-4, IL-5, and IL-13 in addition to IL-10, regulatory cells either develop to exclusively produce IL-10 or may down modulate the production of the proinflammatory Th2 cytokines IL-4, IL-5, and IL-13 while retaining IL-10 production. The cytokine profile of T cells primed with OVA-IC–loaded DCs exhibits an increase in all of the bonafide Th2 cytokines in addition to IL-10. Thus, our data support the notion that upon Fcγ receptor ligation, DCs bias the generation of Th2 responses and not the generation of regulatory T cells.

We propose that FcγRIII may play a "co-stimulatory" role and differentially modulate TLR-induced DC cytokine production. This co-stimulatory function of Fcγ receptors suggests an important link between the innate and adaptive immune responses. Antibody production by B cells could potentially influence the activation and function of innate immune cells such as DCs via ligation of Fcγ receptors. This idea is further supported by the findings that B cell–deficient mice produce reduced Th2 responses (73, 74). As IgG production occurs relatively late after initial sensitization with antigen, the influence of Fcγ receptor signaling on DCs during initial priming is less likely. However, Fcγ receptor signaling could influence the magnitude of ongoing and recall Th2 responses by further augmenting Th2 differentiation during secondary responses. Thus, IgG antibodies induced under Th2 conditions may form a feedback loop by ligating FcγRIII on DCs, thereby enhancing an established inflammatory Th2 response.

Allergies and asthma are chronic disorders whose severity depends upon continued Th2 responses to the allergen. Our data suggest a central role for activating Fcγ receptors in regulating Th2 responses and may provide a molecular basis for the proinflammatory role of IgG in diseases such as allergies and asthma. However, depending on its subclass, in addition to activating Fcγ receptors allergen-specific IgG also has the potential to engage the inhibitory Fcγ receptor FcγRIIb. Interestingly, ligation of the inhibitory receptors has been reported to suppress inflammatory responses. Zhu et al. (13) have described a chimeric Fel d1–human Fcγ fusion protein that suppresses IgE-mediated mast cell activation by coligation of FcγRIIb. Further, FcγRIIb ligation has been shown to augment mucosal tolerance and limit inflammation to innocuous antigens (14). Thus, the relative contributions of different Fc receptor family members in the development and progression of inflammatory diseases may be exceedingly complex.

Allergen-specific IgG may have different functions in distinct inflammatory processes. This complexity is highlighted by a recent report that suggests that in immediate anaphylactic effector responses, which are largely governed by innate cells such as macrophages and mast cells, allergen-specific IgG may have a suppressive role (75). In contrast, our study addresses the role of allergen-specific IgG in the priming of adaptive immune responses that are likely to be of explicit importance in more chronic Th2-mediated diseases.

Additionally, the amount of allergen-specific IgG present in the serum of sensitized individuals may also differentially influence inflammatory processes. In the same report (75), it was demonstrated that the suppressive effects of the inhibitory FcγRIIb were most evident only when the allergen amount was low and the amount of allergen-specific IgG was high. It is quite likely that the conditions, in which allergen-specific IgG may have an inhibitory role, may not always exist under physiological exposure and response to allergens in a diverse group of individuals. It is in fact tempting to speculate that different genetic predispositions and environmental influences may in turn impact on the relative amounts of

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allergen present in an individual as well as their ability to produce allergen-specific IgG antibodies. In any event, these studies are in agreement with our findings that FcγRIIb can limit FcγRIII-mediated augmentation of IL-10 production from BMDCs, and together emphasize the importance of selectively targeting different Fcγ receptors for treatment of allergic diseases.

Translation of our studies to human DC and Th2 diseases remains to be explored. Interestingly, a report by Banki et al. suggests that, similar to our results, ligation of activating Fcγ receptors modulates the function of human DCs (76). They demonstrated that cross-linking of CD32a, an activating Fcγ receptor, on human DCs induces their functional maturation along with an increase in IL-10 production. Due to differences in Fcγ receptor expression and function between humans and mice, it is likely that the exact molecular identity of the specific Fcγ receptor may be different. Nevertheless, it is conceivable that in addition to IgE, allergen-specific IgG may also modulate the severity of allergic responses in humans. In light of these reports and our findings, therapeutic strategies such as "allergen hyposensitization" that aim to switch antibody production from IgE to IgG need to take into account the IgG subclasses induced and their relative affinities for different Fcγ receptors. Inadvertent induction of IgG subclasses that activate FcγRIII may exacerbate rather than ameliorate allergic diseases. Furthermore, therapeutic antibodies clinically used to neutralize proinflammatory molecules are largely of the IgG isotypes. Infusion of large doses of these antibodies may lead to the generation of immune complexes, leading to FcγRIII activation that in turn could bias the $CD4^+$ T cell response toward a Th2 phenotype. Thus, our studies now demonstrate a novel role for FcγRIII in Th2 differentiation that must be considered in the future design of immunotherapies to reduce adverse effects on therapeutic efficacy.

MATERIALS AND METHODS

Mice. $Fc\gamma RI^{-/-}$ (18) and $Fc\gamma RIII^{-/-}$ (17) mice have been backcrossed 6 and 13 generations, respectively, on the B6 background. Female 8–12-wk-old BALB/c and B6 mice were purchased from The Division of Cancer Treatment at the National Cancer Institute or from Charles River Laboratories. $Fc\gamma RIIb^{-/-}$, $FcR\gamma^{-/-}$, and $Fc\gamma RIIb^{-/-}FcR\gamma^{-/-}$ mice were purchased from Taconic. C.C3-*Tlr4*$^{Lps\text{-}d}$ mice congenic for the *Tlr4*$^{Lps\text{-}d}$ interval from C3H/HeJ strain and IL-$10^{-/-}$ mice were purchased from The Jackson Laboratory. MCP-$1^{-/-}$ mice were provided by W.J. Karpus (Northwestern University, Chicago, IL). The OTII TCR (specific for $OVA_{323–339}$ peptide) transgenic mice were bred and maintained at the University of Chicago. Animals were housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. The studies detailed herein conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Reagents. Grade V chicken egg OVA and rabbit anti–chicken egg OVA IgG were purchased from Sigma-Aldrich. IgG–OVA-ICs were made by mixing a 4:1 excess of anti-OVA:OVA at 37°C for 30 min. To control for nonspecific effects from the antisera, an aliquot of the sera was passed over a protein G column to deplete all IgG present in the serum. This aliquot was mixed with OVA (OVA-IgG^{Depl}) and used as the control for OVA-IC. Endotoxin levels present in OVA, OVA-IgG^{Depl}, and OVA-IC were 22.1, 17.8, and 38 EU/mg, respectively, as determined by the Limulus Amebocyte Lysate assay (Cambrex). The monoclonal antibody 2.4G2 and the control rat IgG antibody were purified from culture supernatants as described previously (77).

Production of BMDCs. DCs were generated by a technique described by Lutz et al. (78). In brief, bone marrow was flushed from the femurs of mice, and cells were cultured in bacteriological Petri dishes at 2×10^5 cells/ml. The cultures were supplemented with 20 ng/ml GM-CSF (PeproTech) and replenished on days 3 and 6. On day 8, the suspension cells were harvested and found to contain >85% $CD11c^+$ cells.

Stimulation of BMDCs. For stimulation, 5×10^5 BMDCs were cultured in 24-well plates with OVA-IC or OVA-IgG^{Depl} at a final OVA concentration of 100 μg/ml. For stimulation with 2.4G2, 24-well plates were coated with 500 μl of the indicated concentrations of 2.4G2 or control rat IgG in PBS for 2 h at 37°C. BMDCs were transferred to the coated plates and cultured in the presence of 10 ng/ml LPS. Cytokine levels in the supernatant were estimated at 24 h. The level of cytokine produced in DC cultures that were neither stimulated with LPS or OVA-IC is below the level of detection, which is 32 pg/ml for IL-10, 64 pg/ml for IL-12p70, and 80 pg/ml for MCP-1.

Induction and assessment of airway inflammation. Mice were sensitized and challenged with *S. mansoni* antigens as described previously (41). In brief, mice were immunized i.p. on day 0 with 5,000 inactivated *S. mansoni* eggs. Mice were challenged on day 7 via i.t. delivery of 5 μg SEA and killed 4 d later.

Mice were sensitized to OVA using adoptive transfer of antigen-loaded BMDCs as described previously, with minor modifications (32). 10^6 BMDCs from B6 or $Fc\gamma RIII^{-/-}$ mice were loaded with OVA-IgG^{Depl} or OVA-IC (final OVA concentration of 100 μg/ml) for 24 h, harvested, and instilled i.t. into naive B6 recipients on day 0. On days 7, 8, and 9, the mice were challenged by i.t. aspiration of 100 μg OVA and killed on day 10.

BAL was performed by delivering 0.8 ml cold PBS into the airway via a trachea cannula and gently aspirating the fluid. The lavage was repeated three times to recover a total volume of 2–3 ml. The cells were stained with trypan blue to determine viability, and total nucleated cell counts were obtained using a hemocytometer. The percentage of $CCR3^+$ eosinophils, $CD4^+$ T cells, and $CD8^+$ T cells in the BAL was determined using flow cytometry.

Histology. Lungs were removed from mice after BAL and fixed by immersion into 4% formalin. Lobes were sectioned sagitally, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin for analysis of cellular infiltrates or with periodic acid schiff (PAS) for analysis of mucus-containing cells. The score of peribronchiolar and perivascular inflammation was determined as described previously (44). In brief, for the quantification, the airways were scored on a scale of 0 to 4, with 0 being negative and 1–4 being positive for PAS-staining bronchi. The extent of mucus production in positive bronchi was scored as follows: 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Peribronchiolar and perivascular inflammation was 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; and 4, a ring of inflammatory cells of more than four-cells deep.

Airway responsiveness. Rrs was measured through a computer-controlled small animal ventilator (Flexivent; SCIREQ) as described previously (79). Each mouse was challenged with increasing doses of methacholine (0, 10, 20, 40, and 80 μg in saline) administered i.v. After each challenge, Rrs was recorded during tidal breathing every 10 s for 2 min. Maximum values of Rrs were taken and expressed in terms of percentage change from baseline after saline aerosol.

Isolation of lung cells. Lungs were disassociated by agitating the tissue for 1 h in 20 ml of digestion buffer (85 U/ml hyaluronidase [Sigma-Aldrich],

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50 U/ml DNaseI [Boehinger Mannheim], and 1.0 mg/ml collagenase P [Boehringer Manheim]). The digest was passed through nytex filter, and RBCs were depleted with ammonium chloride-potassium lysing buffer. Dead cells were removed by centrifugation through Ficoll-Hypaque (Sigma-Aldrich).

Cytokine analysis. Cells from the draining LNs were cultured with 5 μg/ml SEA or 100 μg/ml OVA at 4×10^5 cells/well for 48 h, and supernatants were collected and analyzed by ELISA or cytometric bead array according to the manufacturer's protocol (R&D Systems and BD Biosciences). For intracellular cytokine analysis, cells were stimulated overnight with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 1 μg/ml brefeldin A and 2 mM monensin.

ELISPOT. ELISA spot plates (Cellular Technology Limited) were coated with 2 μg/ml rat anti–mouse IL-4, IL-5, and IFN-γ antibodies (BD Biosciences). Plates were blocked with PBS/0.1% BSA and washed with PBS. Lung cells were cultured on the plates for 16 h at 37°C in the presence or absence of 100 μg/ml OVA. The ELISPOT plates were scanned by an ImmunoSpot Series 2 Analyzer (Cellular Technology Limited).

Flow cytometric analysis. Lung cells were suspended in 100 μl of FACS buffer (PBS containing 0.1% sodium azide and 1% BSA) and labeled with APC-, FITC-, or PE-conjugated anti-CD4, CD8 (BD Biosciences), IL-4, IFN-γ, and TNF-α (eBioscience), and CCR3 and IL-5 (R&D Systems) antibodies. For detection of intracellular cytokines, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin in PBS containing 0.1% BSA. After fixation and permeabalization, cells were incubated with fluorochrome-conjugated anti-cytokine antibodies. Flow cytometric analysis was performed with a FACSCalibur or LSRII (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star Inc.).

Measurement of OVA uptake and processing. To quantify antigen processing, cells were pulsed with DQ-OVA (Invitrogen) or DQ-OVA-IC (generated by incubating 4:1 excess of anti-OVA:DQ-OVA at 37°C for 30 min) for 15 min at 37°C to allow internalization. The cells were washed extensively with cold FACS buffer and incubated at 37°C for the indicated time points. Nonspecific binding of DQ-OVA was assessed by incubating with DQ-OVA on ice for 60 min. After extensive washing with ice-cold FACS buffer, cells were stained with anti-CD11c and analyzed by flow cytometry. Amount of OVA internalized and processed by the $CD11c^+$ DCs was quantified as the FITC mean fluorescence intensity.

T cell enrichment. Total LN cells from OTII mice were passed through a nylon wool column, and nonadherent cells were collected. T cells were further purified by complement-mediated cytotoxic treatment with anti–heat-stable antigen (J11D) antibodies (American Type Culture Collection). Dead cells were removed by centrifugation through Ficoll-Hypaque (Sigma-Aldrich). T cell purity was analyzed by flow cytometry was consistently >97% $CD3^+$.

T cell proliferation and differentiation. To determine proliferation, 2.5×10^4 T cells from TCR transgenic OTII mice were labeled with CFSE and co-cultured with 2.5×10^3 OVA- or OVA-IC–stimulated BMDCs for 96 h. To determine the differentiation of the T cells, the purified T cells were co-cultured with the DCs as described above, and after 7 d of culture the cells were harvested and dead cells were removed by centrifugation through a Ficoll gradient. The cells were equalized and restimulated for an additional 48 h on anti-CD3–coated plates.

Statistical analysis. All statistics were done using an unpaired Student's two-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) or two-way ANOVA. Error bars represent SEM.

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