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Fcγ Receptor IIB on Dendritic Cells Enforces Peripheral Tolerance by Inhibiting Effector T Cell Responses[1]

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The uptake of immune complexes by FcRs on APCs augments humoral and cellular responses to exogenous Ag. In this study, CD11c$^+$ dendritic cells are shown to be responsible in vivo for immune complex-triggered priming of T cells. We examine the consequence of Ab-mediated uptake of self Ag by dendritic cells in the rat insulin promoter-membrane OVA model and identify a role for the inhibitory FcγRIIB in the maintenance of peripheral CD8 T cell tolerance. Effector differentiation of diabetogenic OT-I CD8$^+$ T cells is enhanced in rat insulin promoter-membrane OVA mice lacking FcγRIIB, resulting in a high incidence of diabetes. FcγRIIB-mediated inhibition of CD8 T cell priming results from suppression of both DC activation and cross-presentation through activating FcγRs. Further FcγRIIB on DCs inhibited the induction of OVA-specific Th1 effectors, limiting Th1-type differentiation and memory T cell accumulation. In these MHC II-restricted responses, the presence of FcγRIIB only modestly affected initial CD4 T cell proliferative responses, suggesting that FcγRIIB limited effector cell differentiation primarily by inhibiting DC activation. Thus, FcγRIIB can contribute to peripheral tolerance maintenance by inhibiting DC activation alone or by also limiting processing of exogenously acquired Ag. ***The Journal of Immunology,*** **2007, 178: 6217–6226.**

Antigen uptake receptors on APCs, including dendritic cells (DCs),[3] target Ag to processing pathways for delivery of degradative products onto MHC molecules (signal 1), enabling the immunomodulation of T cell responses. In cases in which endocytic uptake occurs in the absence of an activation signal, tolerogenic T cell responses ensue (1), whereas effector responses are triggered when concomitant DC activation signals are provided (signal 2) (2, 3). Dual functionality is provided by engagement of FcγRs that both endocytose Ab:Ag complexes (immune complexes (ICs)) as well as modulate cellular activation responses via their ITIM- or ITAM-containing cytosolic domains. In the case of ITAM-associated activating FcRs, cross-linking on DCs leads to Syk-dependent lysosomal targeting of Ag and up-regulation of DC immunophenotypic maturation markers (4–7). In contrast, coengagement of activating FcγRs with the inhibitory FcR, FcγRIIB, suppresses human and murine ITAM-induced DC maturation (4, 8, 9). FcγRIIB maintains endocytic function (10), but the consequences of Ag internalization through FcγRIIB on T cell outcomes are unclear. For instance, FcRγ$^{-/-}$ mice, which solely express FcγRIIB and lack all activating FcγR expression, have been shown in some (11, 12), but not all (13), studies to support IC-mediated Ag presentation to T cells in vivo.

The two predominant isoforms, FcγRIIB1 and B2, are generated from alternative spliced RNA transcripts (14, 15). Endocytosis of ICs by FcγRIIB is mediated by the dileucine motif in the cytoplasmic tail of FcγRIIB2, but this activity is not shared by FcγRIIB1 (16–18). In keeping with their differential expression patterns in hemopoietic cells, it has been reported that B cells that express FcγRIIB1 fail to endocytose ICs, whereas macrophages do so efficiently through FcγRIIB2 (19). However, the inability of FcRIIB1 to enhance Ag presentation has been challenged in other studies with FcγRIIB1 (20). In cultured bone marrow-derived DCs (BMDCs), internalization of ICs through FcγRIIB leads to an Ag retention compartment that poorly accesses the lysosomal degradative compartment and is capable of cell surface recycling of native Ag for presentation to B cells (10). This suggests the existence of a degradative pathway accessed by FcγRIIB distinct from that accessed by activating FcRs, potentially leading to discrete functional T cell outcomes in vivo.

We examine the consequences of IC engagement of FcγRIIB on DCs on both Ag uptake and inhibitory signaling and establish that although activating and inhibitory FcγRs endocytose ICs at comparable rates (10), FcγRIIB-mediated MHC I- and II-restricted Ag presentation is poor. Thus, on wild-type (WT) DCs, it would be expected that FcγRIIB would competitively interfere with antigenic processing of ICs internalized through activating FcγRs. The functional consequences of this prediction are examined in in vitro and in vivo. We show that DC-mediated, Ab-triggered, cross-priming by self Ag is negatively regulated by FcγRIIB, consistent with an important role for FcγRIIB in the maintenance of peripheral T cell tolerance. Supporting our prediction, we demonstrate that FcγRIIB limits MHC I-restricted cross-presentation. Surprisingly, for MHC II-restricted responses, Ag internalization by FcγRIIB fails

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[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; DTH, delayed-type hypersensitivity; DTR, diphtheria toxin receptor; IC, immune complex; LN, lymph node; mOVA, membrane OVA; RIP, rat insulin promoter; Tg, transgenic; WT, wild type.

to substantially diminish signal 1 intensity. In this case, FcγRIIB exerts its negative regulatory influence instead by dramatically attenuating the IC-mediated activation response.

Materials and Methods

Mice

BALB/c, C57BL/6, and CD11c-diphtheria toxin receptor (DTR) ((Itgax-DTR/GFP)57Lan/J) mice (C57BL/6 background) were purchased from The Jackson Laboratory. *Fcer1g* (FcRγ$^{-/-}$) and *Fcgr2b* (FcγRIIB$^{-/-}$) knockout mice, available on either the C57BL/6 or BALB/c background, were purchased from Taconic Farms. FcγRIII$^{-/-}$ mice (eighth generation backcross to C57BL/6), originally generated by T. Takai (Tohoku University, Sendai, Japan), were provided by J. Ravetch (Rockefeller University, New York, NY). OT-I and OT-II TCR transgenic (Tg) mice specific for OVA/H-2k^{b} and OVA/I-A^{b}, respectively, were provided by J. Nikolich-Zugic (Oregon Health Sciences University, Portland, OR) and A. Frey (New York University, New York, NY). DO11.10 TCR Tg mice specific for OVA/I-A^{d} were purchased from Taconic Farms. Rat insulin promoter (RIP)-membrane OVA (mOVA) mice (C57BL/6 background), originally generated by W. Heath and F. Carbone, Walter and Eliza Hall of Medical Research, Melbourne, Australia (21), were obtained from T. Ratliff (University of Iowa, Iowa City, IA). RIP-mOVA FcγRIIB$^{-/-}$ mice were bred at Columbia University (New York, NY).

Dendritic cells

DCs were prepared, as previously described (5), from bone marrow progenitors by culture in GM-CSF-containing medium. Supernatant from a cell line J558L (provided by A. Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) transfected with the mouse *gm-csf* gene was used as the source of GM-CSF.

Abs and flow cytometry

mAbs specific for CD4 (RM4-5), CD8α (53-6.7), CD11c (HL3), CD16/32 (2.4G2), CD44 (IM7), and CD45.1 (A20) were obtained from BD Pharmingen. The mAb specific for the DO11.10 TCR clonotype (KJ1.26) was obtained from Caltag Laboratories. mAb specific for CD64 (X54-5/7.1) was a gift from P. Hogarth (Austin Research Institute, Heidelberg, Victoria, Australia).

Antigens

OVA was purchased from Worthington Biochemical. OVA was used at a final concentration of 10 μg/ml, unless otherwise indicated. Polyclonal rabbit IgG specific for OVA (anti-OVA) was commercially prepared by Covance Research Products. OVA-containing ICs (OVA-ICs) were made by the admixture of equal volumes of OVA and anti-OVA at final concentration of 10 and 50 μg/ml, respectively. The immunodominant class I-restricted ($OVA_{257–264}$) and class II-restricted peptides ($OVA_{323–339}$) from OVA were synthesized by New England Peptide.

Diabetes induction

Six- to 8-wk-old RIP-mOVA mice were injected i.v. with 5×10^6 OT-I cells either alone or concurrent with i.p. administration of 100 μg of anti-OVA IgG. Diabetes was monitored using Accu-Check Advantage Glucometer and Chemstrip 2 GP urinalysis strips (Roche Diagnostics). Mice were considered diabetic if any single urinary glucose measurement equaled or exceeded 1000 mg/dL. OT-I cells were prepared by CD8$^+$ MACS (Miltenyi Biotec) separation from the spleens of OT-I Tg mice (isolated cells were >95% CD8$^+$Vα2$^+$Vβ5$^+$). Anti-OVA rabbit IgG was purified by protein A affinity chromatography from the serum of a hyperimmunized rabbit (Covance Research Products). Effector responses were assessed 5 days after transfer of OT-I cells and before the onset of hyperglycemia or glycosuria. For intracellular staining, 2×10^6 cells/500 μl complete medium were incubated with 0.1 μM SIINFEKL peptide and GolgiStop solution (BD Biosciences) for 6 h at 37°C. Cell fixation and permeabilization were done using the Cytofix/Cytoperm Plus kit (BD Biosciences) before staining with anti-IFN-γ AlexaFluor-647 (XMG1.2).

Delayed-type hypersensitivity (DTH)

Ag-pulsed DCs (1×10^6/mouse) were adoptively transferred into the front footpad of WT C57BL/6 mice. Seven days after primary immunization, the mice were challenged with 30 μg of OVA in 30 μl of PBS or PBS alone injected into the right and left hind footpads, respectively. The thickness of the foot was measured with a Pocket Thickness Gauge (Mitutoyo American), and DTH severity was assessed 24 h after challenge as the mean difference in swelling (mm) between the OVA- and PBS-challenged feet.

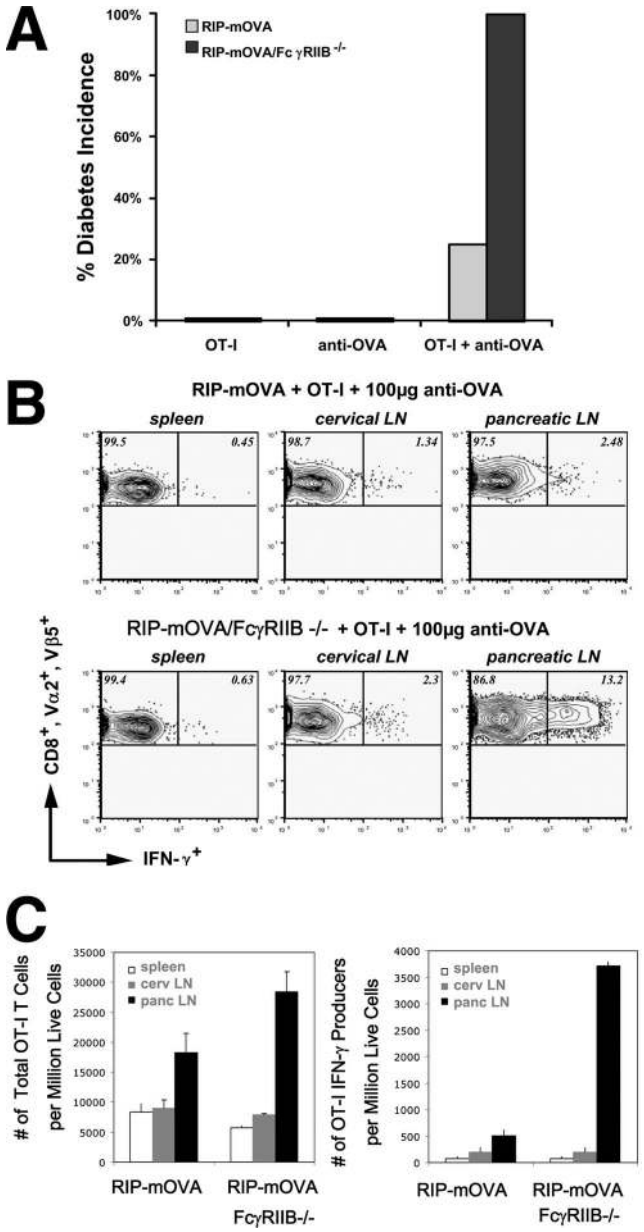

FIGURE 1. FcγRIIB inhibits cross-priming of diabetogenic T cells and the development of Ab-induced diabetes. *A*, Diabetes incidence (as measured by glucosuria) ($n \geq 8$ mice per group) is significantly increased in RIP-mOVA mice that lack FcγRIIB ($p = 0.003$, Fisher's exact *t* test). No diabetes occurred in RIP-mOVA or RIP-mOVA/FcγRIIB$^{-/-}$ mice that received either OT-I T cells or 100 μg of rabbit anti-OVA IgG alone. *B* and *C*, Differentiation of OT-I T cells into IFN-γ-producing effectors was enhanced in draining pancreatic LNs of RIP-mOVA that lacked FcγRIIB. No appreciable IFN-γ production was seen in the spleen or the nondraining cervical LNs. Contour plots in *B* are gated on CD8$^+$, Vα2$^+$, and Vβ5$^+$ cells, and the identical data are shown in *C* as absolute numbers of accumulated CD8$^+$, Vα2$^+$, Vβ5$^+$ OT-I, or OT-I IFN-γ producers (per million total cells).

In vivo proliferation

Naive CD4$^+$ OT-II or CD8$^+$ OT-I T cells (in some cases CD45.1$^+$ allotypically marked) were positively selected by anti-CD4 or anti-CD8 magnetic microbeads (Miltenyi Biotec), respectively, labeled with 10 μM CFSE (Molecular Probes), and injected into the lateral tail vein. Three days after i.v. injection of 5×10^6 T cells, spleens of the recipient animals were analyzed by flow cytometry. To determine a requirement for DCs, CD11c-DTR Tg$^+$ and Tg$^-$ recipients of OT-I and OT-II naive Tg T cells were immunized with OVA or OVA-ICs, 1 day following

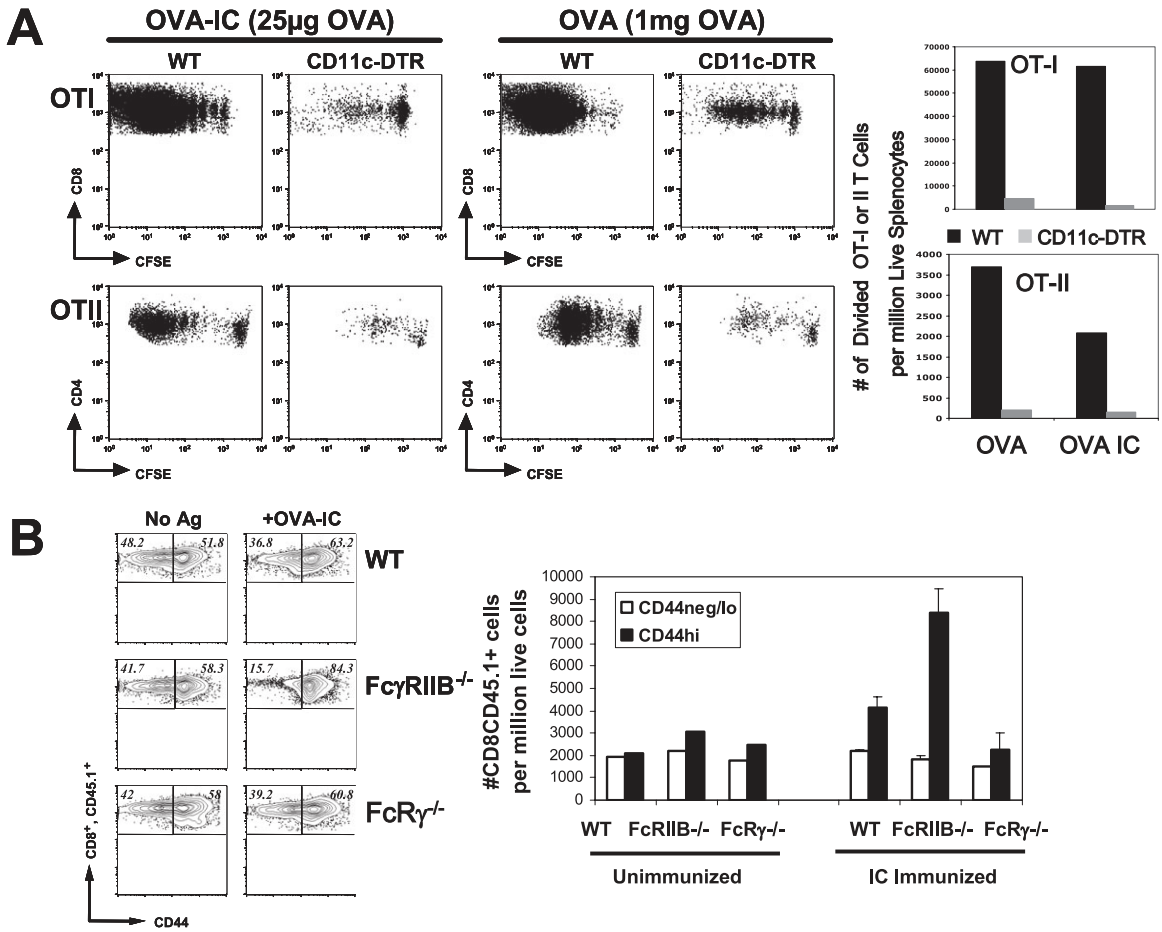

FIGURE 2. T cell priming by soluble ICs in vivo requires CD11c$^+$ cells and is inhibited by FcγRIIB. *A*, Proliferative responses were assessed 3 days after i.p. immunization with OVA-ICs (containing 25 μg of OVA) or 1 mg of OVA in recipients of 5×10^6 CFSE-labeled CD8$^+$ OT-I (*top row*) or CD4$^+$ OT-II TCR Tg T cells (*bottom row*). Absolute number of divided cells/million splenocytes is provided in the bar graph (*right*), whereas the flow cytometric populations of CFSE$^+$ OT-I or II cells are provided on the *left*. In WT mice immunized with OVA-ICs or high-dose OVA, both OT-I and II cells expanded by >10-fold. Diptheria toxin (administered to all mice, 24 h before immunization) abrogated the accumulation of proliferating T cells in CD11c-DTR Tg$^+$ mice (absolute numbers of divided cells shown on the *right*). These data are representative of three independent experiments. *B*, Eight days after i.p. immunization with soluble OVA-ICs, FcγRIIB$^{-/-}$ mice exhibit increased accumulation of CD44$^+$ memory OT-I cells. Flow cytometric analysis of splenocytes is displayed as contour plots (*left panels*) gated on CD45.1$^+$CD8$^+$ showing percentage of memory OT-I T cells (CD44$^+$) or as bar graphs (*right panels*) to illustrate average numbers of total and memory CD45.1$^+$CD8$^+$ OT-I T cells (n = 3/group). Increased numbers of memory OT-I cells accumulated in FcγRIIB$^{-/-}$ mice as compared with WT mice (Student's t test, $p = 0.04$; ANOVA, $p < 0.001$).

treatment with CD11c-depleting doses of diptheria toxin (4 ng/g diphtheria toxin (22)). CD11c$^+$ cells were depleted in Tg$^+$ animals by >95%, as assessed by flow cytometric analysis of splenocyte and lymph node (LN) populations, whereas Tg$^-$ CD11c populations remained unchanged (data not shown). Preliminary experiments in DT-treated WT and CD11c-DTR Tg$^+$ mice showed that transferred OT-I and OT-II cells accumulated and localized to the T cell zones of the LNs and spleen in comparable numbers regardless of the presence or absence of CD11c$^+$ cells (data not shown).

In vitro proliferation

Naive CD4$^+$ OT-II T cells (3×10^5/well) were incubated with OVA-IC, OVA protein, or peptide-pulsed DCs (3.75×10^3/well) in a 96-well round-bottom plate. Proliferating cells were labeled after 48 h with 1 μCi/well [^{3}H]thymidine. Sixteen hours later, the cells were harvested and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. Proliferative responses are expressed as the mean (cpm) ± SD of triplicate wells.

Cytokine measurements

IL-2, IL-4, IL-5, IFN-γ, and TNF-α concentrations were measured using a mouse Th1/Th2 cytokine CBA kit (BD Biosciences). Naive CD4$^+$ OT-II T cells were positively selected by anti-CD4 magnetic microbeads. For the primary stimulation, T cells (1×10^6/well) were coincubated with OVA-IC- or OVA (100 μg/ml)-pulsed DCs (1×10^5/well) in a volume of 1 ml in a 24-well plate. After 7 days, a secondary stimulation was set up identical with the primary stimulation. Supernatant was collected on the third day and assayed. As a positive control for cytokine production, OT-II T cells were added to wells precoated with anti-CD3/anti-CD28 (BD Biosciences).

IL-12p70, IL-6, and TNF-α concentrations were measured using a mouse inflammatory cytokine CBA kit (BD Biosciences). DCs (1×10^6/well) from WT, FcγRIIB$^{-/-}$, and FcRγ$^{-/-}$ mice were pulsed with decreasing amounts of OVA-ICs in a final volume of 1 ml. After 24 h, supernatant was collected and assayed. Cytokine production by DCs pulsed with OVA alone or anti-OVA IgG alone was not greater than that seen with DCs cultured in the absence of Ag (data not shown).

RT-PCR

RNA was extracted from cells by using TRIzol (Invitrogen Life Technologies). cDNA was synthesized using Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies). The following primers were used to assess FcγR expression: FcγRI, 5′-CAATGCCAAGTGACCCTGTGC-3′ and 5′-ACTGCTGTCCTCCGTGGCTACC-3′; FcγRIIB isoforms (23), 5′-GCCTGTCACCATCACTGTCCAAGGGCCCAA-3′ and 5′-AATGTGGTTCTGGTAATCATGCTCTGTTTCTTC-3′; FcγRIII, 5′-TCCGAAGGCTGTGGTGAAACTG-3′ and 5′-CGTAGAAATAAAGGCCCGTGTCC-3′; and FcγRIV, 5′-CTAGGCGATCCAGGGTCTCCAT-3′ and 5′-GCGTGCGCATTGCTGTATCA-3′.

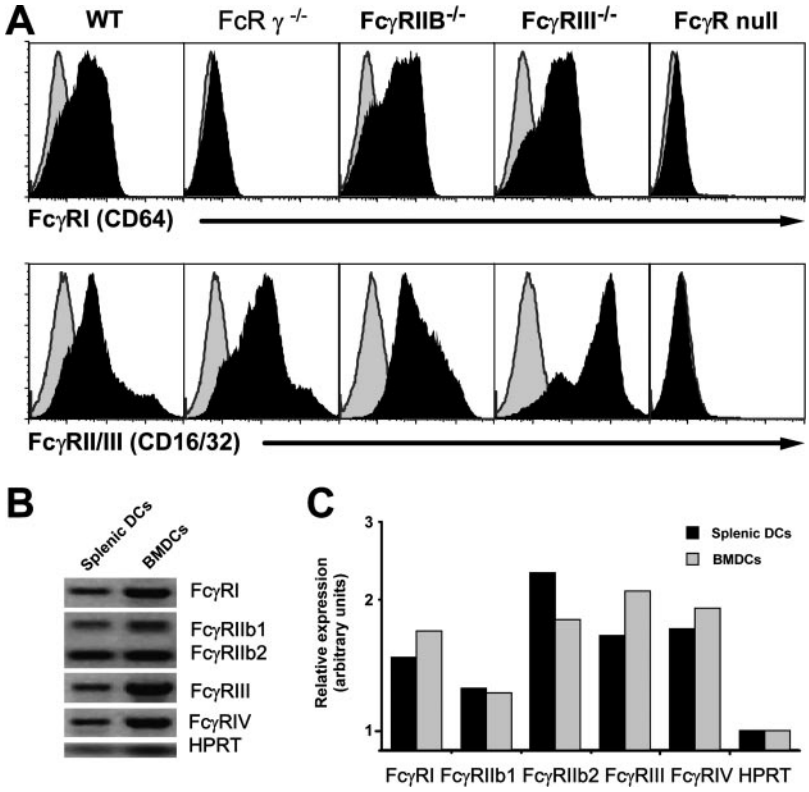

FIGURE 3. FcγR expression on DCs. *A*, Flow cytometric analyses of FcγR expression on BMDCs. BMDCs from WT, FcRγ$^{-/-}$, FcγRIIB$^{-/-}$, FcγRIII$^{-/-}$, and FcR null (γ$^{-/-}$RIIB$^{-/-}$) were stained with mAbs specific for either CD64 (X54-5/7.1, *top panels*) or CD16/32 (2.4G2, *bottom panels*). Black histograms represent specific staining for FcγR expression compared with secondary reagents alone (gray histograms). *B*, RT-PCR was performed on mRNA from splenic and BMDCs using primers specific for FcγRI, II, III, and IV. *C*, Densitometry analysis of the relative mRNA expression of FcγRs normalized to the expression of the housekeeping gene, hypoxanthine phosporibosyltransferase (HPRT). Both splenic DCs and BMDCs express all four FcRs for IgG. The endocytic FcγRIIB2 is the predominant RNA isoform expressed by DCs.

Hybrids

T cell hybridomas from OT-I and OT-II mice were generated by a previously described method (24). Briefly, splenocytes from these mice were stimulated twice in vitro with $OVA_{257–264}$ or $OVA_{323–339}$ peptides, respectively. Three days after the second stimulation, the expanded T cells were fused with the BWZ.36 fusion partner (provided by N. Shastri, University of California, Berkeley, CA). BWZ.36 cell line was generated by transfecting the BW5147 thymoma with a reporter NF-AT-*lacZ* construct (25). Productive T cell hybrids that grew under hypoxanthine, aminopterin, and thymidine selection were screened for peptide specificity and subsequently cloned by limiting dilution. The OTZ-I T cell hybrid recognizes the class I-restricted $OVA_{257–264}$ peptide, and the OTZ-II T cell hybrid recognizes the class II-restricted $OVA_{323–339}$ peptide. Activation of these T cell hybridomas results in the production of β-galactosidase, which can be measured spectrophotometrically by the cleavage of the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (Calbiochem).

Immunofluorescence endocytosis assays

DCs were pulsed with OVA:polyclonal rabbit anti-OVA (5:25 μg/ml) or 5 μg/ml FcOxyburst ICs (Molecular Probes) for 1 h at 4°C (binding assays) or 37°C (uptake assays). DCs were fixed in 2% paraformaldehyde. No additional steps were required for detection of FcOxyburst fluorescence, which fluoresces spontaneously and persistently in oxidative organelles. For detection of OVA-ICs, cells were permeabilized and blocked with RPMI 1640, 10 mM glycine, 0.05% saponin, and 5% goat serum (Sigma-Aldrich). OVA ICs were detected using OVA Alexa-488 or anti-rabbit IgG Alexa-488 (Molecular Probes). The percentage of fluorescent cells was measured by counting the number of fluorescent cells among 400–600 total cells in each of three representative fields.

Results

FcγRIIB maintains peripheral tolerance by inhibiting cross-priming by self Ag

To examine the role of self-reactive Ab in altering T cell responses to self, we have investigated RIP-mOVA mice that express the model Ag OVA as self in the thymus, the β cells of the pancreas, and the proximal tubules of the kidney (21). Thymic expression of OVA leads to central tolerance; however, studies of peripheral tolerance are enabled by transfer of OVA-specific naive T cells. Transferred OT-I CD8$^+$ Tg T cells undergo initial proliferative responses in the draining pancreatic LNs, but subsequently fail to differentiate into cytotoxic effectors, and instead deletional tolerance ensues (26). OT-I T cell tolerance can be overcome synergistically by the presence of self-reactive Abs. In RIP-mOVA mice provided with 1 mg of polyclonal anti-OVA IgG, OT-I priming is enhanced in an FcγR-dependent manner and diabetogenic effector T cell differentiation occurs in 100% of WT RIP-mOVA mice (27). To assess the role of FcγRIIB in the regulation of Ab-mediated cross-priming, 100 μg of anti-OVA IgG and OT-I cells was transferred into RIP-mOVA FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice (Fig. 1*A*). At this reduced dose of anti-OVA IgG, diabetes developed in only two of eight FcγRIIB$^{+/+}$ mice, whereas all FcγRIIB$^{-/-}$ recipients developed diabetes by 8 days. No animals developed diabetes when injected with either anti-OVA IgG or OT-I cells alone, demonstrating that Abs and T cells induced disease synergistically. To directly examine whether cross-priming by Abs was enhanced in FcγRIIB$^{-/-}$ mice, pancreatic LNs were obtained 5 days after transfer of OT-I cells, before the onset of overt diabetes. OT-I effector T cell differentiation was greatly enhanced in anti-OVA IgG-treated FcγRIIB$^{-/-}$ mice (Fig. 1*B*). Furthermore, OT-I effector T cell differentiation appeared to be restricted to the site of Ag presentation (i.e., draining pancreatic LNs) because IFN-γ-producing OT-Is were not appreciably detected at other sites, including the spleen and nondraining cervical LNs. Thus, FcγRIIB, most likely on cross-priming DCs, negatively regulates the induction of peripheral T cell tolerance to self Ags.

CD11c$^+$ DCs are responsible for T cell priming by soluble OVA and OVA-ICs

Circulating ICs are bound in vivo by many potential APCs, including marginal zone B cells, macrophages, as well as DCs. To directly examine the requirement for DCs in the priming of T cells by ICs in vivo, CD11c-DTR Tg mice were investigated that express a human high-affinity DTR on the surface of

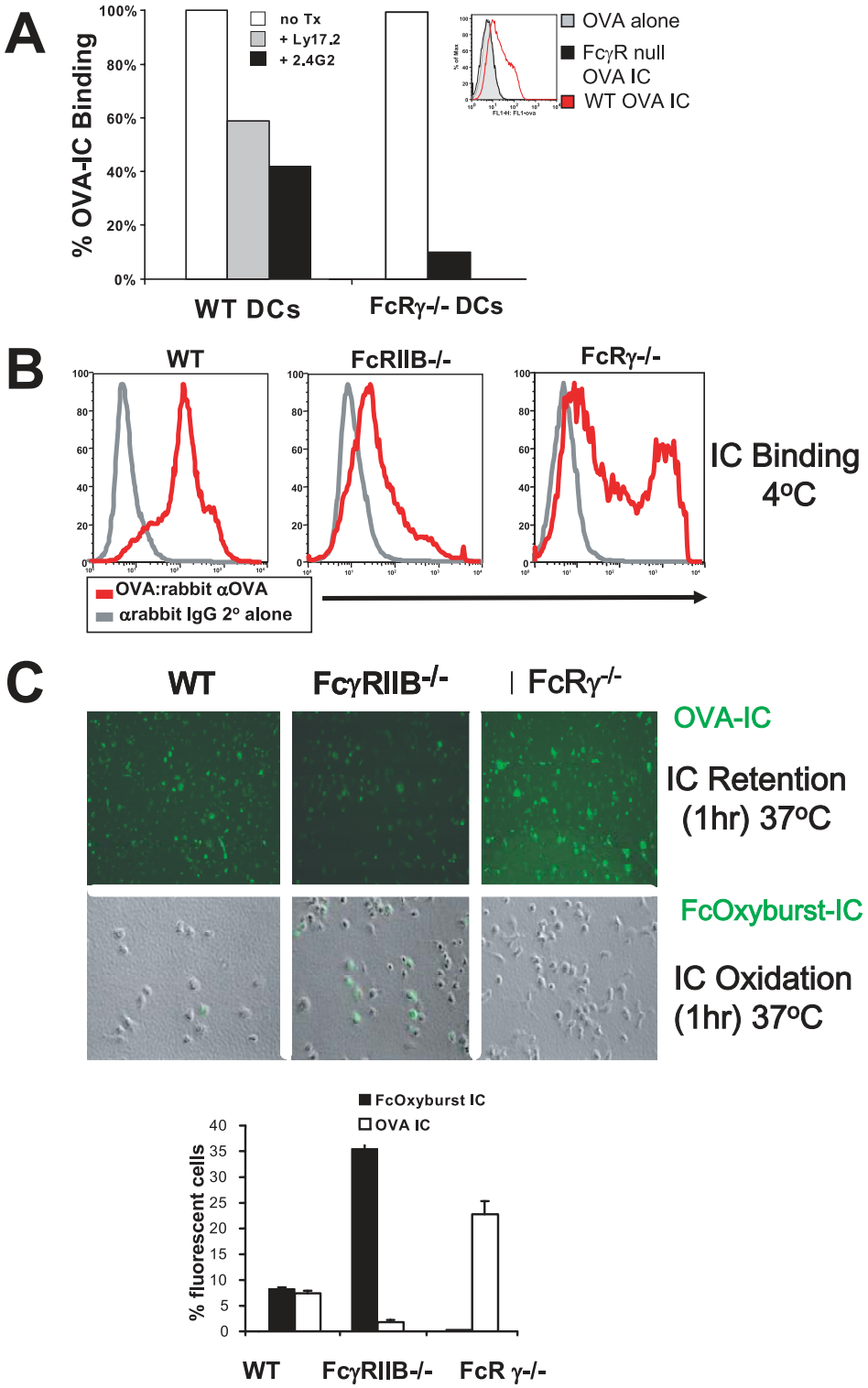

FIGURE 4. ICs internalized by FcγRIIB are inefficiently degraded and fail to reach an oxidative compartment. *A*, The majority of bound ICs on WT DCs are engaged to FcγRIIB. WT and FcRγ$^{-/-}$ BMDCs were either untreated or preblocked with 10 μg of Ly17.2 (specific for FcγRIIB) or 10 μg of 2.4G2 (specific for FcγRII/III) for 45 min on ice, then incubated with Alexa-488-labeled OVA-ICs for 30 min on ice. Cells were washed twice, and Alexa-488-labeled OVA-IC binding was assessed by flow cytometry. The percentage of OVA-IC binding is normalized to the level of OVA-IC binding seen on untreated BMDCs (see IC binding of WT vs FcR null (γ$^{-/-}$ RIIB$^{-/-}$), *inset*). Blocking FcγRIIB (Ly17.2 mAb) on WT BMDCs reduced OVA-IC binding by 40%, whereas blocking both FcγRIIB and FcγRIII (2.4G2 mAb) reduced binding by 60%, indicating that 20% of ICs are bound to FcγRIII. The remaining 40% of OVA-IC binding, after blocking with 2.4G2, is most likely the shared contribution of binding to both FcγRI and/or FcγRIV. *B*, Day 7 immature BMDCs from WT, FcγRIIB$^{-/-}$, or FcRγ$^{-/-}$ mice were pulsed at 4°C for 1 h with ICs made from OVA and polyclonal rabbit anti-OVA. Cells were then washed and stained with CD11c and secondary Abs to rabbit IgG. A large fraction of ICs binds FcγRIIB. *C*, After a 2-h pulse chase with OVA-ICs, the DCs were fixed, permeabilized, and stained with anti-rabbit F(ab′)$_2$-Alexa-488 (green) to detect cell-associated ICs (*top row*). For FcOxyburst pictures (*lower row*), fluorescence and phase-contrast images are overlayed. The mean percentage of cells exhibiting fluorescence for each condition is shown in the bar graph as calculated from counts of >300 cells. OVA-ICs are more efficiently degraded and oxidized in FcγRIIB$^{-/-}$ DCs.

CD11c$^+$ cells, making them specifically sensitive to DT-mediated apoptosis (22). Using this depletion model, DCs have been previously noted to be required for the cross-presentation of cell-associated (22) and soluble exogenous Ag (28) as well as for the presentation of bacterial (22) and viral Ags (29, 30). Therefore, DT-treated CD11c-DTR Tg$^-$ and Tg$^+$ mice were transferred with 5 × 10^6 CFSE-labeled OT-I cells or OT-II cells, and proliferation was assessed 3 days after i.v. injection with ICs containing 25 μg of OVA (Fig. 2*A*). In CD11c-DTR Tg$^-$ animals, OT-I and OT-II proliferative responses were robust. Strikingly, accumulation of proliferating OT-I and OT-II cells was greatly reduced in DC-depleted CD11c-DTR Tg$^+$ recipient mice. Similarly, the accumulation of divided OT-I and OT-II cells in response to high doses of i.v. soluble OVA (1 mg/mouse) was greatly attenuated in DC-depleted mice, suggesting that CD11c$^+$ DCs are required for effective priming of naive CD4$^+$ and CD8$^+$ T cells to exogenous soluble Ag. Although this model implicates DCs as the responsible APCs, contributions of subpopulations of DT-sensitive marginal zone macrophages cannot be excluded (31). The loss of splenic DCs

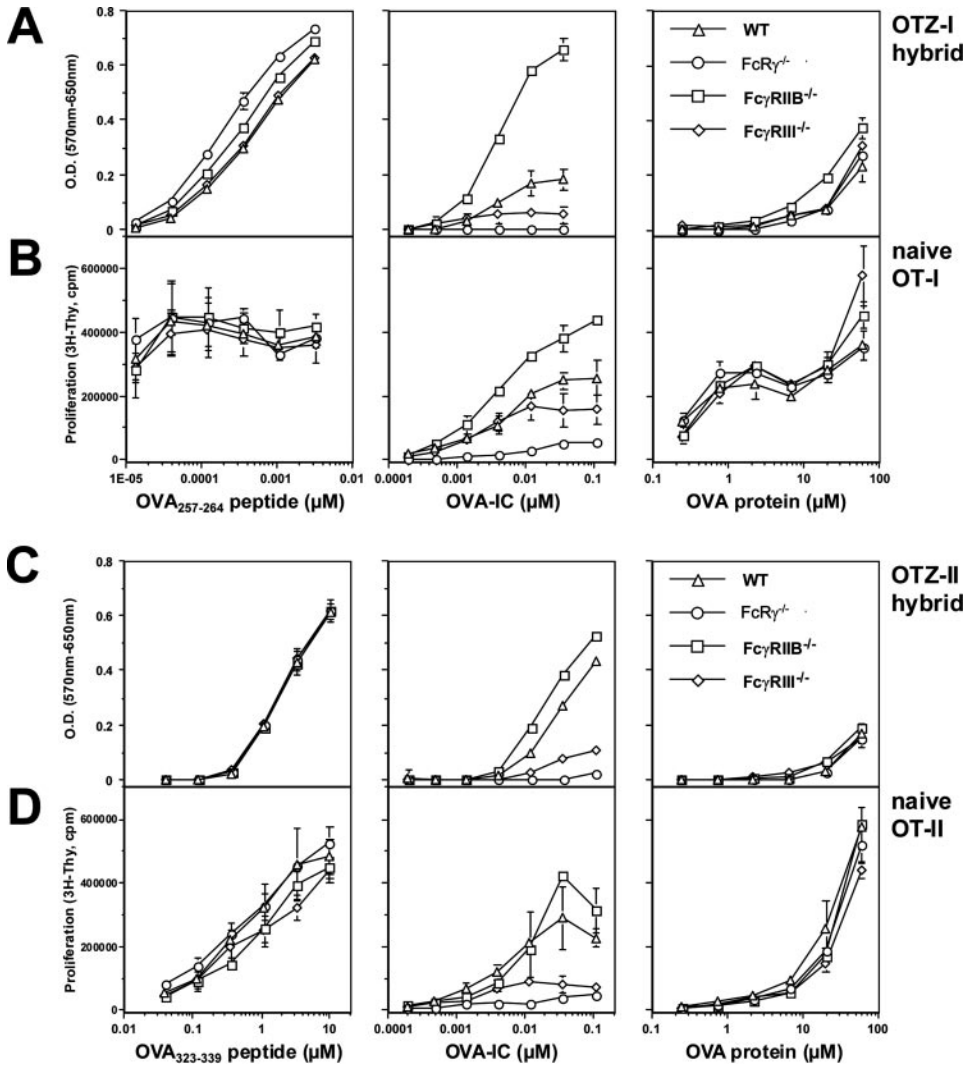

FIGURE 5. Limited Ag presentation occurs after uptake through FcγRIIB, but this does not substantially impact Ag presentation through activating FcγRs. BMDCs from WT, FcRγ$^{-/-}$, FcγRIIB$^{-/-}$, and FcγRIII$^{-/-}$ were pulsed with the indicated concentrations of peptide, OVA-IC, or OVA protein. Ag presentation to OTZ-I (*A*) and OTZ-II (*C*) T cell hybrids was measured by the LacZ assay. Ag presentation and subsequent proliferation of naive CD8$^+$ OT-I (*B*) and CD4$^+$ OT-II (*D*) T cells were measured by thymidine incorporation. The means ± SD of triplicate wells are displayed.

and macrophage subpopulations did not lead to reduced localization of injected ICs to splenic marginal zone/red pulp areas (data not shown). With regard to IC-mediated Ag presentation, despite the availability of many other endocytically competent FcγR and complement receptor-bearing APCs in mice, including monocytes and B cells, these remaining cell types are shown to be much less capable of sustaining efficient IC-mediated CD4$^+$ or CD8$^+$ T cell proliferative responses.

To directly examine whether FcγRIIB inhibits CD8$^+$ T cell responses to Ag-containing soluble ICs, WT, FcγRIIB$^{-/-}$, and FcRγ$^{-/-}$ recipients of OT-I cells were injected i.v. with soluble OVA-ICs (Fig. 2*B*). Eight days after immunization, significantly greater numbers of memory OT-I cells accumulated in FcγRIIB$^{-/-}$ mice than in WT mice.

FcγRIIB-mediated inhibition of DC function could be due to negative regulatory effects on Ag uptake/processing and/or cellular activation. To distinguish between these possible mechanisms, we have investigated the comparative roles of activating and inhibitory FcγRs on IC internalization, functional Ag presentation, DC maturation, and generation of inflammatory cytokines and chemokines.

Endocytic capacities of activating and inhibitory FcγRs

FcγR expression on BMDCs was evaluated by flow cytometry and on splenic DCs by RT-PCR (Fig. 3). Consistent with prior reports, DCs express both activating FcγRs (I, III, and IV) and the inhibitory FcγRIIB, including the endocytically competent isoform FcγRIIB2. As expected, FcγRI is expressed on WT DCs and is dramatically reduced on FcRγ$^{-/-}$ BMDCs (32). FcγRIIB expression was detected using an anti-FcγRII/III Ab to stain FcγRIII-deficient BMDCs (either FcγRIII$^{-/-}$ or FcRγ$^{-/-}$), and similarly, FcγRIII expression was determined with anti-FcγRII/III staining of FcγRIIB$^{-/-}$ BMDCs. FcγRIV expression was demonstrated by RT-PCR at the RNA level in both BMDCs and splenic DCs (Fig. 3, *B* and *C*). To assess the proportion of ICs binding to inhibitory FcγR vs activating FcγRs, IC binding to WT, FcγRIIB$^{-/-}$, and FcRγ$^{-/-}$ BMDCs was compared (Fig. 4*B*). Binding was completely abolished in FcR null BMDCs (Fig. 4*A*, *inset*). FcγRIIB$^{-/-}$ BMDCs bound ICs to a lesser degree than FcRγ$^{-/-}$ BMDCs, arguing for a greater contribution by FcγRIIB. To directly assess the proportion of ICs bound to FcγRIIB in WT DCs, binding was blocked with the anti-FcγRIIB Ab (anti-Ly17.2) and anti-FcγRII/III mAb, 2.4G2. Anti-Ly17.2 blocked ~40% of IC binding, implying that activating FcγRs (I, III, and IV) taken together bind the remaining 60% (Fig. 4*A*). We have shown previously that both activating and inhibitory FcRs internalize ICs at comparable rates; however, ICs endocytosed through FcγRIIB are excluded from the lysosomal compartment and remain cell associated, whereas those that enter through activating FcγRs are degraded (10). Thus, ICs endocytosed through FcγRIIB remain

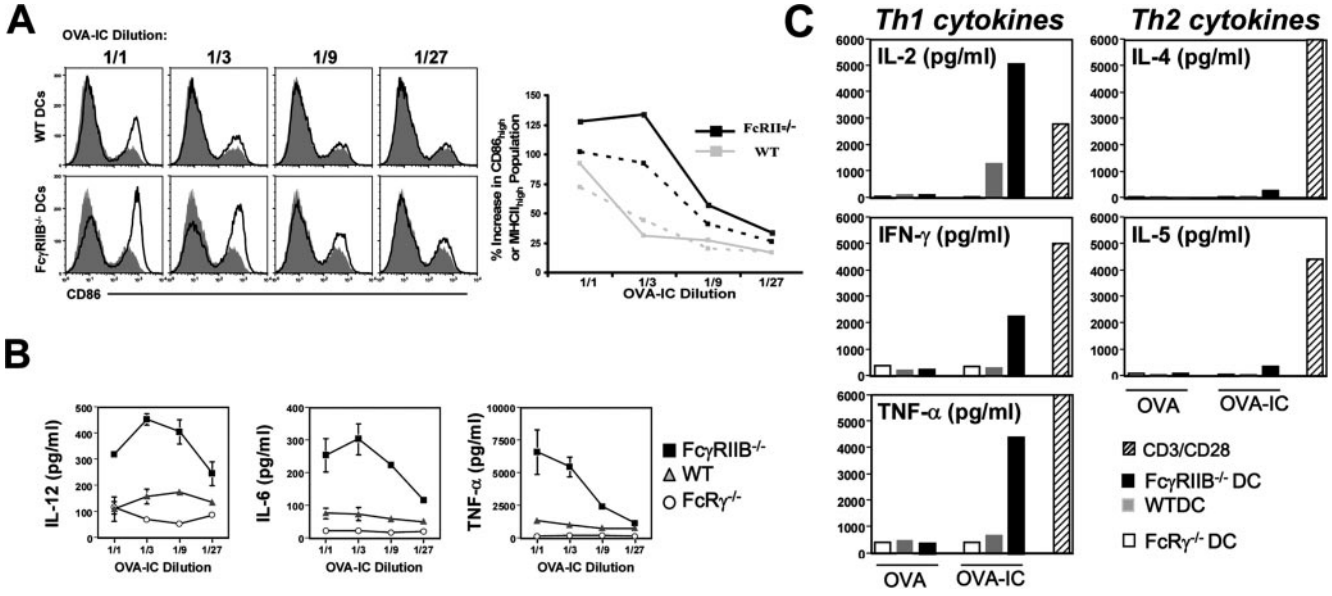

FIGURE 6. FcγRIIB negatively regulates DC maturation, proinflammatory cytokine production, and Th1 cell differentiation. *A*, CD86 expression on WT (*top row*) and FcγRIIB$^{-/-}$ (*bottom row*) BMDCs pulsed for 2 days with decreasing amounts of OVA-ICs (black open histograms). DCs that were not pulsed with OVA-ICs are indicated by the gray filled histograms. Threefold IC dilutions began at a starting concentration of 50 μg/ml anti-OVA:10 μg/ml OVA. Treatment with either OVA (10 μg/ml) or anti-OVA alone (50 μg/ml) did not result in increased maturation (data not shown). Percentage of maturation is shown quantitatively in the adjacent panel as both %CD86^{high} (dotted lines) and %MHC II^{high} (bold lines) populations. *B*, BMDCs from WT (triangles), FcγRIIB$^{-/-}$ (squares), and FcRγ$^{-/-}$ (circles) mice were pulsed with decreasing amounts of OVA-ICs. Data are expressed as the mean ± SD. Cytokine production by DCs pulsed with OVA alone or anti-OVA IgG alone was not greater than that seen with DCs cultured in the absence of Ag (data not shown). *C*, Cytokine production after secondary stimulation of CD4$^+$ OT-II T cells by DCs from WT (gray), FcγRIIB$^{-/-}$ (black), and FcRγ$^{-/-}$ (white) mice that were prepulsed with either OVA (100 μg/ml) or OVA-ICs. The potential of CD4$^+$ OT-II T cells to make both Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-4 and IL-5) cytokines was assessed by CD3/CD28 stimulation (hatched bars).

detectable 1 h after endocytosis in FcRγ$^{-/-}$, whereas ICs endocytosed through activating FcγRs are degraded after 1 h and are undetectable in FcγRIIB$^{-/-}$ (Fig. 4*C*). In this study, we compared the detection of internalized OVA-ICs with internalized FcOxyburst ICs, which consist of BSA-ICs coupled to a fluorochrome active only in an oxidative environment, e.g., an organelle containing an active NADPH oxidase (33) (Fig. 4*B*). In contrast to the situation seen when detecting unlabeled OVA-ICs with a secondary fluorescent Ab, FcγRIIB$^{-/-}$ DCs fluoresce brightly after pulsing with FcOxyburst ICs, whereas FcRγ$^{-/-}$ DCs show minimal fluorescence. In WT DCs, which express both activating and inhibitory FcRs, an intermediate level of fluorescence is observed. The failure of FcγRIIB-internalized ICs to reach an oxidative compartment could not be rescued by addition of LPS, which induces vesicular acidification (34) and augments NADPH oxidase activity (35) (data not shown). These results indicate that ICs endocytosed through activating FcγRs (in FcγRIIB$^{-/-}$ DCs) enter an oxidative and degradative pathway, whereas ICs endocytosed instead through FcγRIIB (in FcRγ$^{-/-}$ DCs) reach a nonoxidative and nondegradative compartment, suggesting the possibility that FcγRIIB might alter T cell responses by competitively interfering with the magnitude of signal 1 provided by Ag presentation through activating FcγRs.

FcγRIIB reduces cross-presentation, but not exogenous OVA presentation by DCs in vitro

To determine the consequences of uptake through FcγRIIB on Ag presentation to T cells, IC-pulsed BMDCs from WT, FcγRIIB$^{-/-}$, and FcRγ$^{-/-}$ mice were compared for their abilities to stimulate MHC I- and II-restricted T cell activation using both naive cells and hybrids derived from the TCR Tg OT-I and OT-II mice (Fig. 5). After uptake exclusively through activating FcγRs (by FcγRIIB$^{-/-}$ BMDCs), impressive IC enhancement (>1000-fold) of both MHC I- and II-restricted Ag presentation is seen in comparison with soluble OVA alone. FcγRIII$^{-/-}$ BMDCs exhibited substantial reductions in both MHC I and MHC II Ag presentation (MHC II > I), indicating a dominant role for this activating FcγR, as opposed to the remaining FcγRs, FcγRI and IV, in IC-mediated Ag presentation by BMDCs.

In contrast, exclusive antigenic uptake through FcγRIIB (by FcRγ$^{-/-}$ BMDCs) led to modest enhancement of OVA presentation to naive CD4 and CD8 T cells and almost no detectable presentation to MHC I- and II-restricted hybrids. Thus, by itself, entry of ICs through FcγRIIB inefficiently primes T cell responses. This was not further augmented by addition of LPS, indicating that the failure of Ags to be processed for Ag presentation after internalization through FcγRIIB is not due to the lack of induction of endosomal acidification occurring during DC maturation (34) (data not shown). This contrasts with some previous studies using transfected B cell systems as APCs; targeting of hen egg lysosome to FcγRIIB2 led to presentation of a subset of potential MHC II-restricted T cell epitopes, whereas a complete repertoire of potential T cell epitopes was presented after uptake via activating FcRs (20, 36). Our observations in DCs, that FcγRIIB-internalized Ags were inaccessible to the degradative processing pathway, led to the prediction that its expression on WT BMDCs would strongly compete with Ag presentation mediated by activating FcγRs. Indeed, FcγRIIB$^{-/-}$ DCs were more potent activators of OT-I CD8 T cells than WT DCs, indicating that the presence of FcγRIIB limits cross-presentation to both OT-I Tg T cells and hybrids (Fig. 5). Surprisingly, however, in comparing WT and FcγRIIB$^{-/-}$ BMDCs, the loss of FcγRIIB only modestly affects MHC II-restricted Ag presentation to OT-II CD4 cells. Similarly, BALB/c congenic WT and

FcγRIIB$^{-/-}$ BMDCs were also found to comparably induce IC-mediated proliferative responses of I-Ad-restricted, OVA-specific DO11.10 Tg CD4 T cells (data not shown). The limited ability of FcγRIIB to interfere with the activating FcγR exogenous pathway is not due to low levels of expression, because FcγRIIB is highly xpressed on BMDCs accounting for 40% of the fractional binding of total ICs bound to FcγRs on DCs (Figs. 3 and 4).

FcγRIIB strongly inhibits immunophenotypic DC activation and elaboration of chemokines and Th1-promoting cytokines

Having determined that initial OT-II CD4 proliferative responses were not quantitatively enhanced by FcγRIIB$^{-/-}$ DCs, we next pursued whether loss of FcγRIIB makes a more dramatic impact on the qualitative effector CD4 T cell outcome by modulating DC activation. ICs were added to 6-day immature BMDCs in increasing doses and DC maturation was assessed immunophenotypically. FcRγ$^{-/-}$ BMDCs were not sensitive to IC-mediated DC activation (data not shown). As seen from Fig. 6*A*, FcγRIIB$^{-/-}$ BMDCs are more sensitive than FcγRIIB$^{+/+}$ to IC-induced maturation, exhibiting comparable degrees of CD86 induction at 9-fold reduced quantities of ICs. Similar results were seen for other markers of DC maturation, including MHC II (Fig. 6*A*) as well as ICAM-1, CD40, and CD80 (data not shown). Thus, the presence of the inhibitory receptor FcγRIIB raises the threshold of cellular activation by ICs, implying that at low concentrations of ICs the presence of FcγRIIB down-modulates the strength of costimulation, a key requirement for the induction of effector T cell responses.

In addition to up-regulating costimulatory molecules, maturing DCs elaborate both chemokines and cytokines, which contribute to the recruitment and activation of effector T cells. IC-triggered FcγRIIB$^{-/-}$ DCs recruited 2- to 3-fold more splenic CD44^{low} naive CD4 and CD8 T cells in transwell migration assays than comparably treated FcγRIIB$^{+/+}$ DCs (data not shown). To address the impact of the inhibitory FcγRIIB pathway on the production of proinflammatory cytokines, WT, FcRγ$^{-/-}$, and FcγRIIB$^{-/-}$ BMDCs were cultured with OVA-ICs for 24 h and cytokines in the supernatant were quantified (Fig. 6*B*). ICs triggered the production of IL-12, TNF-α, and IL-6 by WT DCs, but not by FcγRγ$^{-/-}$ DCs. Thus, engagement of activating FcγRs induces DCs to produce all three Th1-promoting cytokines. Production of IL-12, TNF-α, and IL-6 was dramatically enhanced in the absence of FcγRIIB, revealing the importance of the inhibitory receptor in mediating suppression of these inflammatory cytokines. FcγRIIB-mediated inhibition of IL-12 production was not due to increased production of IL-10 (data not shown). Unbridled production of proinflammatory cytokines by IC-stimulated FcγRIIB$^{-/-}$ DCs would be predicted to have important consequences on the induction of cellular immune responses, including both Th1 polarization and memory cell generation of responder CD4 and CD8 T cells.

FcγRIIB inhibits the induction of Th1-polarized T cell responses

Previously, we have shown that IC-stimulated WT DCs polarize Th1-type responses. To directly examine the contribution of FcγRIIB engagement on Th1/Th2 polarization of responder T cells, OVA- or OVA-IC-pulsed DCs were cultured with OT-II T cells. After 7 days, the OT-II T cells were restimulated, and 3 days later supernatant was assessed for cytokine production. Under nonpolarizing conditions, CD3/CD28 stimulation induced OT-II T cell production of both Th1 and Th2 type cytokines, whereas stimulation with IC-loaded WT BMDCs induced production of IL-2 and a limited amount of Th1-type cytokines, including TNF-α and IFN-γ (Fig. 6*C*). In contrast, elaboration of effector Th1-type cytokines by OT-II T cells was dramatically increased after stimulation with IC-loaded FcγRIIB$^{-/-}$ BMDCs. Thus, IC-stimulated DCs preferentially polarize Th1-effector responses in a manner that can be strongly inhibitable by FcγRIIB.

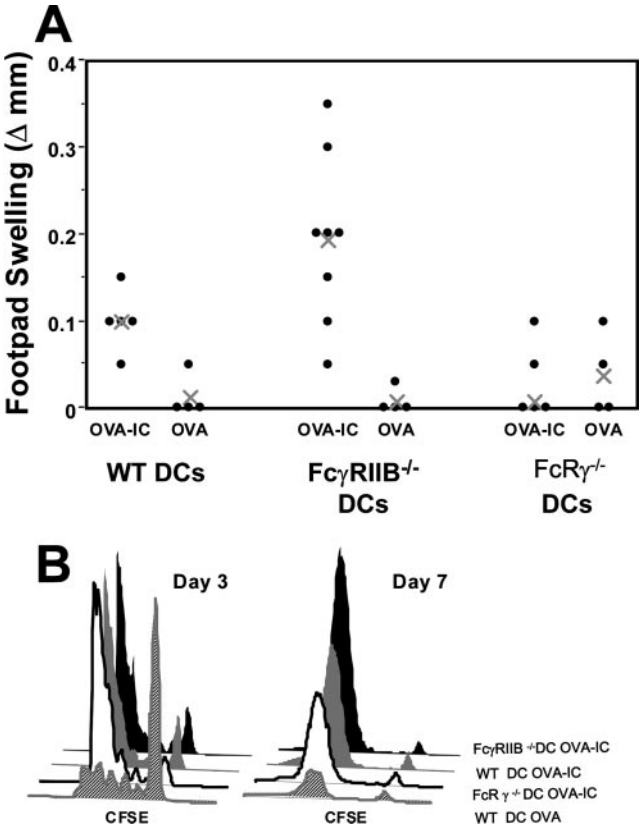

FIGURE 7. FcγRIIB expressed on DCs inhibits the development of memory Th1-type effector responses in vivo, without inhibition of the initial T cell proliferative response. *A*, WT mice were immunized with BMDCs from WT, FcγRIIB$^{-/-}$, and FcRγ$^{-/-}$ mice pulsed with either OVA-ICs or OVA. After 7 days, sensitized mice were challenged with OVA, and the severity of the DTH response was measured 24 h later. Each group contained four to eight mice (●); the mean for each group is also shown (X). The DTH induced in mice that received OVA-IC-pulsed FcγRIIB$^{-/-}$ DCs was significantly higher than the DTH induced in mice that received OVA-IC-pulsed WT DCs ($p < 0.02$, Student's *t* test). These data are representative of at least three independent experiments. *B*, Proliferation (3 days) and memory cell expansion (7 days) of CFSE-labeled CD45.1$^+$CD4$^+$ OT-II T cells were monitored in WT mice adoptively transferred with OVA- or OVA-IC-pulsed BMDCs. OT-II cells proliferated briskly 3 days after immunization with IC-loaded BMDCs, without appreciable differences seen between genotypes of immunizing DCs. However, accumulation of memory CD4$^+$ OT-II T cells after 7 days was greatest in the LNs of WT mice that received OVA-IC-pulsed FcγRIIB$^{-/-}$ BMDCs (3.2% CD45.1$^+$ of total LN cells) compared with WT (2.2%) or FcRγ$^{-/-}$ mice (1.9%). CD45.1$^+$CD4$^+$ OT-II T cells constituted only 0.6% of the CD4$^+$ cells in the LNs of WT mice that received OVA-pulsed WT BMDCs.

In vitro T cell proliferative responses do not necessarily reflect the situation in vivo in which Ag-loaded DCs must migrate to the T cell areas of lymphoid organs and present Ag to cognate T cells. Thus, OVA-specific Th1-mediated effector responses were assessed in WT mice immunized with OVA-IC-pulsed WT, FcγRIIB$^{-/-}$, or FcRγ$^{-/-}$ BMDCs. Recall responses were elicited 7 days later upon injection with s.c. OVA to induce DTH responses (Fig. 7*A*). DCs loaded with OVA (10 μg/ml) alone did not sensitize. However, potent DTH responses were elicited in mice injected with IC-loaded WT BMDCs. IC-loaded FcRγ$^{-/-}$ BMDCs

failed to elicit DTH responses, indicating that uptake through activating FcγRs is required for effective sensitization. Conversely, elimination of the inhibitory pathway enhanced sensitization, because DTH responses were exaggerated in recipients of IC-loaded FcγRIIB$^{-/-}$ BMDCs ($p < 0.02$). Thus, preferential engagement of the activating FcγRs on DCs promotes Th1-effector cell responses in vivo.

T cell priming by immunizing DCs was examined in WT recipients of OT-II T cells. CFSE-dilutional analysis of transferred naive OT-II Tg CD4 T cells demonstrated robust and indistinguishable OT-II proliferative responses seen 3 days after transfer of either WT or FcγRIIB$^{-/-}$ BMDCs (Fig. 7*B*), confirming our observations with in vitro primed T cells (Fig. 5) and suggesting that the differences in Th1 priming by WT and FcγRIIB$^{-/-}$ BMDCs are not simply due to quantitative differences in antigenic processing. Despite comparable initial proliferative responses, the accumulation of CD44^{+} memory OT-II T cells at 7 days after priming in vivo was significantly enhanced after immunization with FcγRIIB$^{-/-}$ DCs (Fig. 7*B*). Thus, FcγRIIB on DCs limits the development of activated CD4 memory Th1-type effectors without impacting their initial proliferative response, most likely the consequence of inhibition of DC activation without alteration of antigenic processing.

Discussion

ICs can be endocytosed through either complement receptors or FcγRs present on a variety of cell types, including DCs, myeloid-lineage cells, follicular DCs, and B cells. DCs isolated from OVA-IC-injected mice have been shown to present Ag ex vivo to both OT-I and OT-II T cells (12); however, the contributions of other cell types have not been determined. Using the CD11c-DTR mouse model, we show that specific depletion of the CD11c^{+} DC compartment abrogated the presentation of ICs to both CD4 and CD8 cells in vivo (Fig. 2). In this model, DT treatment also depletes marginal zone macrophage subsets (31), yet immunofluorescence studies demonstrated that injected ICs localized readily to the marginal zone/red pulp (data not shown). Although roles for subpopulations of marginal zone splenic macrophages cannot be ruled out (31), these data demonstrate that DCs are also required for T cell priming by endocytically acquired soluble Ag. Thus, regulation of IC-mediated Ag presentation by activating and inhibitory FcγRs on DCs would be expected to critically modulate not only autoantibody-triggered loss of T cell tolerance, but also recall T cell responses to foreign Ag.

We have developed an Ab-triggered model for T cell-mediated diabetes to evaluate the roles of activating and inhibitory FcγRs in the cross-priming of diabetogenic T cells (27). We show in this study that augmented effector OT-I responses develop in FcγRIIB$^{-/-}$ RIP-mOVA mice, resulting in a lowered threshold for diabetes development (Fig. 1). Thus, FcγRIIB-deficient mice have enhanced Ab-triggered cross-priming by self Ag. The enhanced priming of OT-I T cells in FcγRIIB$^{-/-}$ RIP-mOVA is due to the loss of FcγRIIB on DCs because DCs are required for both CD4 and CD8 proliferative responses to exogenous IC and islet OVA (Fig. 2) (27). These results demonstrate that FcγRIIB on DCs can control autoimmunity by limiting T cell priming by self Ag-containing ICs. Thus, in addition to modulating the pathogenic effects of the autoimmune humoral response (37–39), FcγRIIB may also limit the development of autoimmunity in which self-reactive cells, rather than autoantibodies, are the major effectors of injury.

In dissecting the inhibitory mechanisms of FcγRIIB on DCs, we have examined the role of FcγRIIB on both Ag uptake/presentation and cellular activation. We show that after uptake of Ags exclusively through FcγRIIB, there are limited degradation and modest or absent Ag presentation to OT-I and OT-II T cell hybrids and naive Tg T cells. We predicted that in WT DCs, the presence of FcγRIIB would compete with activating FcγRs for limiting amounts of internalized ICs, resulting in diminishing Ag presentation. Indeed, this is apparently true for cross-presentation of OVA to OT-I T cells, but is not relevant for MHC II-restricted Ag presentation to OT-II cells. Thus, FcγRIIB$^{-/-}$ and WT BMDCs exhibited very similar functional capacity to activate both MHC II-restricted hybrids in vitro (Fig. 5) and induce similar T proliferative responses in vivo (Fig. 7*B*), suggesting that activating and inhibitory exogenous processing pathways are parallel and noncompetitive. Although these data have been confirmed using another OVA-specific MHC II-restricted Tg CD4 cell, namely D011.10 Tg CD4 T cells, the generalizability of these observations will need verification in other Ag systems.

Loss of FcγRIIB on DCs makes a significantly greater impact on the OVA-specific Th1-effector outcome through regulation of DC activation than on quantitative Ag presentation. Immunophenotypic maturation and chemokine/cytokine production were dramatically enhanced in FcγRIIB$^{-/-}$ DCs, resulting in Th1-polarized responses in vitro (Fig. 6). Thus, acquisition of ICs through activating FcγRs, in the absence of coengaged FcγRIIB, led to greater accumulation of long-lived T cells and Th1-type polarized responses in vivo without altering initial proliferative responses (Fig. 7). As a consequence, we find that FcγRIIB negatively regulates both the Th1-type CD4 response to exogenous Ag (DTH, Fig. 7*A*) and the CD8 cellular response to self Ag and tumor Ag (Fig. 1, and data not shown). The preferential induction of a Th1-type cytokine profile by engagement of activating FcRγ is consistent with recent studies using human cultured DCs (5, 8, 9), but contrasts with the findings of Anderson and Mosser (40) and Anderson et al. (41), who noted a Th2 profile with combined TLR and FcγR stimuli.

Taken together, these data demonstrate that FcγRIIB plays a prominent role in preventing IC-mediated activation of DCs and potently inhibits the elaboration of costimulatory molecule expression (signal 2) and cytokine production (signal 3), which are required to potently induce Th1-type and CD8-mediated cellular immunity. In contrast, FcγRIIB appears to inhibit antigenic processing through the exogenous pathway to a lesser extent than through the cross-presentation pathway. Thus, we provide two illustrative examples of the ability of FcγRIIB to modulate the quality and quantity of the T cell response. In the case of OT-I activation, FcγRIIB limits T cell activation through inhibiting both antigenic processing and DC activation, whereas OT-II Th1-effector responses are restrained primarily by preventing DC activation. This suggests the possibility that, at least for some antigenic systems, FcγRIIB may limit DC activation while leaving signal 1 intact for the maintenance of peripheral tolerance. Indeed, FcγRIIB-deficient mice were shown recently to generate reduced numbers of Ag-specific regulatory T cells in a mucosal tolerance model (42).

FcγRIIB has been shown previously to function at multiple levels to limit the induction and consequences of inflammatory responses triggered by autoantibody. For instance, FcγRIIB on innate myeloid effectors inhibits Ab-triggered effector responses (37), whereas its presence on B cells limits the generation of self-reactive plasma cells (38, 39). In this study, we show that FcγRIIB on DCs blocks a proximal step in inflammatory responses mediated by self-reactive T cells by inhibition of their priming by DCs.

In vivo DCs coexpress both activating and inhibitory FcγRs, suggesting that their regulated expression may contribute to autoimmune disease susceptibility in cases in which autoantibodies are present, but T cells are the dominant effectors, e.g., multiple sclerosis, type I diabetes, and rheumatoid arthritis.

Disclosures

The authors have no financial conflict of interest.

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