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FcγRIIB Regulates Nasal and Oral Tolerance: A Role for Dendritic Cells

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Mucosal tolerance prevents the body from eliciting productive immune responses against harmless Ags that enter the body via the mucosae, and is mediated by the induction of regulatory T cells that differentiate in the mucosa-draining lymph nodes (LN) under defined conditions of Ag presentation. In this study, we show that mice deficient in FcγRIIB failed to develop mucosal tolerance to OVA, and demonstrate in vitro and in vivo a critical role for this receptor in modulating the Ag-presenting capacity of dendritic cells (DC). In vitro it was shown that absence of FcγRIIB under tolerogenic conditions led to increased IgG-induced release of inflammatory cytokines such as MCP-1, TNF-α, and IL-6 by bone marrow-derived DC, and increased their expression of costimulatory molecules, resulting in an altered immunogenic T cell response associated with increased IL-2 and IFN-γ secretion. In vivo we could show enhanced LN-DC activation and increased numbers of Ag-specific IFN-γ-producing T cells when FcγRIIB$^{-/-}$ mice were treated with OVA via the nasal mucosa, inferring that DC modulation by FcγRIIB directed the phenotype of the T cell response. Adoptive transfer of CD4$^+$ T cells from the spleen of FcγRIIB$^{-/-}$ mice to naive acceptor mice demonstrated that OVA-responding T cells failed to differentiate into regulatory T cells, explaining the lack of tolerance in these mice. Our findings demonstrate that signaling via FcγRIIB on DC, initiated by local IgG in the mucosa-draining LN, down-regulates DC activation induced by nasally applied Ag, resulting in those defined conditions of Ag presentation that lead to Tr induction and tolerance. *The Journal of Immunology,* **2005, 174: 5279–5287.**

Mucosae, as found in the nose and the gastrointestinal tract, are continuously in contact with exogenous Ag. The mucosal immune system is adapted to choose between T cell activation and tolerance, depending on pathogenicity of the Ag, to avoid unnecessary immune responses against exogenous Ags that are not detrimental to the host (1, 2). A decisive step in the development of mucosal tolerance is the induction of Ag-specific regulatory T cells (Tr)[3] that suppress the activation and differentiation of potentially harmful responder T cells (3–8). Recently, we have shown that mucosal Tr are highly discriminative, suppressing inflammatory responses exclusively to the Ag used for tolerization and expanding their suppressive capacity by infectious tolerance (9). These mucosal Tr differentiate from naive CD4$^+$ T cells in the mucosa-draining lymphoid tissue within 72 h post-Ag application and reside in both CD25$^+$ as well as CD25$^-$ subsets (10, 11). The precise mechanisms underlying the induction of mucosal Tr and subsequent development of tolerance are the focus of extensive research that may yield important clues on the development of selective immunomodulatory therapies for T cell-mediated inflammatory diseases, ranging from autoimmunity to transplant rejection and allergy.

The mucosa-draining lymph nodes (LN) are essential for the induction of mucosal tolerance, based on the observation that removal of these LN in mice abrogates mucosal tolerance (8). However, nasal tolerance could not be achieved when nose-draining cervical LN (CLN) were replaced by peripheral LN (8), suggesting that neither uptake of Ag through the mucosal surface, nor the uptake and transport of Ag APCs, such as dendritic cells (DC), from the mucosal surface to the draining CLN is sufficient for mucosal tolerance induction. Therefore, we hypothesize that a defined local microenvironment within the specific mucosa-draining LN is essential for the development of mucosal tolerance. As a consequence, specific conditions for Ag presentation by DC may be created in the mucosa-draining LN, resulting in Tr differentiation and tolerance. These conditions are mostly unidentified, but may include the failure or attenuation of DC maturation, as defined by low expression of costimulatory markers and decreased release of cytokines such as IL-12 (12–15).

Comparison of the gene expression profile of whole CLN and peripheral LN from naive mice revealed a difference in gene expression of IgG2b, which correlated with an increased number of IgG2b-producing plasma cells in the CLN compared with peripheral LN (16). We hypothesize that such mucosa-specific Ig expression may differentially modulate DC function by selective engagement of Ig FcR (17). FcR constitute a family of surface molecules expressed on hemopoietic cells that are capable of stimulating or inhibiting cellular responses upon being immobilized by their ligand, Ab-Ag complexes (18, 19). Types I and III FcR for IgG

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[3] Abbreviations used in this paper: Tr, regulatory T cell; BM-DC, bone marrow-derived DC; CLN, cervical LN; DC, dendritic cell; DTH, delayed-type hypersensitivity; LN, lymph node; MFI, mean fluorescence intensity; WT, wild type.

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(FcγRI and FcγRIII) are expressed primarily on cells of the myeloid lineage and can mediate effector functions such as phagocytosis, Ab-dependent cytotoxicity, and release of inflammatory mediators (18). Signaling via these receptors leads to activating signals through intracellular ITAM motifs (20). In contrast, the type II receptor (FcγRII), which is found on both myeloid and lymphoid cells, provides inhibitory signals via ITIMs (21). When stimulated, DC can express both activating FcγRIII and inhibitory FcγRIIB receptors. Therefore, the functional outcome of immune complex-induced stimulation should reflect a balance between opposing stimulatory and inhibitory signals. The implications of such a balance for subsequent $CD4^+$ T cell responses have not yet been elucidated. However, absence of FcγRII renders mice more susceptible to inducible forms of autoimmunity and spontaneous autoimmunity, suggesting that this receptor plays a role in establishing tolerance to self Ags (18, 19). Based on these findings, we questioned whether, in the mucosa-draining LN, signaling via FcγRIIB plays a role in inhibiting Ag presentation and DC maturation, leading to Tr induction and subsequent mucosal tolerance to exogenous Ag.

Materials and Methods

Mice

BALB/c mice were obtained from Charles River Laboratories and kept in our animal facility under routine laboratory conditions. Eight- to 10-wk-old FcγRIIB$^{-/-}$ mice on 129/C57BL/6 background and wild-type (WT) controls were provided by S. Verbeek (Leiden University Medical Center, Leiden, The Netherlands). FcγRIIB$^{-/-}$ mice on BALB/c background were obtained from Taconic Farms. OVA-specific TCR transgenic mice on BALB/c background (DO11.10 mice) were bred at the Vrije Universiteit University Medical Center. All experiments performed were approved by the Animal Experiments Committee of the Vrije Universiteit University Medical Center.

Mucosal tolerance induction and delayed-type hypersensitivity (DTH) reaction

For induction of nasal tolerance, FcγRIIB$^{-/-}$ mice and WT controls received three doses of 100 μg of OVA protein (OVA type VII; Sigma-Aldrich)/10 μl of saline intranasally on each of 3 consecutive days. For oral tolerance induction, mice received a single feed of 25 mg of OVA intragastrically. The development of tolerance was read out by sensitizing the mice 8 days after the last intranasal or oral administration by injecting 100 μg of VA/25 μl of saline/25 μl of IFA (Difco Laboratories) s.c. in the tail base. As challenge for the DTH response, 10 μg of OVA/10 μl of saline was injected 5 days later in the auricle of each ear. The increase of ear thickness was measured with an engineer's micrometer (Mitutoyo) at 24 h after the challenge and compared with the ear thickness, as measured before OVA injection. Measurements were performed in blinded fashion. Values are expressed as the mean increase in ear thickness of both ears at 24 h postchallenge.

Flow cytometry

Single cell suspensions of CLN from WT and FcγRIIB$^{-/-}$ were prepared by straining the tissues through a 100-μm gauze. LN cells, or DC that had been cultured in vitro, were washed in PBS containing 2% heat-inactivated FCS (BioWhittaker) (FACS buffer), and aliquots were incubated with Abs: rat anti-CD86 (clone GL-1), rat anti-CD40 (clone 3/23), FITC-conjugated rat anti-MHCII (clone M5/114), PE-conjugated rat anti-CD80 (clone 1G10), PE-conjugated hamster anti-CD11c (HL3), rat anti-B220 (clone 6B2), and rat anti-αβTCR (clone H57-597) at the appropriate concentrations. Supernatant obtained from the murine K9361 hybridoma (22) specific for the Ly-17.1 allele of the FcγRIIB, a kind gift of M. Daeron (Institut Curie, Paris, France), was purified, biotinylated according to manufacturer's protocol (Molecular Probes), and used to detect FcγRIIB. Subsequently, the cells were washed three times with FACS buffer and, when conjugate staining was necessary, the cells were incubated on ice for 30 min with PE-conjugated donkey anti-rat Ig (The Jackson Laboratory) or streptavidin-PE (Vector Laboratories), as appropriate. After incubation, the cells were washed and resuspended in FACS buffer, and fluorescence was measured using a FACSCalibur (BD Biosciences). A total of 50,000, 70,000, or 250,000 events per sample was analyzed. Cells that had been incubated with concentration matched isotype control and, when required with conjugate, served as negative controls.

DC culture

Bone marrow-derived DC (BM-DC) were cultured from BALB/c or FcγRIIB$^{-/-}$ mice. Briefly, on day 0, femurs and tibia of adult donor mice were flushed, and the resulting BM suspension was passed through a 100-μm gauze to obtain a single cell suspension. The cells were seeded at 2×10^6 per petri dish in IMDM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 50 U/ml sodium penicillin-G (BioWhittaker), 5×10^{-5} M 2-ME (Merck), and 20 ng/ml murine rGM-CSF (X63-GM-CSF-producing cell line supernatant (23)). On days 3 and 6, 20 and 10 ng/ml murine rGM-CSF was added, respectively, in 10 ml of fresh IMDM. On day 8, the nonadherent cells consisting of immature and mature BM-DC were harvested and used for subsequent experiments.

In vitro Ig stimulation and FcγRIIB neutralization

Twenty-four-well plates (Greiner Bioscience) were coated with purified, endotoxin-low, mouse IgG1 anti-trinitrophenyl (BD Pharmingen) at 10 μg/ml in PBS, or PBS alone as a control, at 37°C. After a 60-min incubation, the coating solution was removed and DC were seeded in the wells at 1×10^6/ml. In several experiments, FcγRIIB signaling was neutralized by first preincubating the DC in suspension with K9361 mAb or the isotype control mAb ED6 that stains rat stromal reticular cells and has no known reactivity against mouse tissues (24), both at a concentration of 10 μg/ml in PBS on ice for 60 min. After incubation, the cells were centrifuged, resuspended in IMDM, and seeded in the coated 24-well plates at 1×10^6/ml in the presence or absence of 10 μg/ml K9361 or ED6. After a 60-min incubation, the cells were stimulated with LPS from *Escherichia coli* 0111:B4 (Difco Laboratories) at a concentration of 1 ng/ml. The cells were cultured at 37°C and 5% CO_2, and after 24 h, the supernatant was collected and stored at −80°C, and the cells were recovered for further analysis.

Kinetics of OVA-specific T cell response in vitro and in vivo

OVA-TCR transgenic T cells were isolated and labeled, as described previously (10, 11). Briefly, at 8–12 wk of age, DO11.10 transgenic mice were sacrificed, and LN and spleens were collected. Single cell suspensions of spleens and LN were prepared separately by straining the tissues through a 100-μm gauze. Erythrocytes in the splenocyte suspension were lysed by incubation with lysis buffer (150 mM NH_4Cl, 1 mM $NaHCO_3$, pH 7.4) for 5 min on ice. Cell suspensions were depleted of B cells, $CD8^+$ T cells, and APC by incubation with a mixture of mAb containing anti-B220, anti-CD8, MAC-2, F4/80, and anti-MHC-II, and subsequently removing labeled cells with sheep anti-rat IgG Dynabeads, according to manufacturer's instructions (Dynal Biotech). Thereafter, the remaining $CD4^+$ T cell-enriched fraction was labeled with CFSE (Molecular Probes). The proportion of OVA-TCR transgenic$^+$ $CD4^+$ T cells in suspension was determined by flow cytometric analysis of an aliquot of cells using the clonotypic Ab KJ1-26 (anti-OVA transgenic TCR) and anti-CD4 (GK1.5). For in vitro T cell differentiation studies, 2×10^5 CFSE-labeled KJ1-26$^+$$CD4^+$ T cells were cocultured with 1×10^5 BM-DC in the presence or absence of 100 nM $OVA_{323–339}$ peptide. At 72 h postculture, T cells and supernatants were harvested for analysis.

For in vivo T cell differentiation studies, the cells were diluted to 1×10^7 KJ1-26$^+$$CD4^+$ T cells/100 μl in saline. Naive FcγRIIB$^{-/-}$ or WT acceptor mice (on BALB/c background) were injected i.v. with 1×10^7 KJ1-26$^+$$CD4^+$ T cells. One day after adoptive transfer of KJ1-26$^+$$CD4^+$ T cells, mice were tolerized by administration of 15 μl of saline containing 400 μg of OVA (type VII; Sigma-Aldrich) intranasally. At 48 h posttolerization, nose-draining CLN were removed, and single cell suspensions were analyzed for cell division by flow cytometry.

Measurement of cytokine-producing cells and cytokine release

The percentage of OVA-specific T cells secreting IL-2, IL-4, IL-10, and IFN-γ was determined using the appropriate murine cytokine secretion assays (Miltenyi Biotec), according to manufacturer's instructions.

The concentrations of murine MCP-1, TNF-α, IL-6, IL-2, IL-10, IFN-γ, and IL-12p70 in the culture supernatants were determined using a cytometric bead array from BD Pharmingen. Reagents for the IL-12p40 ELISA were obtained from BioSource International. For staining of intracellular IL-12, single cell suspensions of CLN were prepared, as described earlier. The cells were first stained for CD11c and thereafter fixed and permeabilized with the Cytofix/Cytoperm Plus kit with Golgistop (BD Pharmingen) and stained with PE-labeled rat anti-mouse IL-12 p40/p70 (clone C15.6) or PE-labeled rat-IgG1 isotype control (clone R3-34), according to manufacturer's instructions.

Adoptive transfer of total or subsets of CD4$^+$ splenocytes from tolerized mice to naive recipients

FcγRIIB$^{-/-}$ or WT acceptor mice (on BALB/c background) were tolerized via the nasal mucosa and sensitized for a DTH response, as described above. Measurement of the DTH response confirmed that WT mice had become tolerant, whereas FcγRIIB$^{-/-}$ mice had not. Seven days after challenge, spleens from WT and naive control mice were collected. Single cell suspensions were obtained by mincing the spleens and straining them through 100-μm gauze. The cell suspensions were enriched for CD4$^+$ T cells, as described above. CD4$^+$-enriched cells were adoptively transferred to naive BALB/c acceptor mice by the i.v. injection of 5×10^5 CD4$^+$ T cells. For transfer of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells, CD4$^+$ cells were labeled with biotinylated anti-CD25 Ab (clone PC61; BD Pharmingen) for 20 min on ice, followed by anti-biotin immunomagnetic beads (MACS; Miltenyi Biotec) for 20 min at 4°C, and separated using an MS column, according to the manufacturer's protocol. Purified CD4$^+$CD25$^+$ (98% CD25$^+$) and CD4$^+$CD25$^-$ (98% CD25$^-$) T cells were transferred at 2×10^5 cells/mouse. One day after adoptive transfer, all mice were sensitized, and 5 days thereafter, mice were challenged according to the DTH protocol described above.

Statistics

For ear-swelling responses, the mean increase in ear thickness of both ears was determined for each mouse per group. For all experiments, groups were compared by ANOVA, followed by a two-sided Tukey-Kramer multiple comparisons test. A p value of <0.05 was considered significant.

Results

FcγRIIB is essential for mucosal tolerance

We recently detected a difference in gene expression of IgG2b in the CLN compared with peripheral LN of naive mice, which correlated with an increased number of IgG2b-producing plasma cells in the CLN (16). If such differential Ig expression is involved in modulation of DC function mediating tolerance at mucosa-draining sites, it would require a role for Ig FcR. We therefore established whether the inhibitory FCR, FcγRIIB, regulates mucosal tolerance induction. FcγRIIB$^{-/-}$ mice and WT controls received intact OVA via the nasal mucosa, and were subsequently subjected to a standard systemic DTH challenge (9, 11) to assess whether tolerance to OVA had developed. In contrast to WT controls, FcγRIIB$^{-/-}$ mice were not able to suppress a DTH response after nasal pretreatment with OVA, as can be seen from Fig. 1*A*, in which comparable increases in ear thickness in FcγRIIB$^{-/-}$-tolerized and nontolerized groups are seen.

It is unclear whether nasal and oral tolerance develop according to similar mechanisms, and so development of tolerance in FcγRIIB$^{-/-}$ mice by ingestion of OVA via the gastrointestinal tract was also assessed. As shown in Fig. 1*B*, the intragastric ingestion of OVA by FcγRIIB$^{-/-}$ mice also failed to induce suppression of a DTH response to OVA, whereas WT mice became tolerant.

In conclusion, these findings show that FcγRIIB is essential for mucosal tolerance induction via both nasal and oral routes.

Absence of FcγRIIB function on BM-DC alters Ag presentation, leading to an altered T cell response in vitro

FcγRIIB functions by providing inhibitory signals through ITIMs (21). The balance between inhibitory signals from FcγRIIB and stimulatory signals from FcγRI and FcγRIII may determine the outcome of the response of DC to exogenous Ag: tolerance or activation. We therefore investigated whether the demonstrated requirement for FcγRIIB in the establishment of nasal and oral tolerance (Fig. 1) could be due to the inhibitory effect of FcγRIIB on DC phenotype and function.

Immature BM-DC from FcγRIIB$^{-/-}$ and WT mice were therefore stimulated in vitro with immobilized IgG1, and either neutralizing anti-FcγRIIB Ab or isotype control, where appropriate, and subsequent changes of costimulatory molecule expression and cytokine secretion were investigated. Stimulation was elicited by the presence of low doses (1 ng/ml) of LPS to mimic priming of BM-DC as occurs upon Ag encounter in vivo.

Stimulation with immobilized IgG1 in the presence of LPS significantly increased the expression of CD80 (data not shown), MHC class II (data not shown), CD86, and CD40 on BM-DC from both FcγRIIB$^{-/-}$ and WT mice when compared with DC incubated with LPS alone (Fig. 2, *A* and *B*). However, when the signaling via the FcγRIIB on DC was either absent or neutralized using the mAb K9361, stimulation with immobilized IgG and LPS dramatically further increased the expression of CD86 and CD40 expression (Fig. 2, *A* and *B*), whereas the expression of CD80 and MHCII was only slightly or not affected (data not shown). LPS priming was not essential to demonstrate the induction of enhanced costimulatory molecule expression on FcγRIIB-deficient BM-DC, as incubation with immobilized IgG with and without OVA also led to enhanced CD86 and tended to increase CD40 expression in FcγRIIB-deficient BM-DC (Fig. 2*D*). The up-regulation of costimulatory molecules was accompanied by an enhanced secretion of the inflammatory cytokines MCP-1, TNF-α, and IL-6 in the absence of FcγRIIB function (Fig. 2*C*).

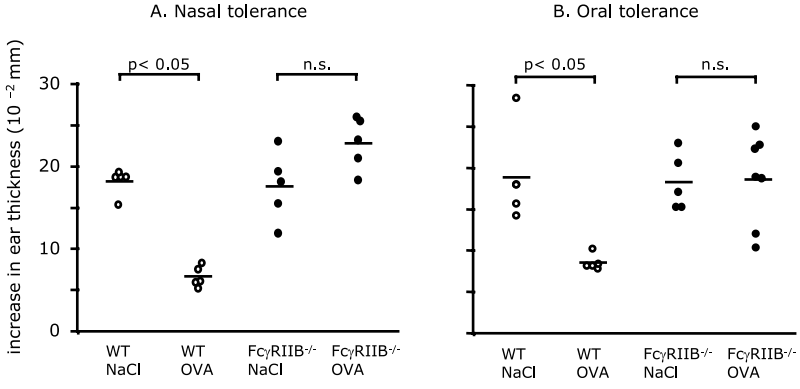

FIGURE 1. FcγRIIB is essential for the induction of mucosal tolerance. Nasal tolerance was induced in FcγRIIB$^{-/-}$B6 (●) mice and WT (○) controls by the intranasal administration of OVA on each of 3 consecutive days (*A*). Oral tolerance was induced by giving mice a single feed of 25 mg of OVA intragastrically (*B*). Controls received saline via respective routes. The establishment of tolerance was assessed by sensitizing the mice with OVA/IFA s.c. in the tail base and 5 days later injecting 10 μg of OVA protein in the auricle of each ear as a challenge DTH response. The increase of ear thickness was measured at 24 h after the challenge and compared with the ear thickness as measured before OVA injection. Values are expressed as the mean increase in ear thickness of both ears of one mouse at 24 h postchallenge relative to the mean ear thickness before challenge (n.s. = nonsignificant). These data are representative of three separate experiments.

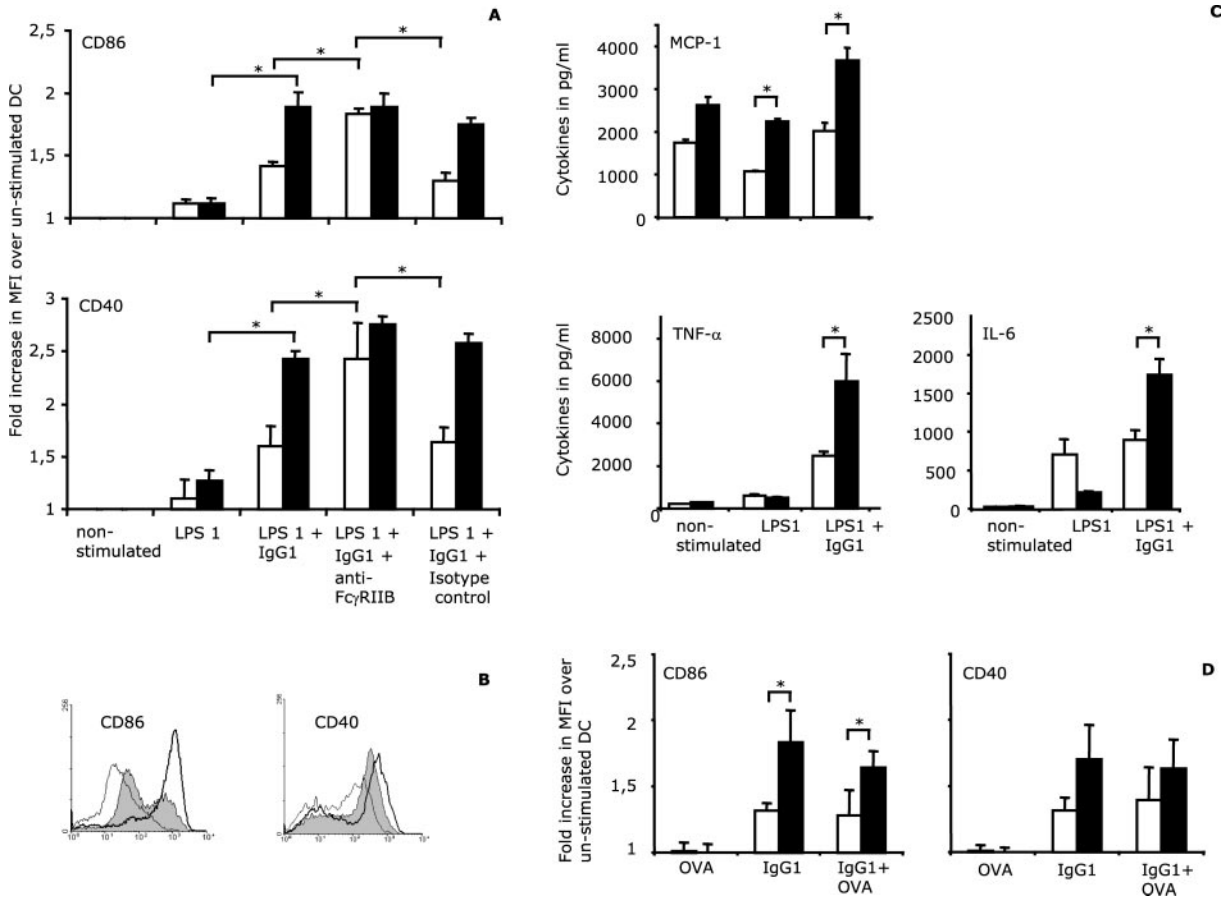

FIGURE 2. FcγRIIB regulates the profile of costimulatory molecule expression by BM-DC in vitro. Twenty-four-well plates were coated with mouse IgG1 anti-trinitrophenyl, or with PBS as a control at 37°C. After 60 min, the coating solution was removed, and either WT or FcγRIIB$^{-/-}$ BM-DC were seeded in the wells at 1×10^6 cells/ml. FcγRIIB signaling was neutralized in WT BM-DC by preincubating the BM-DC in suspension with K9361 mAb or isotype control ED6 mAb. After preincubation, the cells were washed and seeded in the coated 24-well plates in the presence or absence of K9361 or ED6, as appropriate. After a 60-min incubation, the cells were stimulated with 1 ng/ml LPS or 1 mg/ml OVA and cultured during 24 h. Thereafter, the cells were recovered for flow cytometric analysis. *A* and *B*, FcγRIIB inhibits IgG-induced stimulation of costimulatory molecules on BM-DC. *A*, Increase of costimulatory molecule expression expressed as MFI over unstimulated DC; $n = 3 \pm$ SD; □ = WT; ■ = FcγRIIB$^{-/-}$. *B*, Histograms are representative of $n = 3$ separate experiments in WT mice; thin line, LPS treatment; shaded histogram, LPS + immobilized IgG1; thick line, LPS + immobilized IgG1 + K9361. *C*, Cytokine concentrations in supernatant at $t = 24$ h. *D*, Increase of costimulatory molecule expression in the absence of LPS priming; $n = 3$; mean + SD; ∗, indicates $p < 0.05$.

The possible translation of this observed increased BM-DC activation into an altered T cell response was assessed by loading BM-DC with OVA peptide, and determining T cell proliferation after 72 h of coculture with CFSE-labeled OVA-TCR transgenic (DO11.10) T cells. The absence of FcγRIIB on BM-DC did not affect the number of dividing cells nor their kinetics of division relative to observations for WT BM-DC (Fig. 3*A*). However, the division of FcγRIIB$^{-/-}$-BM-DC-stimulated T cells was associated with a vast increase in IL-2 secretion, and a slight, but consistently enhanced IFN-γ release, relative to the division of WT-BM-DC-stimulated T cells (Fig. 3*B*).

These results clearly demonstrate that stimulation of BM-DC with IgG in the absence of FcγRIIB function enhances BM-DC function, with consequent changes in the elicited T cell response. The inhibitory function of FcγRIIB in this respect is clear, in line with its requirement in the generation of nasal and oral tolerance.

Altered DC activation in the nose-draining CLN of FcγRIIB$^{-/-}$ mice results in a different T cell response

We next investigated whether the in vitro observed enhancement of BM-DC activation and resulting altered T cell response would lead to similar functional changes in the in vivo generation of nasal tolerance. We have previously shown that mucosal Tr differentiate from naive T cells in the nose-draining CLN within 72 h post-Ag application (11). Within that time, the Tr attain all requirements for their suppressive function, even though their phenotype is highly similar to that of nonregulatory T cells (11). We therefore hypothesized that any balancing effect of FCγRIIB on Ag presentation required for mucosal Tr induction would be exerted in the mucosa-draining CLN within the first 72 h post-OVA treatment. In CLN, both DC and B cells express FcγRIIB irrespective of saline or OVA treatment (data not shown).

Indeed, at 24 h postnasal OVA application, secretion by DC of the inflammatory cytokines TNF-α, IL-6, and IL-12p70 was significantly higher in the CLN of FcγRIIB$^{-/-}$ mice than WT mice (Fig. 4, *A* and *B*). This increase in secretion was not due to enhanced DC influx into the node, as total numbers of CD11c$^+$ cells in the CLN were comparable for FcγRIIB$^{-/-}$ and WT mice (data not shown). Furthermore, based on the number of CD11c$^+$ cells within the node that expressed IL12p40, and the amount of this cytokine released in the culture, it was calculated that the amount of IL-12p40 released per CD11c$^+$cell was significantly enhanced in FcγRIIB$^{-/-}$ mice (Fig. 4*C*). MCP-1 secretion also tended to increase (Fig. 4*A*), but this was not significant.

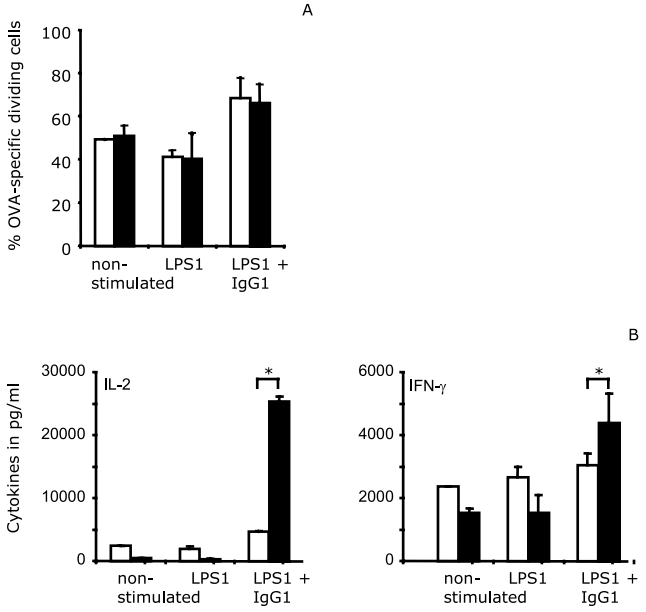

FIGURE 3. BM-DC lacking FcγRIIB function induce an alternative T cell differentiation after stimulation with IgG. BM-DC were stimulated, as described in Fig. 2, and 1×10^5 cells were incubated with 2×10^5 CFSE-labeled OVA-TCR transgenic (DO11.10) T cells in the presence or absence of 100 nM $OVA_{323–339}$ peptide. At 72 h postincubation, cells and supernatants were harvested, and the proliferation and secretion of cytokines were determined. *A*, The percentage of OVA-specific dividing T cells, that is cells that had undergone at least one division based on CFSE dilution, was assessed by flow cytometry ($n = 3$). *B*, Cytokine release at 72 h postculture ($n = 3$); mean + SD; □ = WT; ■ = FcγRIIB$^{-/-}$. ∗, Indicates $p < 0.05$.

After nasal OVA administration in FcγRIIB$^{-/-}$ mice, no increased up-regulation of costimulatory molecules on DC in the CLN could be detected in vivo, as demonstrated by the finding that at 24 h post-OVA the mean fluorescence intensity (MFI) of CD40 (2172.9 ± 224.4) and CD86 (3259.9 ± 22.4) on CD11c$^+$ cells in WT mice was comparable to the MFI of CD40 (2100.0 ± 131.7) and CD86 (3307.6 ± 194.9) in FcγRIIB-deficient mice. These data suggest that local IgG in the mucosa-draining LN modulates DC activity in an FcγRIIB-dependent manner.

Having established that enhanced DC activation occurs in the CLN of intranasally treated FcγRIIB$^{-/-}$ mice, we proceeded to investigate the consequences for the resulting T cell response. A possible change in the OVA-specific T cell response was assessed by adoptively transferring FcγRIIB$^{-/-}$ mice and WT mice on BALB/c background with OVA-specific TCR transgenic (DO11.10) CD4$^+$ T cells that had been labeled with CFSE. One day later, these mice were treated with OVA intranasally, and 72 h thereafter, the kinetics of division of CFSE-labeled OVA-specific T cells in the CLN were measured. OVA-specific CD4$^+$ T cells of both WT and FcγRIIB$^{-/-}$ mice had undergone between zero and four divisions (Fig. 5*A*). The percentage of OVA-specific T cells within each division did not differ between WT and FcγRIIB$^{-/-}$ mice, indicating that the kinetics of division of OVA-specific T cells was not altered (Fig. 5*A*), as was also observed in Fig. 3*A*.

Previously, we have shown that the differentiation of OVA-specific T cells at this time point in the mucosa-draining LN yields two populations of Tr that can also be retrieved at later time points from the spleen. One population contains adaptive CD25$^+$ Tr that suppress more than only the responses to the Ag applied via the mucosa and that may share characteristics with the CD25$^+$ thymus-derived Tr. The second population contains CD25$^-$ Tr that only suppress responses to the Ag that was applied via the mucosa (11). To obtain a direct indication on whether the frequency of the CD25$^+$ Tr was changed in FcγRIIB$^{-/-}$ mice, we determined the percentage of CD25$^+$ OVA-specific T cells among the dividing cells in the CLN. As shown in Fig. 5*B*, there was no difference in the percentage of CD25$^+$-dividing OVA-specific T cells in the CLN of the FcγRIIB$^{-/-}$ mice, excluding that there is a complete lack of these cells. We are cautioned when drawing this conclusion by the fact that at this time point of differentiation expression of CD25 may also be required to receive an IL-2 signal, irrespective of whether these cells are Tr or effector T cells. Because there are no other markers available to distinguish mucosal Tr from effector T cells, we assessed whether the enhanced DC activation in FcγRIIB$^{-/-}$ led to altered differentiation with respect to cytokine

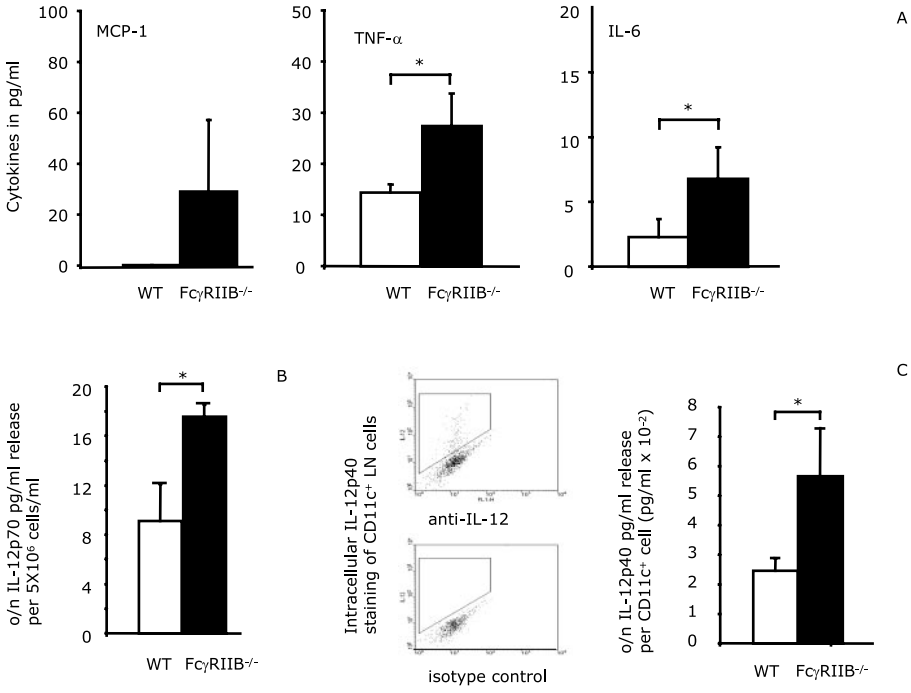

FIGURE 4. FcγRIIB regulates inflammatory cytokine expression by DC in vivo. FcγRIIB$^{-/-}$ and WT mice on BALB/c background were treated with 400 μg of OVA/10 μl of saline intranasally, and 24 h later, the CLN were removed. Single cell suspensions were cultured overnight in IMDM, and cytokine release into the supernatant was determined (*A* and *B*) (11). IL-12p40 cytokine secretion per cell was determined by staining permeabilized LN cells with CD11c in combination with anti-IL-12 p40 (*C*). $n = 5$; mean + SD; □ = WT; ■ = FcγRIIB$^{-/-}$. ∗, Indicates $p < 0.05$.

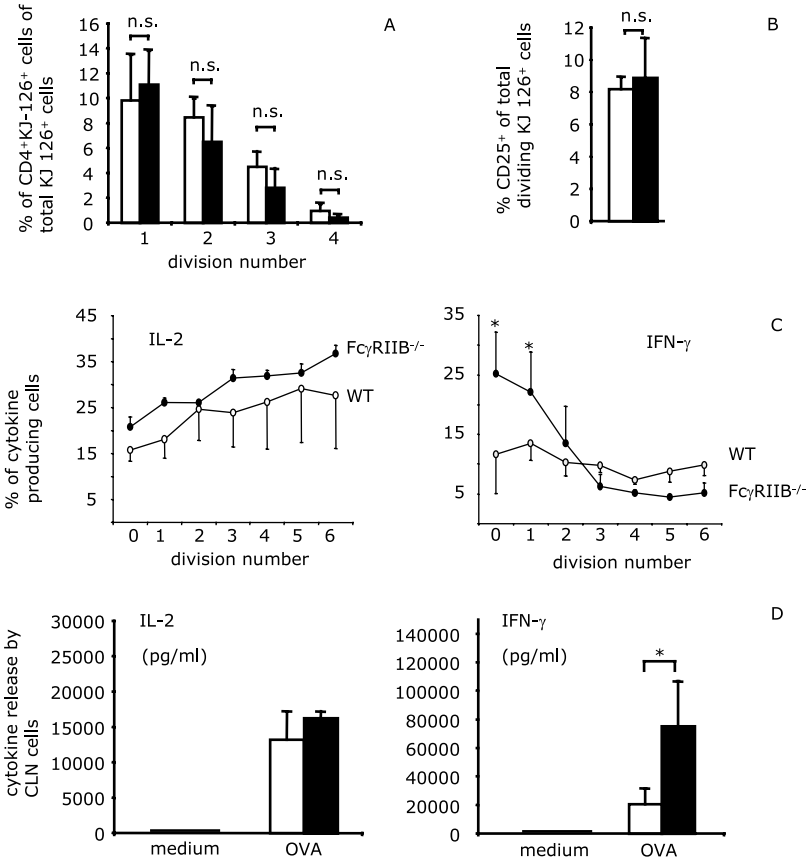

FIGURE 5. Kinetics and phenotype of the OVA-specific T cell response in the nose-draining CLN of FcγRIIB$^{-/-}$ mice. The kinetics of OVA-specific T cell responses to nasally applied OVA in FcγRIIB$^{-/-}$ mice and WT mice on BALB/c background were investigated by adoptively transferring OVA-specific TCR transgenic (DO11.10) CD4$^+$ T cells that had been labeled with CFSE. One day later, these mice were treated with 400 μg of OVA/15 μl of saline intranasally, and 48 h thereafter, the kinetics of dividing OVA-specific T cells in the CLN were determined, as described previously (11). *A*, Mean + SD percentage of OVA-specific T cells per peak of division is depicted in WT (□) and FcγRIIB$^{-/-}$ (■) mice. *B*, Percentage of CD25$^+$ cells within the population of dividing cells. *C*, The phenotypes of the OVA-specific T cell responses in the CLN were assessed by collecting specific T cells and restimulating them in vitro with either 1 mg/ml OVA or medium as a control. Cells were harvested after 18 h, and the percentage of OVA-specific T cells secreting IL-2 and IFN-γ was determined using the appropriate murine cytokine secretion assays. The percentage of cytokine-secreting CD4-positive-KJ1-26-positive cells per division peak was calculated for WT (□) and FcγRIIB$^{-/-}$ (■) CLN cells in comparison with medium-stimulated controls. *D*, The concentrations of murine IL-2 and IFN-γ in the culture supernatants of total CLN cells of WT (□) or FcγRIIB$^{-/-}$ mice (■) were determined using the cytometric bead array assay. $n = 4$; mean + SD; ∗, indicates $p < 0.05$; n.s. = nonsignificant.

secretion. The percentage of cytokine-producing OVA-specific T cells and the release of cytokines by total CLN cells were also determined in WT and FcγRIIB$^{-/-}$ mice (Fig. 5, *B* and *C*). The percentage of IL-2-secreting OVA-specific T cells was not detectably higher in FcγRIIB$^{-/-}$ mice compared with WT mice (Fig. 5*B*); this finding is reflected by the similar concentrations of IL-2 in the supernatants of total LN cell restimulation cultures from WT and FcγRIIB$^{-/-}$ mice (Fig. 5*C*). However, the percentage of IFN-γ-secreting undivided cells or one-time-divided T cells was significantly higher in FcγRIIB$^{-/-}$ mice than in WT mice (Fig. 5*B*); this increase is reflected by a 4-fold increase in the concentration of IFN-γ in the supernatant of total CLN cell restimulation cultures of FcγRIIB$^{-/-}$ mice compared with WT (Fig. 5*C*). No IL-4 or IL-10 OVA-specific T cells could be detected in either WT or FcγRIIB$^{-/-}$ mice.

In conclusion, these data show that the down-regulating effect of FcγRIIB in the induction of nasal tolerance results in a significantly reduced IFN-γ secretion by Ag-specific T cells, but not in an alteration in IL-2 secretion or kinetics of T cell division.

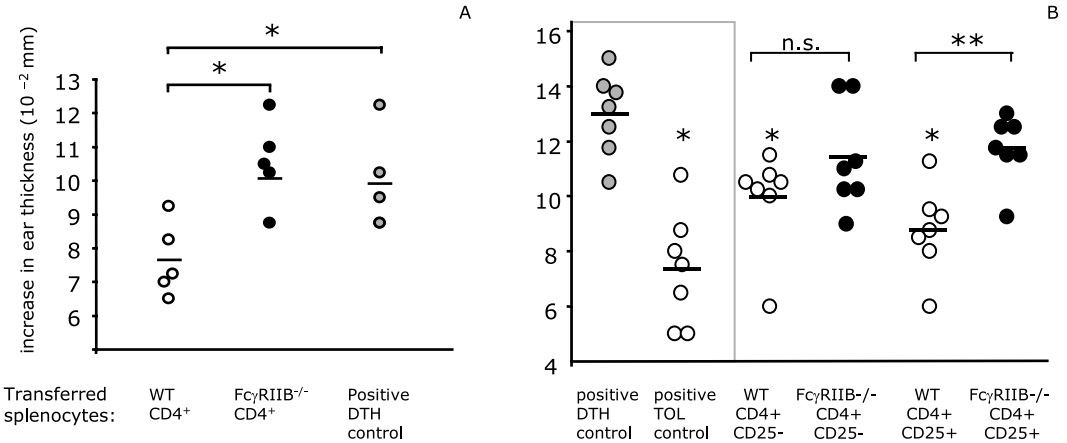

FIGURE 6. Absence of functional CD4$^+$ Tr in FcγRIIB$^{-/-}$ mice. FcγRIIB$^{-/-}$ and WT mice were tolerized, sensitized, and challenged, as described in the legend of Fig. 1. One week after challenge, CD4$^+$ T cells were isolated from the spleens of these WT (tolerant) and FcγRIIB$^{-/-}$ (nontolerant) mice and transferred as a whole (*A*) or separated into CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ subsets (*B*) to naive recipients. Transfer of tolerance by the CD4$^+$ T cells from WT (○) and FcγRIIB$^{-/-}$ (●) mice to the naive recipients was assessed by sensitizing and challenging the latter for a DTH response, and comparing with mice that were merely sensitized and challenged (gray filled circle) (positive DTH control). ∗, Indicates $p < 0.05$ when compared with positive DTH control. ∗∗, Indicates $p < 0.05$ when FcγRIIB$^{-/-}$ is compared with WT controls.

Absence of functional CD4$^+$ Tr in FcγRIIB$^{-/-}$ mice

A decisive step in the development of mucosal tolerance is the induction of Ag-specific Tr that suppress subsequent systemic challenges with the same Ag (3–8). Previously, we have demonstrated that after Tr differentiation in the mucosa-draining LN, these cells leave the LN and can be isolated from the spleen. These Tr are CD4$^+$ and fall in both CD25$^+$ and CD25$^-$ subsets (10, 11). The OVA-specific T cells in the CLN of FcγRIIB$^{-/-}$ mice had an increased proinflammatory profile relative to those in WT mice (Fig. 5), and so we hypothesized that as a result of unbalanced Ag presentation and subsequent altered T cell differentiation, no Tr were generated in FcγRIIB$^{-/-}$ mice.

To test this hypothesis, FcγRIIB$^{-/-}$ mice and WT mice received OVA via the nasal mucosa and were subsequently sensitized and challenged. As predicted by the experiment described in Fig. 1, FcγRIIB$^{-/-}$ mice did not become tolerant, whereas WT mice did (data not shown). One week after challenge, CD4$^+$ T cells were isolated from the spleens of WT and FcγRIIB$^{-/-}$ mice and transferred to naive recipients. Transfer of tolerance by the CD4$^+$ T cells to the naive recipients was assessed by sensitizing and challenging the latter for a DTH response, and comparing the outcome with that of mice that were merely sensitized and challenged (positive DTH control). As depicted in Fig. 6*A*, mice that received CD4$^+$ T cells from tolerized WT mice were tolerant, as exhibited by a significantly lower ear thickness than control DTH mice. However, mice that had received CD4$^+$ T cells from OVA intranasally treated FcγRIIB$^{-/-}$ mice were not tolerant, as evidenced by an increase in ear thickness similar to that of the positive DTH control (Fig. 6*A*). To assess whether lack of FcγRIIB affects a specific subset of Tr-purified splenic CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells from WT and FcγRIIB$^{-/-}$ mice were transferred separately. As shown in Fig. 6*B*, function of both subsets derived from FcγRIIB$^{-/-}$ mice was perturbed, as they were unable to suppress a DTH response. It should be noted that when compared with the WT CD4$^+$CD25$^-$ T cells, the CD4$^+$CD25$^-$ T cells from FcγRIIB$^{-/-}$ mice seemed to retain some residual suppressive ability (Fig. 6*B*).

We conclude that FcγRIIB balances Ag presentation that is required for both functional CD25$^+$ as well as CD25$^-$ Tr induction in the mucosa-draining LN.

Discussion

This study demonstrates, using mice deficient in FcγRIIB, that signaling via this receptor is essential for the induction of nasal tolerance. In vitro, as well as in the nose-draining LN, absence of Ig-initiated signaling via FcγRIIB on DC disturbs the normal balance of costimulatory molecule expression and cytokine secretion, resulting in a proinflammatory condition whose outcome is an altered CD4$^+$ T cell differentiation and prevention of functional Tr cell formation. From these results, we conclude that signaling via FcγRIIB on DC may prevent maximal costimulatory molecule expression and proinflammatory cytokine secretion, a balance required for Tr induction during the generation of mucosal tolerance.

Our findings are the first evidence that a member of the FcγR family is involved in Tr induction in mucosal tolerance. They are in line with the observation that FcγRIIB$^{-/-}$ mice are susceptible to both inducible forms of autoimmunity, such as collagen-induced arthritis and Goodpasture's syndrome (25, 26), and also to spontaneous autoimmunity (27). Although spontaneous autoimmunity due to lack of FcγRIIB function can largely be attributed to a defect in B cell function (28), the results presented in this work question a role of FcγRIIB in regulating Tr that maintain tolerance to self Ags (29–31).

What is the mechanism underlying a central role of FcγRIIB in mucosal tolerance? The OVA applied on the nasal mucosa is most likely taken up by local DC that line the mucosa, as has been demonstrated for buccal (32) and gastrointestinal (15) mucosal immunizations. Resting DC predominantly express the inhibitory FcγRIIB, which accounts for ~75% of total FcγR expression (17). However, whether this also holds true for mucosal DC is unclear, although the results reported may imply this is the case. After Ag uptake, the DC migrate to the mucosa-draining LN while undergoing activation and/or differentiation. Upon migration and activation, DC may up-regulate the stimulatory FcγRI and FcγRIII, which, upon encountering immune complexes, initiates many cellular changes, including the up-regulation of costimulatory molecules and proinflammatory cytokine secretion, resulting in enhanced T cell activation (20); indeed, we have confirmed that WT BM-DC that were stimulated with immobilized IgG enhanced their costimulatory molecule expression and cytokine secretion (Fig. 2). However, immune complexes also trigger signaling of the inhibitory ITIM containing FcγRIIB, leading to association of this receptor with activated FcγRI and III and down-modulation of their stimulatory ITAM-mediated signals (21). Similarly to our murine experiments, human monocyte-derived DC primarily express the IIB form of FcγR, which, upon cross-linking with immobilized IgG, induces DC maturation associated with IL-10 secretion that can be blocked with a neutralizing Ab against FcγRII (33). Clearly, our data show that in the absence of FcγRIIB function, this down-modulation does not occur, because stimulation of FcγRIIB$^{-/-}$ BM-DC with immobilized IgG resulted in increased costimulatory molecule expression relative to WT BM-DC, most notably CD86 and CD40, and substantially enhanced proinflammatory cytokine secretion (Fig. 2). This is in line with a previous report that BM-DC of FcγRIIB$^{-/-}$ mice show more MHC class II and CD86 expression than WT BM-DC when stimulated with immune complexes consisting of OVA with anti-OVA rabbit IgG (17). Crucially, this FcγRIIB-mediated down-regulation of DC activation has functional consequences in vivo, because the application of OVA via the nose-induced deregulated proinflammatory cytokine release in the CLN of FcγRIIB$^{-/-}$ mice, which, in the case of IL-12p70, could be related to increased IL-12p40 release per CD11c$^+$ cell (Fig. 4).

In contrast to the in vitro experiments with myeloid BM-DC, the expression of costimulatory molecules on DC in the CLN was not detectably increased in FcγRIIB-deficient mice. This may be explained by the fact that Ag presentation in vivo is more complex, most likely involving DC belonging to different lineages that are either resident in the LN or migrate into the LN after picking up Ag at the mucosa. The response of these cells to OVA is likely to be less homogeneous than in the in vitro system, which may limit detection of the enhanced costimulatory molecule expression.

We have previously shown that increased numbers of IgG2b-producing B cells are present in the mucosa-draining LN of naive mice (16). In the experiments described in this work, it is unclear whether there is an additional increase in IgG that is specifically directed against OVA, especially considering the short period of 72 h after protein administration. Such monomeric IgG and/or immune complexes may modulate the function of the migrated or resident DC by ligation of FcR similarly to the effects described after i.v. Ig treatment (34): independent of its Ag specificity, i.v. γ-globulin treatment has effective anti-inflammatory activity against a variety of immune-mediated disorders, due to the induction of inhibitory FcγRIIB on macrophages (35).

It should be noted that naive FcγRIIB$^{-/-}$ LN did not contain resting DC or B cells with enhanced costimulatory molecule expression (data not shown), implying that resting FcγRIIB$^{-/-}$ LN

DCs have a phenotype comparable to WT DC. It seems therefore that it is the inability of FcγRIIB$^{-/-}$ mice to develop tolerance in response to tolerogenic Ag that explains their normal DTH response (Fig. 6), rather than the spontaneous deregulated generation of stimulatory conditions in the absence of tolerogenic Ag.

Characteristics of DC that are associated with the generation of a tolerogenic T cell response include specific subsets of DC, such as CD8α-positive DC (36); surface ligands including ICOS-L (37) and receptor activator of NF-κB (38); and/or the degree of maturity of the DC (14). These studies attest that defined conditions of Ag presentation determine whether the outcome of a T cell response is tolerogenic or immunogenic. Therefore, deregulated DC activation in the mucosa-draining LN following OVA treatment in FcγRIIB$^{-/-}$ mice could be the basis of the subsequent observed T cell response, from tolerogenic to immunogenic. The effects of deficient FcγRIIB signaling on CD4$^+$ responses in vitro and in vivo were characterized in the OVA-specific CD4$^+$ T cell response in coculture or in the nose-draining LN after nasal OVA application, respectively. Surprisingly, no difference in the number of divisions or proportion of dividing OVA-specific CD4$^+$ T cells could be observed in FcγRIIB$^{-/-}$ mice (Figs. 3*A* and 5*A*).

In vitro, in the absence of FcγRIIB on DC, Ag-induced T cell proliferation leads to enhanced IL-2 release, but is not accompanied by enhanced T cell proliferation when compared with T cells incubated with WT DC. This is surprising, as it has been known for a long time that IL-2 has potent T cell growth factor activity in that it generates clonal expansion of T cells. The simplest interpretation of these findings is that the T cells are unable to respond to the higher amounts of IL-2. This seems unlikely, as within each peak of division there are dividing cells that express the IL-2Rα chain CD25. However, the IL-2 may also have other activities besides affecting the clonal expansion. A more enticing, but speculative explanation is that IL-2 may control effector cell differentiation. Recently, it has been proposed that signaling through the IL-2R can be a crucial checkpoint for substantial clonal expansion and development into effector cells. The IL-2-mediated signal is denoted as signal 3, after ligation of the TCR (signal 1) and engagement of costimulatory molecules (signal 2) during Ag presentation by the DC (39). IL-2 may control effector T cell differentiation by, for example, stabilizing accessibility of cytokine genes, as has been demonstrated for the *Il-4* gene (40). Although under homeostatic conditions Tr induction is dependent on IL-2 release (39), the excessive IL-2 release as seen in our model may skew the T cell differentiation away from a Tr phenotype. Whether such a IL-2-mediated regulation may explain the observed IFN-γ production (Fig. 3*B*) is unclear.

Although this in vitro system is useful to detect the effect of FcγRIIB on DC-T cell interaction, it should be viewed with caution. This is best illustrated by our finding that in vivo enhanced IL-2 secretion was not evident in LN cells from FcγRIIB-deficient mice when compared with the in vitro data shown in Fig. 3*B*. In vivo Ag presentation in the LN takes place within a complex microenvironment in which movement of Ag, APCs, and T cells is restricted to specific anatomical locations. As recently described, there are resident and migrating DC in the LN (41). Both types of DC have the capacity to acquire Ag and generate peptide-MHCII complexes, but may have different functions. Migrating DC have up-regulated CD86/CD80 and may be highly stimulatory T cells, whereas resident DC can be tolerogenic associated with lower expression of costimulatory molecules. In theory, the T cells in the nose-draining CLN could encounter both migrating and resident DC that differ with respect to FcγRIIB expression and in consequence have a different status of activation. Moreover, in contrast to the myeloid DC used in vitro, such CLN-DC may also belong to other lineages such as the plasmacytoid DC, as found in human nasal mucosa (42), and in murine lungs, where this similar DC phenotype was shown to have different T cell-stimulating properties (43). Due to this complexity of DC-T cell interaction in vivo, the enhanced IL-2 release may possibly not be detected or may be overruled by inhibitory signals from the natural environment.

Crucially, both in vitro and in vivo absence of FcγRIIB on DC favored the development of IFN-γ-secreting T cells: in particular, more IFN-γ-producing cells were observed, and more IFN-γ protein was detected in the supernatant of total LN cell cultures after restimulation with Ag (Figs. 3*B* and 5*C*). These findings suggest a skewing of the T cell response toward an immunogenic Th1-like phenotype and away from a tolerogenic Tr phenotype.

What are the consequences of these changes toward a more Th1-like proinflammatory response to OVA in the mucosa-draining lymph node of FcγRIIB$^{-/-}$ mice? The differentiation of mucosal Tr within 72 h of nasal OVA application is inhibited. We have previously demonstrated that after differentiation in the mucosa-draining LN, Tr reside within the CD4$^+$ splenic T cell pool in both the CD25$^+$ and CD25$^-$ compartments (9). Therefore, we assessed whether these splenic regulatory T cells could be retrieved from FcγRIIB$^{-/-}$ mice. Undeniably, our data show that CD4$^+$ splenic T cells from tolerized FcγRIIB$^{-/-}$ mice fail to transfer tolerance to naive mice and that both CD4$^+$CD25$^+$ as well as CD4$^+$CD25$^-$ Tr are affected in their function, clearly demonstrating that there is abortive Tr induction in FcγRIIB$^{-/-}$ mice.

In summary, we report new evidence that signaling via FcγRIIB is essential for Tr induction in the generation of mucosal tolerance. Signaling via FcγRIIB on DC in the mucosa-draining LN, initiated by local IgG, down-regulates costimulatory molecule expression and cytokine secretion induced by stimulatory FcγRI and FcγRIII, resulting in the defined conditions necessary for Tr induction and subsequent tolerance as opposed to immune response. Further studies on the regulation of FcγRIIB signaling will be important for understanding the intricate processes that underlie Tr induction and will have consequences for application of tolerance induction in autoimmune disease and allergy.

Disclosures

The authors have no financial conflict of interest.

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