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Important role for Fcγ IIB receptor on B lymphocytes for mucosal antigen-induced tolerance and Foxp3^{+} regulatory T cells

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ABSTRACT

FcγRIIB, which is the only Fcγ receptor expressed on B cells, is important in the maintenance of immunological tolerance to self antigens (Ags). In this study, we investigated the role of FcγRIIB in Ag-specific CD4 T cell tolerance induced by mucosally administered Ag (OVA) coupled to cholera toxin B subunit (Ag/CTB) or given alone. We found that sublingual administration of Ag/CTB conjugate or intragastric administration of a >100-fold higher dose of Ag alone efficiently suppressed parenteral immunization-induced Ag-specific T cell proliferation and delayed-type-hypersensitivity responses in FcγRIIB-expressing wild-type (WT), but not FcγRIIB$^{-/-}$ mice. Tolerization, associated with induction of Ag-specific Foxp3^{+} regulatory T (Treg) cells, was restored in FcγRIIB$^{-/-}$ mice by adoptive transfer of either WT B cells or WT dendritic cells (DCs) before the mucosal Ag/CTB treatment, and was pronounced even further in μMT mice receiving FcγRIIB-overexpressing B cells before treatment. Furthermore, cell transfer in both WT and μMT mice of WT but not FcγRIIB$^{-/-}$ B cells pretreated for 1h in vitro with Ag/CTB conjugate, induced Ag-specific immunological tolerance, which was further enhanced by adoptive transfer of WT B cells pretreated with anti-Ag IgG immune complexed Ag/CTB. We conclude that FcγRIIB expression on B cells, in addition to DCs, is important for mucosal induction of Ag-specific immune tolerance.

Introduction

Oral tolerance is known as a systemic immune unresponsiveness to a normally immunogenic parenteral injection of antigen (Ag) induced by oral (or other mucosal) administration of the same Ag. Physiologically, oral tolerance may be important to avoid developing allergic responses to ingested food or inhaled environmental Ags. Experimentally, mucosal administration of selected autoreactive tissue antigens or allergens has been found to induce peripheral tolerance that can prevent or ameliorate autoimmune and allergic inflammatory diseases and has therefore attracted attention as a possible means for Ag-specific immune therapy in such disorders (for reviews see 1, 2). Oral tolerance primarily affects effector T (Teff) cells, and antibody responses are in general not much reduced unless very high Ag doses are used for tolerization. High-dose tolerization may also lead to clonal depletion of T cells, in contrast to tolerization using lower Ag doses which is mediated mainly by regulatory T (Treg) cells, both $Foxp3^+$ and $Foxp3^-$ cells, suppressing the development and function of Teff cells (1, 2).

We and others have demonstrated that mucosal administration of various types of Ags chemically conjugated or genetically fused to cholera toxin B subunit (Ag/CTB) can more efficiently and at more than 100-fold reduced dosages compared to Ag alone or Ag mixed with CTB induce peripheral T cell tolerance (3–8). Similar to other low-dose tolerance this effect is associated with induction of $Foxp3^+$ as well as $Foxp3^-$ Treg cells (9). The coupling of Ag to CTB, which binds to GM1 ganglioside receptors present on many cell types including all known antigen-presenting cells (APCs), strongly potentiates the mucosal tolerizing effect (10) by both facilitating Ag transport across the mucosal barrier and, most importantly, increasing Ag uptake and presentation by APCs such as dendritic cells (DCs), B cells and macrophages (11, 12).

Recent work has identified a previously poorly recognized role for B cells in immunological tolerance, including oral tolerance. While it is well known that B cells can be pathological in autoimmune diseases by producing autoantibodies, recent studies have shown that several experimentally induced autoimmune or inflammatory conditions, such as colitis (13), encephalitis (14) and collagen-induced arthritis (15), have a more aggressive course in B cell-deficient compared to normal mice, which can be corrected by adoptive transfer of B cells (15, 16). Studies have shown that under certain conditions, activated B cells can suppress Teff cell responses through providing signals, including IL-10 and TGF-β which promote the development and expansion of $Foxp3^+$ Treg and/or Tr1 cells (16–20). Consistent with a regulatory role of B cells, B cell-deficient mice have shown defective ability to develop oral tolerance (21) as well as Treg cells in response to mucosal Ag administration including Ag conjugated to CTB, which defect is also corrected by adoptive transfer of B cells (10). Indeed, our studies (10, 22) have shown that B cells play an especially important role as tolerogenic APCs after mucosal exposure to Ag/CTB conjugate, probably due to the fact that the majority of B cells through the CTB-mediated binding to GM1 ganglioside receptors become effective regulatory APCs for any contacted Ag coupled to CTB irrespective of their B cell receptor (BCR) specificity.

In this study, we have investigated the role of the IgG Fc-receptor FcγRIIB, and specifically its expression on B cells, in oral tolerance induced by mucosally administered Ag (OVA) conjugated to CTB or given alone. Fc receptors for IgG (FcγR) constitute a family of surface molecules expressed on hemopoietic cells that can bind and be aggregated by the Fc domains of IgG immune complexes thereby either stimulating or inhibiting cellular responses (23). The inhibitory receptor FcγRIIB has been found to play an important role both in the maintenance of immunological tolerance to self Ags and in regulating immune responses to exogenous Ags (24–28). Most cells of the myeloid lineage express both activating FcγRs and the inhibitory

FcγRIIB; however B cells only express FcγRIIB which, dependent on its co-ligation together with the BCR, controls the magnitude and persistence of the response to the Ag (23). The significance of FcγRIIB-mediated negative regulation of immune responses has been established largely through the use of mice being either deficient in or having enhanced expression of this receptor (25). FcγRIIB deficiency is associated with increased inflammation, allergy and development of chronic autoimmunity (26, 29, 30). Moreover, these knockout mice have been described to display impaired mucosal tolerance after nasal administration of Ag (OVA) resulting in reduced suppression of eosinophilia and IgE production in murine allergic rhinitis and delayed type hypersensitivity (DTH) reactions, which was assumed to reflect the lack of FcγRIIB expression on DCs (30, 31).The role of FcγRIIB expressed on B cells in oral tolerance has not been examined.

In this study, using OVA as a model Ag, we show that FcγRIIB expression is critical for induction of Ag-specific oral tolerance after mucosal administration of high-dose free Ag as well as low-dose Ag linked to CTB. Focusing on the latter system we demonstrate that FcγRIIB-expressing B cells are both efficient and sufficient for induction of functional tolerance as well as development of $Foxp3^+$ Treg cells, although partial tolerization (but not any increase in Treg cells) could also be achieved in FcγRIIB-deficient mice after engraftment with FcγRIIB-expressing DCs.

Materials and Methods

Mice

Female 6-8 wk old FcγRIIB-deficient mice on a pure C57BL/6 background were purchased from Taconic Europe (Ejby, Denmark). Female C57BL/6 mice of similar age were also used (B & K Universal AB, Stockholm, Sweden) together with OT-IIxLy5.1 ($CD45.1^+$) mice on a C57BL/6 background (kind gift from Dr. Mary Jo Wick, Gothenburg) which expressed a class II TCR specific for the peptide 323–339 fragment of OVA (OVAp) on nearly 50% of the $CD4^+$ T cells. In addition, we also used 6-8-wk-old female B cell-deficient μMT mice (kind gift from Dr. Nils Lycke, Gothenburg) on a C57BL/6 background. Transgenic mice overexpressing FcγRIIB on B cells (B-TG) and littermate nontransgenic mice (B-NTG) expressing normal WT levels of FcγRIIB were generated as previously reported (25). Transgenic and gene-deficient mice were bred or kept under specific pathogen-free conditions at the experimental animal facility of the University of Gothenburg. The studies were approved by the University of Gothenburg Ethical Committee for Animal Experimentation.

Antigens and conjugation of OVA to CTB

OVA protein (grade VII) was purchased from Sigma (St. Louis, MO, USA) and OVA 323-339 peptide (OVAp, sequence ISQAVHAAHAEINEAGR) of >95% purity was obtained from TAG Copenhagen A/S (Klampenborg, Denmark). Highly purified recombinant CTB was kindly provided by Crucell-Sweden AB (Stockholm, Sweden). OVA protein was chemically coupled to CTB using N-succininmidyl (3-[2-pyridyl]-dithio) propionate (Pierce Biotechnology, Inc., Rockford, IL, USA) as a bifunctional coupling reagent, as described elsewhere (9). The OVA/CTB conjugate was purified by fast protein liquid chromatography (FPLC) gel filtration on a Superdex 200 16/60 column (Pharmacia Biotech) using the Biologic Workstation FPLC system (Bio-Rad Laboratories, Richmond, CA, USA). The

purified conjugate was analyzed by the ganglioside-monosialic acid (GM1)-ELISA and shown to have strong GM1-binding activity as well as high reactivity with antibodies to either OVA or CTB (9). The conjugate also had a strong capacity to induce OVA-specific T cell proliferation when tested on OT-II splenocytes; further, in the latter assays, the activity of the conjugate was not significantly inhibited by pre-incubation and co-culture with polymyxin, but was completely inhibited by pre-incubation and co-culture with highly purified GM1 (gift from the late Prof. Lars Svennerholm).

Mucosal tolerization and s.c. immunization

Mucosal administrations of CTB/OVA conjugate for tolerization were given by the s.l. route using a previously well-established regimen (10). In short, mice were given 10-µl s.l. administrations of 40 µg OVA/CTB conjugate (or for control purposes PBS) on 3 occasions at 2-d intervals, and then the extent of tolerization was tested by immunizing the mice, 2-d after the last s.l. dose, by s.c. injections in two dorsal positions on either side of the spine close to the tail with a total of 100 µg of OVA emulsified in 100 µl CFA. As an alternative to the described standard low-dose s.l. tolerization regimen with OVA/CTB, mucosal high-dose tolerization was also induced by a single administration of 20 mg OVA intragastrically in 0.3 ml 3% (w/v) sodium bicarbonate solution using a baby feeding catheter, and then tolerization was tested as described for the s.l. tolerization by Ag/CTB.

Isolation of B cells and DCs and in vitro treatment with Ag/CTB

Spleens and indicated lymph nodes (CLNs or PLNs) were filtered through nylon nets, erythrocytes were removed by lysis, and single-cell suspensions were prepared by filtration through a 40 µm nylon net (BD Falcon^{TM}; BD, Franklin Lakes, NJ, USA). $CD19^+$ B cells were isolated from either C57BL/6 mice or the various gene-deficient mice by positive

selection using MACS microbeads coated with Ab against mouse CD19 (Miltenyi Biotec, Auburn, CA, USA). Purified $CD19^+$ B cells (purity >99%) at 10^6 cells/ml were then either adoptively transferred to mice (see below) or incubated *in vitro* with 0.1 μg/ml OVA/CTB conjugate, or OVA/CTB pre-mixed with anti-OVA IgG (2.5 μg/ml; Sigma-Aldrich), or 1 μg/ml OVA or PBS for 1 h at 37°C in 5% CO_2, followed by two rounds of thorough washing with PBS and resuspension in PBS or medium (complete IMDM) to the desired cell concentration.

$CD11c^+$ DCs were prepared by first digesting minced spleens and LNs of mice in 25 μg/ml Liberase (Roche Applied Science, Mannheim, Germany) and 400 U/ml DNase I (Roche) at 37°C for 30 min followed by filtration through a nylon net and centrifugation; $CD11c^+$ DCs were then isolated by positive selection using MACS microbeads coated with Ab against mouse CD11c (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's recommendation and the DCs (purity >90%) used in similar transfer and *in vitro* studies as described for the purified B cells.

T cell purification

Single-cell suspensions were prepared from spleens and LNs as described above. $CD4^+$ T cells were then purified routinely by positive selection, or in cases requiring further isolation of $CD25^+$ T cells by negative selection. In either method, MACS microbeads labeled with various mAbs (Miltenyi Biotec, Auburn, CA, USA) were used in accordance with the manufacturer's recommended protocol. Magnetic separation was performed with a positive selection column, according to the manufacturer's recommendation (Miltenyi Biotec, Auburn, CA, USA).

Adoptive transfer of cells

Purified $CD19^+$ B cells (1×10^7 cells), purified $CD4^+$ T cells (5×10^6 cells) or purified $CD11c^+$ DCs (2×10^6 cells), all administered in 200 μl PBS per mouse, were adoptively transferred to syngeneic mice by i.v. injection into the tail vein.

DTH test and preparation of cells from DTH-tested ears

After mucosal tolerization and two wk after the subsequent s.c. immunization, six mice in each group were given an intradermal (i.d.) injection in the left ear with 10 μg of OVA in 20 μl of PBS. The DTH reaction was monitored by measuring the ear thickness using a caliper meter (Mitutoyo) before and 24 h after the i.d. injection, and the differences were calculated and used as measures of DTH responses in a blinded fashion. Mice were sacrificed immediately after the ear thickness measurement and single cell suspensions of challenged ears were prepared by digesting minced ears in 25 μg/ml Liberase (Roche Applied Science, Mannheim, Germany) and 400 U/ml DNase I (Roche) at 37°C for 30 min followed by filtration through a nylon net and centrifugation and resuspension.

T cell proliferation and IFN-γ assays

Single cell suspensions from PLNs or spleen were prepared and erythrocytes removed by lysis. For studies of cell proliferation, 2×10^5 cells/well were cultured in a 200-μl volume of IMDM supplemented with 10% FCS, 1% L-glutamine, 1% gentamicine, and 50 μM mercaptoethanol for 3 d in 96-well plates with or without 0.2 or 1 μg/ml OVAp. [^{3}H]thymidine (1 μCi/well) was added for the last 16-18 h of culture, whereafter [^{3}H] incorporation was measured as previously described (10) Production of IFN-γ released from the cultured cells was measured in culture supernatants by ELISA according to the manufacturer's instructions (Duo Set kit; R&D Systems, Minneapolis, MN, USA).

Flow cytometry staining and analyses

For staining of surface markers, cells were incubated with FITC-, PE-, or APC-labelled mAbs (BD Biosciences Pharmingen, San Jose, CA, USA) to mouse CD4, CD25, or CD45.1 (for identifying OVAp-specific OT-IIxLy5.1 cells). For detection of intracellular Foxp3, cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience, San Diego, CA, USA) according to the manufacturer's recommended protocol, followed by incubation with APC-conjugated anti-Foxp3 FLK-16 mAb (1 μg/10^6 cells) (Nordic Biosite, Taby, Sweden) at 4°C for 30 min in the dark. Cells were washed and analyzed by a LSRII FACS machine (BD Pharmingen, San Jose, CA, USA). Apoptotic cells were detected by staining with Annexin V-APC and 7-amino-actinomycin D (7-AAD) (BD Biosciences Pharmingen, San Jose, CA, USA) according to the manufacturer's protocol. For detection of cells expressing LAP/TGF-β, cells were first incubated for 2 d in the presence of OVAp, and then stained with biotinylated anti-LAP Ab (R & D Systems, Minneapolis, MN) followed by Streptavidin-APC, in addition to staining with Abs against selected surface markers as described above.

Statistical analysis

Results are expressed as mean ± SEM. Unpaired Student's *t*-tests (two-tailed) were used to calculate statistical differences between experimental and control groups. *P*-values < 0.05, < 0.01, and < 0.001 are referred to in the figures by the symbols *, **, and ***, respectively.

Results

Mucosal administration of low-dose Ag/CTB conjugate or high-dose free Ag fails to induce oral tolerance in FcγRIIB$^{-/-}$ mice

Sublingual tolerization with OVA/CTB. Previous studies have shown that intragastric, sublingual (s.l.) or intranasal mucosal administration of OVA and other Ags in mice, given alone or with increased efficiency when linked to CTB, can induce peripheral T cell tolerance ("oral tolerance") along with Ag-specific Treg cells (8, 9). We examined whether FcγRIIB is required for such mucosal induction of tolerance, initially by comparing the effect on the development of DTH in FcγRIIB$^{-/-}$ mice and WT mice after s.l. mucosal administration of OVA/CTB conjugate. DTH is a typical T cell-dependent inflammatory response which is readily established by s.c. immunization with Ag together with complete Freund´s adjuvant (CFA) followed 2 wk later by an i.d. challenge with the same Ag. As shown in Fig. 1*A*, consistent with our previous findings (10), DTH (measured by ear thickness increase 24 h after i.d. challenge in the ear with OVA) was efficiently suppressed in WT mice that had received 3 s.l. administrations of OVA/CTB before a parenteral OVA/CFA immunization; in contrast, similarly treated FcγRIIB$^{-/-}$ mice showed no evidence of tolerance. Furthermore, infiltration of both total CD4^{+} T cells (data not shown) and Foxp3^{-}CD4^{+} Teff cells (Fig. 1*B* and Supplemental Fig. 1*A*) was strongly reduced in challenged ears following s.l. administration of OVA/CTB in the WT but not FcγRIIB$^{-/-}$ mice. To determine whether the lack of mucosal tolerance in OVA/CTB-treated FcγRIIB$^{-/-}$ mice was associated with reduced development or infiltration of Treg cells, we also examined Foxp3^{+}CD4^{+} Treg cells in the challenged ears 24 h after DTH challenge. We found that s.l. treatment with OVA/CTB significantly increased the frequency of these cells in WT but not FcγRIIB$^{-/-}$ mice (Fig. 1*C* and Supplemental Fig. 1*B*).

In parallel, we examined the popliteal lymph node (PLN) reaction of the mice following the s.l. OVA/CTB treatment and s.c. immunization. Sublingual OVA/CTB treatment prior to s.c. immunization significantly inhibited the proliferative response of PLN T cells from WT mice but not FcγRIIB$^{-/-}$ mice (Supplemental Fig. 2*A*). The treatment also resulted in an increase in apoptotic PLN T cells from WT mice (Supplemental Fig. 2*B*), a reduction in Foxp3^{-}CD25^{-} CD4^{+} Teff cells (Supplemental Fig. 2*C*) and an increase in Foxp3^{+} CD4^{+} Treg cells (Supplemental Fig. 2*D*), effects that were not seen in PLN cells from FcγRIIB$^{-/-}$ mice.

We also examined the levels of anti-OVA IgG antibodies in serum 1 d before and 2 wk after the s.c. immunization with OVA in CFA. Contrary to the T cell responses, no suppression of antibody responses was evident in either the WT or FcγRIIB$^{-/-}$ mice after the s.l. administrations of OVA/CTB (data not shown).

FcγRIIB is also required for high-dose oral tolerance induction. We also tested the importance of FcγRIIB for induction of oral tolerance after a single high-dose Ag administration. WT and FcγRIIB$^{-/-}$ mice were given an intragastric 20-mg dose of OVA, and 1 wk later the mice were s.c. immunized with OVA in CFA. Another two wk later the mice were sacrificed and PLN cells examined in vitro for OVA-specific T cell proliferation and frequencies of Teff and Treg cells. The proliferation of PLN T cells from OVA-treated WT mice was markedly suppressed (by 73 ± 1.3 %) compared to cells from the PBS-treated controls, whereas the proliferation was instead increased in cells from similarly treated FcγRIIB$^{-/-}$ mice (Fig. 2*A*). Consistent with these findings, PLN cells from the OVA-treated WT but not FcγRIIB$^{-/-}$ mice showed a decrease in Teff cells (Fig. 2*B*) and an increase in Foxp3^{+} Treg cells (Fig. 2*C*) compared to cells from the PBS-treated controls. We conclude that similar to the findings after s.l. administration with the low-dose Ag/CTB conjugate, FcγRIIB is also required for high-dose oral tolerization with Ag alone.

Lack of Treg cell response and of tolerogenic Ag-presenting B cells in FcγRIIB$^{-/-}$ mice after mucosal treatment with Ag/CTB

Our previous work using WT mice has shown that oral tolerance induced by s.l. treatment with Ag/CTB conjugates is associated with increased numbers of Foxp3^{+} Treg cells in the draining cervical lymph nodes (CLNs), and also with Ag-presenting tolerogenic B cells that both in vitro and in vivo can induce Treg cell development and suppress effector T cell responses (10). We examined to which extent these effects did depend on FcγRIIB. To this end, OVA-specific CD4^{+} T cells from OT-IIxLy5.1 mice were adoptively transferred into WT or FcγRIIB$^{-/-}$ recipient mice to facilitate the development of OVA-specific T cell responses and, starting 1 d later, the mice were treated three times s.l. every 2nd d with OVA/CTB conjugate or PBS. Another 3 d later CLNs and spleens were collected and their CD4^{+} T cells and CD19^{+} B cells isolated and examined as described in the following sections.

Defective Treg cell response in FcγRIIB$^{-/-}$ mice. Isolated CD4^{+} cells from CLNs of the s.l. treated WT and FcγRIIB$^{-/-}$ mice were examined for Ag-specific Treg cells 3d after the last s.l. treatment. As shown in Fig. 3*A* and 3*B*, the frequencies of both OVA-specific Foxp3^{+} Treg cells and regulatory latency-associated polypeptide (LAP)/TGF-β^{+} T cells among CD4^{+} T cells were significantly increased in the WT mice following s.l. treatment with OVA/CTB compared with PBS. In contrast, no significant increase in either of these regulatory T cell populations was observed in the OVA/CTB-treated FcγRIIB$^{-/-}$ mice (Fig. 3 *A, B*).

We also examined whether mucosal treatment with Ag/CTB in FcγRIIB$^{-/-}$ mice would generate CD4^{+} T cells that upon adoptive transfer into WT recipients could develop into Treg cells and suppress Teff cell responses to parenteral immunization. We isolated CD4^{+} T cells from pooled CLN and spleen cells of WT or FcγRIIB$^{-/-}$ mice after the standard s.l. treatments with CTB/OVA or PBS, and these cells were then adoptively transferred to WT recipients that 1 d later were immunized s.c. with OVA in CFA. Another 2 wk later, the immunized mice were sacrificed, their draining PLNs collected and the PLN cells examined for OVA-specific T cell proliferation in vitro and frequency of Foxp3^{+} Treg cells. The results show that T cell proliferation upon Ag restimulation was significantly suppressed in PLN cells from the mice that had received CD4^{+} T cells from OVA/CTB-treated WT mice; in contrast, mice that had received T cells from OVA/CTB-treated FcγRIIB$^{-/-}$ mice did not demonstrate any suppression (Fig. 3*C*). Consistent with this, OVA-specific Foxp3^{+} Treg cells were increased in the PLNs of mice that had received CD4^{+} T cells isolated from the mucosally OVA/CTB-treated WT but not FcγRIIB$^{-/-}$ mice (Fig. 3*D*).

B cells from mucosally OVA/CTB-treated FcγRIIB$^{-/-}$ mice fail to suppress T cell response and to induce Treg cells in vitro. We and others have shown that B cells are important in the induction of oral tolerance and Treg cell development (1, 10, 32). Thus, we examined if isolated B cells from mucosally OVA/CTB-treated FcγRIIB$^{-/-}$ mice could induce OVA-specific Treg cells and suppress OVA-specific T cell proliferation when co-cultured with OVA-stimulated T cells in vitro. To test this, we cultured CD19^{+} B cells isolated from pooled CLN and spleen cells of the s.l. treated WT or FcγRIIB$^{-/-}$ mice for 3 d together with freshly isolated CD4^{+} T cells from naive OT-IIxLy5.1 mice in the presence of OVAp323-339 peptide (OVAp), and examined OVA-specific T cell proliferation, IFN-γ production and induction of Treg cells

in the cultures. As shown in Fig. 4*A*, B cells from OVA/CTB-treated WT mice suppressed *in vitro* T cell proliferation, whereas no such effect was seen using B cells from FcγRIIB$^{-/-}$ mice. Consistent with these findings, the levels of IFN-γ production were reduced (Fig. 4*B*), and OVA-specific Foxp3^{+} Treg cells were increased (Fig. 4*C*) in the culture with B cells coming from OVA/CTB-treated WT but not FcγRIIB$^{-/-}$ mice. These results indicate that during co-culture with T cells *in vitro* only B cells from Ag/CTB-treated mice that express FcγRIIB can induce the development of Ag-specific CD4^{+} Treg cells and suppress Teff cell proliferation and cytokine production.

Engraftment with FcγRIIB-expressing B cells restores mucosal tolerization responsiveness in FcγRIIB$^{-/-}$ mice

To further validate the importance of FcγRIIB expressed on B cells for the induction of Ag-specific Treg cell expansion and mucosal tolerance, we carried out engraftment experiments in which WT B cells were transferred into WT or FcγRIIB$^{-/-}$ mice; to facilitate the development of OVA-specific T cell responses, the recipient mice also received CD4^{+} OT-IIxLy5.1 T cells. Next, the mice received the standard three-dose s.l. treatment with OVA/CTB or PBS, followed by the initiation of DTH responses as described above (see Fig. 1). Similarly treated WT and FcγRIIB$^{-/-}$ mice not receiving any exogenous B cells served as controls. Confirming the findings of the experiments shown in Fig. 1*A*, FcγRIIB$^{-/-}$ mice that did not receive any WT B cells failed to respond to OVA/CTB-mediated tolerization and thus did not suppress either the Ag-specific DTH response (Fig. 5*A*) or proliferation of PLN T cells (Fig. 5*B*). In contrast, engrafting FcγRIIB$^{-/-}$ mice with WT B cells fully restored their capacity to suppress both of these responses after mucosal OVA/CTB treatment (Fig. 5*A*, *B*). Furthermore, PLN cells from these chimeric mice, similar to cells from WT mice, contained an increased number of Treg

cells following tolerization and immunization (Fig. 5*C*). In addition, there was also a marked decrease in the number of Ag-specific Teff ($Foxp3^-CD25^-CD4^+$) cells and an increase in apoptotic Ag-specific T cells ($CD45.1^+$ Annexin V^+ $7AAD^-$ $CD4^+$) in the PLNs of the chimeric mice (data not shown).

Increased tolerogenic effect of engraftment of B cell-deficient μMT mice with B cells overexpressing FcγRIIB compared with B cells expressing normal levels of FcγRIIB

It is known that μMT mice, which lack B cells but contain other cell types including DCs expressing normal levels of FcγRIIB, have a defective responsiveness to mucosal tolerization (10, 32, 33). The findings in Fig. 5 showing that transfer of WT B cells to $FcγRIIB^{-/-}$ mice restored the ability of the latter mice to develop tolerance and Treg cells in response to mucosal OVA/CTB administration prompted us to investigate whether a similar transfer of FcγRIIB-expressing B cells to μMT mice would restore the induction of mucosal tolerance and Treg cell development also in these mice after s.l. treatment with OVA/CTB. Further, we wished to determine whether transfer of B cells overexpressing FcγRIIB would be even more efficacious in these respects.

Therefore, $CD19^+$ B cells were isolated from 3 strains of mice: B-TG mice that transgenically overexpress FcγRIIB on B cells (25), littermate control non-transgenic mice (B-NTG) with normal WT expression levels of FcγRIIB, and $FcγRIIB^{-/-}$ mice not expressing any FcγRIIB. These isolated B cells together with naive $CD4^+$ OT-IIxLy5.1 T cells were i.v. transferred into different groups of μMT mice which were then treated with 3 s.l. administrations of OVA/CTB or PBS as previously described, followed by a s.c. immunization with OVA/CTB. Two wk later the mice were sacrificed and their PLN cells examined.

The results in Fig. 6*A* show that Ag re-encounter-stimulated T cell proliferation was strongly inhibited in PLN cells from the OVA/CTB-treated μMT mice that had received B cells from

either B-TG or B-NTG mice; this stood in sharp contrast to the complete lack of inhibition after transfer of B cells from OVA/CTB-treated FcγRIIB$^{-/-}$ mice. When the effects of engraftment using B cells from B-TG and B-NTG mice were compared in greater detail, it was clear that B cells from B-TG mice demonstrated an even stronger suppressive effect (99.2 ± 3.1%, $p < 0.001$ when comparing treated mice vs controls) than did the B cells from B-NTG mice (88.9% ± 3.7%, $p < 0.01$) (Fig. 6*A*).

A similar pattern was observed in the percentages of Foxp3^{-} Teff cells in the PLNs (Fig. 6*B*). Again, not only did B cells from FcγRIIB$^{-/-}$ mice totally lack suppressive capacity, but also did B cells from the FcγRIIB-overexpressing B-TG mice transfer a stronger suppressive effect compared with that of B-cells from B-NTG mice (97.4 ± 2.5% versus 87.4 ± 4.2% for treated mice vs controls (Fig. 6*B*).

These observations were found to be correlated with the generation of Treg cells. Thus, as shown in Fig. 6*C*, engrafting B cells from B-TG, B-NTG and FcγRIIB$^{-/-}$ mice provided as a mean 240% ($P < 0.01$), 140% ($P < 0.05$) and 30% ($P < 0.20$) increase in Foxp3^{+} Treg cells, respectively. We previously demonstrated that OVA/CTB tolerization induced Teff cell apoptosis and increased Foxp3^{+} (and LAP/TGF-β^{+}) CD4^{+} Treg cells in WT but not FcγRIIB$^{-/-}$ mice (Supplemental Fig. 2*B*; Fig. 3*B*). Here we show that tolerization of the chimeric mice besides increasing Foxp3^{+} Treg cells (Fig. 6*C*) also resulted in apoptosis of Teff cells (Fig. 6*D*) and an increase in LAP/TGF-β^{+} CD4^{+} Treg cells (Fig. 6*E*) in the PLNs if the recipient μMT mice had received B cells from either B-TG or B-NTG mice, with the former exhibiting the highest frequency of apoptotic T cells. As expected, no induction of Teff cell apoptosis (Fig. 6*D*) or LAP/TGF-β^{+} CD4^{+} Treg cells (Fig. 6*E*) were seen in the mice that had received cells from FcγRIIB$^{-/-}$ mice.

We also investigated the PLN cells from the recipient mice for their expression of the co-stimulatory/activation molecules CD80, CD83 and CD86 on B cells and DCs. Not surprisingly,

PLN cells from mice that had received B cells from FcγRIIB$^{-/-}$ mice contained 3-5 times as many B cells and DCs expressing these molecules as compared to PLN cells from mice that had received B cells from B-TG and B-NTG mice irrespective of whether or not the mice had received any preceding OVA/CTB treatment (Supplemental Fig. 3*A* and 3*B* and data not shown).

Adoptive transfer of WT but not FcγRIIB$^{-/-}$ B cells pretreated in vitro with Ag/CTB to either WT or μMT mice induce Ag-specific tolerance and Treg cells and these effects are further increased using B cells pretreated with IgG Ab immune complexed Ag/CTB

Our recent findings demonstrated that a short *in vitro* incubation of B cells with OVA/CTB conjugate before adoptive transfer of the treated cells into mice resulted in tolerance and Treg cell induction in the recipients even without any *in vivo* mucosal tolerization treatment (22). To determine if FcγRIIB is critical in this process, and also to study the effect of IgG immune complexes interacting with FcγRIIB on B cells for the induction of immunological tolerance, we isolated B cells from WT and FcγRIIB$^{-/-}$ mice, pulsed them *in vitro* with PBS, OVA/CTB, or OVA/CTB preincubated with anti-OVA IgG antibody. After washing, the differently treated B cells were then transferred i.v. together with CD4^{+} OT-IIxLy5.1 T cells to either WT or μMT mice followed by s.c. immunization of the mice with OVA in CFA. Two wk later, the mice were sacrificed and their PLN cells analysed; in addition, serum was collected and examined for antibodies to OVA and CTB.

As shown in Fig. 7 and supplemental Table I, OVA-stimulated T cell proliferation *in vitro* was suppressed in PLN cells from both WT and μMT mice that had received WT B cells

treated *in vitro* with OVA/CTB, and this effect was further increased when the donor B cells had been pretreated with OVA/CTB plus IgG anti-OVA antibody. The suppressive effects were more pronounced in WT mice compared with the μMT mice as recipients (cf Fig. 7*A* and *B*), possibly owing to the fact that the WT mice in contrast to the B cell-deficient μMT mice had developed serum anti-OVA IgG antibodies in response to the s.c. immunization (Supplemental Table I), which may also form OVA/CTB-IgG complexes that could further promote the aggregation of FcγRIIB. In contrast, no suppression in T cell proliferation was seen in mice that had received B cells from FcγRIIB$^{-/-}$ mice irrespective of whether these cells had been treated with OVA/CTB alone or with OVA/CTB plus anti-OVA IgG; in the μMT mice the T cell proliferation was in fact rather increased (Fig. 7*A* and *B*). In addition, the results show that the serum levels of anti-OVA IgG were reduced in WT recipients that had received WT B cells treated with OVA/CTB plus IgG anti-OVA antibody compared with controls suggesting a partial tolerization also of the OVA-specific antibody response (Supplemental Table I).

Also DCs require expression of FcγRIIB to induce T cell tolerance

Given the important role of expression of FcγRIIB on B cells for mucosal induction of Ag-specific T cell tolerance and the previous report of Samson et al. (30) suggesting a similar role of FcγRIIB-expressing DCs, we tested in a similar way as reported for B cells (Fig. 4) the ability of DCs isolated from WT or FcγRIIB$^{-/-}$ mice after the standard three-dose s.l. treatment with OVA/CTB to suppress OVAp-specific Teff cells and promote Treg cells in vitro. The results in Fig. 8*A* show that Ag-specific T cell proliferation (*left panel*) as well as IFN-γ production (*middle panel)* were suppressed using DCs from OVA/CTB-treated WT mice as APCs but were rather increased using DCs from similarly treated FcγRIIB$^{-/-}$ mice. Consistent

with this, only the DCs from OVA/CTB-treated WT mice induced Foxp3^{+} Treg cells in vitro (*right panel).*

We also carried out engraftment experiments similar to those undertaken with B cells (Fig. 5), in which WT DCs together with CD4^{+} OT-IIxLy5.1 T cells were transferred into FcγRIIB$^{-/-}$ mice, which were then given the standard three-dose s.l. treatment with OVA/CTB or PBS followed by an s.c. immunization with OVA in CFA and measurement of DTH and other T cell responses; mice with CD4^{+} OT-IIxLy5.1 T cells and s.l. treated WT mice served as controls. As shown in Fig. 8*B*, engrafting FcγRIIB$^{-/-}$ mice with FcγRIIB-expressing WT DCs much in the same way as when such mice were engrafted with B cells (Fig. 5) significantly restored their capacity to suppress both DTH (*left panel*) and T cell proliferative (*middle panel*) responses. Notably, however, there was no significant increase of Treg cells in FcγRIIB$^{-/-}$ mice that had received FcγRIIB-expressing DCs following tolerization and immunization (Fig. 8 *B, right panel*), which is in contrast both to OVA/CTB-treated WT mice (Fig. 8 *B, right panel*) and to FcγRIIB$^{-/-}$ mice engrafted with FcγRIIB-expressing B cells (cf Fig. 5*C*). Moreover, at further difference from FcγRIIB$^{-/-}$ mice engrafted with WT B cells there was no increase in apoptotic Ag-specific T cells (CD45.1^{+} Annexin V^{+} 7AAD^{-} CD4^{+}) in the PLNs of FcγRIIB$^{-/-}$ mice engrafted with WT DCs (data not shown).

Discussion

The pathways through which mucosally administered Ags induce oral tolerance remain incompletely understood, although the generation of Treg cells inactivating Teff cells has been identified as the main effector mechanism. Our recent work has identified B cells as playing an important role as tolerogenic APCs in mucosally induced tolerization (10, 22); B cells appear

to be especially prominent in low-dose T cell tolerance induced by mucosal administration of Ags linked to CTB, which latter molecule through binding to the GM1 ganglioside receptor can render most B cells effective tolerogenic APCs irrespective of their BCR specificity.

In this study, we have found the inhibitory IgG receptor FcγRIIB to be critical for the induction of oral tolerance and also that the expression of this receptor on Ag-presenting B cells is both important and sufficient for tolerance induction. Using OVA as model Ag, we found that mucosal administration of either low doses of CTB-linked OVA or a high dose of OVA alone strongly suppressed parenteral immunization-induced OVA-specific T cell proliferation and DTH responses in FcγRIIB-expressing WT mice, but completely failed to do so in FcγRIIB-deficient FcγRIIB$^{-/-}$ mice. We confirmed a previous study that found a contribution of FcγRIIB expression on DCs to induction of mucosal tolerance (30). However, we further extended the study by demonstrating that FcγRIIB expression on B cells is sufficient for induction of Ag-specific immune tolerance and Foxp3^{+} Treg cells after mucosal Ag/CTB administration. In contrast to other myeloid cells including DCs, which express several types of both activating and inhibitory Fcγ receptors, the inhibitory FcγRIIB is the only Fcγ receptor expressed on B cells. Both the tolerogenic effect and the induction of Ag-specific Foxp3^{+} Treg cells were fully restored in FcγRIIB$^{-/-}$ mice that received FcγRIIB-expressing WT B cells before the mucosal Ag/CTB treatment, and adoptive transfer of transgenic FcγRIIB-overexpressing B cells demonstrated even greater tolerogenic effects compared with B cells expressing normal receptor levels.

Purified B cells pulse-treated with OVA/CTB *in vitro* and extensively washed before adoptive transfer to either WT or μMT mice also conferred oral tolerance not only in WT but also in μMT mice provided that the transferred Ag/CTB-treated B cells expressed FcγRIIB. The μMT mice, apart from their lack of B cells, contain functional FcγRIIB-expressing DCs and other types of APCs (34) and yet they have a defective oral tolerance responsiveness

unless adoptively receiving FcγRIIB-expressing B cells (21). At the same time, as first described by Gonnella et al. (2006) (32) and confirmed by us, μMT mice normally can develop a reduced, modified form of tolerance in response to mucosally administered Ag, being mediated by Ag-specific Foxp3$^-$LAP$^+$ Treg cells rather than Foxp3$^+$ cells (10). It has been reported (35) that B cells can modulate the Ag presentation to T cells by DCs in μMT mice by promoting IL-4 and decreasing IL-12 production thus providing a more balanced Th1/Th2 response. Based on this, the complete lack of oral tolerance after adoptive transfer of FcγRIIB$^{-/-}$ B cells may reflect an active role of the FcγRIIB-lacking B cells in inactivating the residual tolerization capacity in the μMT mice (Fig. 6*A*; Fig. 7*B*).

Yet, while demonstrating an important and sufficient role for FcγRIIB-expressing B cells in oral tolerance these studies do not rule out a parallel role for FcγRIIB-expressing DCs. Samsom et al. (30) based their conclusion that oral tolerance depends on FcγRIIB-expressing DCs on *in vitro* studies in which they showed that co-culture of naive OVA-specific T cells with Ag-presenting WT but not FcγRIIB$^{-/-}$ DCs promoted the induction of regulatory CD4$^+$ T cells. In support of a role also for of FcγRIIB-expressing DCs we found that in WT mice but not FcγRIIB$^{-/-}$ mice mucosal Ag/CTB treatment induced not only tolerogenic B cells but also DCs which after isolation and subsequent in vitro culture with Ag and T cells promoted the expansion of Foxp3$^+$ Treg cells and suppressed Ag-specific Teff cell proliferation. In addition, we found that adoptive transfer of FcγRIIB-expressing WT DCs to FcγRIIB$^{-/-}$ mice could restore the in vivo tolerogenic effect of Ag/CTB-treatment in these FcγRIIB$^{-/-}$ mice although apparently to a lesser extent than seen after transfer of FcγRIIB-expressing B cells, and also different from the B cells the transferred DCs failed *in vivo* to induce Ag-specific Foxp3$^+$ Treg cells.

Normally, like other Fcγ receptors FcγRIIB is activated only after being crosslinked by IgG-Ag immune complexes (26, 36). Our results, however, suggest that FcγRIIB on B cells may be activated by exposure to Ag/CTB even in the absence of any IgG Ab, even though the FcγRIIB-expressing B cells became even more effective in inducing tolerance and Treg cells following treatment with IgG Ab immune-complexed Ag/CTB as compared to Ag/CTB alone. It could be argued that when FcγRIIB-expressing WT mice were first mucosally treated with Ag/CTB and then parenterally immunized, they developed IgG Ab that could form immune complexes with the tolerizing Ag/CTB or immunizing Ag thus providing a regulatory mechanism controlling Fcγ activating receptors by the ligation of FcγRIIB. However, this possibility is unlikely in μMT mice completely devoid of IgG, the adoptive transfer of OVA/CTB-pretreated FcγRIIB-expressing B cells significantly suppressed the T cell response to a subsequent parenteral immunization with OVA in CFA (Fig. 7). Furthermore, ELISA analyses confirmed the complete lack of detectable IgG anti-OVA or anti-CTB antibodies in sera from these mice also after immunization (Supplemental Table I). It remains to be determined by which mechanism the CTB-linked Ag can activate the FcγRIIB on B cells in the absence of IgG Ab and render the B cells tolerogenic; studies are in progress to determine possible direct interaction between CTB-bound GM1 ganglioside receptors and FcγRIIB on the B cell surface as well as the in vitro activation of cytoplasmic tyrosine inhibitory motif and other downstream molecules in FcγRIIB-expressing B cells exposed to Ag/CTB conjugate and/or CTB alone. In this regard, it is noteworthy that Floto et al. (2005) (37) found that the inhibitory function of FcγRIIB depends on its aggregation and integration into cell membrane lipid rafts, which could be induced *in vitro* also in the absence of IgG by treating FcγRIIB-expressing cells with PMA and was then found to result in co-localization of FcγRIIB with fluorescently labeled CTB (which is an established marker of lipid rafts).

APCs initiate either pro-inflammatory Teff or anti-inflammatory Treg cell responses, depending on their activation status which is significantly controlled by autologous inhibitory signals generated via FcγRIIB. Samsom and coworkers (30) showed that the absence of FcγRIIB can convert tolerogenic T cell responses into immunogenic responses associated with increased IL-2 and IFN-γ secretion, and they suggested that the disappearance of tolerance resulted from the production of proinflammatory cytokines such as MCP-1, TNF-α and IL-6 together with increased expression of co-stimulatory molecules by DCs lacking the inhibitory FcγRIIB (30). Such a process, although mediated not only by DCs but also and perhaps even more importantly via FcγRIIB$^{-/-}$ B cells, is also suggested by our findings that μMT mice, that had adoptively received FcγRIIB$^{-/-}$ B cells and were then s.c. immunized with OVA in CFA, contained 3- to 5-fold increased numbers of activated DCs and B cells expressing various co-stimulatory molecules (e.g. CD83; Fig. S3) in their PLNs compared to similarly treated mice that had instead received FcγRIIB-expressing B cells. These results suggest that the proimmunogenic activation of APCs in these mice by immunization is largely due to the absence of FcγRIIB on B cells changing the balance between activating and inhibitory signaling on non-B cell APCs such as DCs, and increasing their propensity for stimulating Teff rather than Treg cell responses. These results may also suggest that the refractoriness of the FcγRIIB-deficient mice to mucosal tolerization could result from abnormal baseline activation of APCs in these mice, which is normalized by the adoptive transfer of FcγRIIB-expressing B cells and to a lesser extent by similar transfer of FcγRIIB-expressing DCs. In this regard it is also worth noting that, as illustrated in Fig. 6, the adoptive transfer of transgenic (B-TG) B cells overexpressing FcγRIIB into μMT mice did not only induce the strongest tolerizing effect and the highest levels of Foxp3^{+} and LAP/TGF-β^{+} Treg cells after mucosal OVA/CTB treatment, but did also by themselves (after mucosal treatment only with PBS) induce an approximate doubling of both of these Treg cell populations in comparison with similar

transfer of WT-like B-NTG B cells. These findings not only underline the important role of FcγRIIB expression on B cells for regulating tolerance and Treg numbers in response to specific mucosal Ag/CTB administration, but also suggest that the levels of FcγRIIB expression on B cells (and possibly also other APCs) may have a more general impact for regulating Treg cell levels and LAP/TGF-β expression.

Finally, as mentioned above, while our results indicate that incubation of FcγRIIB-expressing B cells with Ag/CTB conjugate in the absence of any IgG was enough to render the B cells effectively tolerogenic, it is also clear that the tolerogenic effect was further increased by incubating the cells with IgG immune-complexed Ag/CTB conjugate as compared to Ag/CTB conjugate alone. This finding could have direct implications as a means to maximize the efficacy of B cells in cell therapy against autoimmune and related inflammatory diseases. We recently described, as was also evident in the present study, that a highly efficient way to generate tolerogenic B cells is by incubating naive B cells with a relevant Ag conjugated to CTB (22). This allows most B cells, irrespective of BCR, to take up and present the CTB-linked Ag and induce their expression of LAP/TGF-β and after adoptive transfer also their in vivo production of IL-10 (22). Our previous study further suggested that B cells pulsed *in vitro* with relevant Ag/CTB conjugates may be used in cell therapy to induce Ag-specific suppression of autoimmune disease. Thus, adoptive transfer of B cells pulsed with an autoreactive myelin oligodendrocyte glycoprotein peptide conjugated to CTB prevented the development of experimental autoimmune encephalomyelitis (EAE) and increased the number of $Foxp3^+$ Treg cells; similar effects were seen when B cells were given "therapeutically" to mice with early-stage EAE (22). Our present findings suggest that the protective effect against autoimmune and related inflammatory diseases that may be achieved by infusion of B cells pretreated *in vitro* with relevant Ag/CTB formulations can be further increased by adding an

IgG Ab to the Ag/CTB formulation used for the *in vitro* treatment of the B cells before the *in vivo* infusion.

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Disclosures

The authors have no financial conflicts of interest.

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Footnotes

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Abbreviations used in this paper: CLN, cervical lymph node; CTB, cholera toxin B subunit; FcγRIIB, Fcγ Receptor IIB; Foxp3, the forkhead-winged helix family transcription factor 3; LAP, latency-associated peptide; OVAp, $OVA_{323-339}$ peptide; PLN, popliteal/inguinal lymph node; Teff, effector T cell; Treg, regulatory T cell.

The version of this article contains a data supplement.

LEGENDS

FIGURE 1. Mucosal administration of low-dose OVA/CTB conjugate induces immunological tolerance in WT but not FcγRIIB$^{-/-}$ mice. WT or FcγRIIB$^{-/-}$ mice received three s.l. treatments every 2nd d with OVA/CTB or PBS followed by a s.c. immunization with OVA/CFA and 2 wk later the mice were challenged i.d. in one ear with OVA. Twenty four h after challenge DTH reactions were recorded by measuring the increase in ear thickness whereafter single cell suspensions from the challenged ears were prepared for examination of Teff and Treg cells. (*A*) *DTH ear thickness increases*; (*B*) *Teff* (Foxp3^{-}CD25^{-}CD4^{+}) cells among CD4^{+} T cells; and (*C*) *Foxp3^{+}CD4^{+} Treg cells* among CD4^{+} T cells; Data are presented as mean values ± SEM for six animals per group. ***, $p < 0.001$ versus PBS. Data are from one of two independent experiments giving similar results.

FIGURE 2. FcγRIIB is also needed for high-dose oral tolerance induction. WT and FcγRIIB$^{-/-}$ mice were intragastrically given a single 20-mg dose of OVA or PBS, followed by a s.c. immunization with OVA/CFA; 2 wk later the mice were sacrificed and PLN cells examined in vitro for (*A*) *OVA-specific T cell proliferation*; and by flow cytometry for percentages of (*B*) *Teff* (Foxp3^{-}CD25^{-}CD4^{+}) cells among (gated) CD4^{+} T cells; and (*C*) *Treg* (Foxp3^{+}CD62L^{+} CD4^{+}) cells among CD4^{+} T cells. Data are expressed as mean ± SEM (5 mice per group) and values in parentheses show mean changes between OVA-treated and PBS-treated (but immunized) control mice. **$p < 0.01$ and *$p < 0.05$ versus PBS, respectively.

FIGURE 3. Poor Treg cell responses in FcγRIIB$^{-/-}$ mice after mucosal treatment with OVA/CTB. *Defective development of Foxp3^{+} and LAP/TGF-β^{+} Treg cells in treated FcγRIIB$^{-/-}$ mice (A* and *B).* OVAp specific transgenic CD4^{+} T cells from OT-IIxLy5.1 mice were i.v. transferred into either WT or FcγRIIB$^{-/-}$ mice, and starting one d later the mice received three

s.l. administrations of OVA/CTB conjugate or PBS at 2-d intervals. Three d after the last s.l. treatment the mice were sacrificed, single cell suspensions from their CLNs prepared, and percentages of (*A*) Foxp3$^+$ and (*B*) LAP/TGF-β$^+$ Treg cells among CD4$^+$ OT-IIxLy5.1 cells were determined by FACS. *Adoptively transferred T cells from OVA/CTB-treated FcγRIIB$^{-/-}$ mice fail to convey tolerance in vivo (C* and *D)*. CD4$^+$ T cells from OT-IIxLy5.1 mice were i.v. transferred into either WT or FcγRIIB$^{-/-}$ mice which were then subjected to the standard three-dose s.l. OVA/CTB or PBS treatment. Three d after the s.l. treatment, the mice were sacrificed and CD4$^+$ T cells isolated from pooled CLN and spleen were adoptively transferred together with freshly isolated CD4$^+$ OT-IIxLy5.1 cells into naive C57BL/6 recipients, which were then s.c. immunized with OVA/CFA. Two wk later PLNs were collected from the immunized mice and PLN cells examined for *in vitro T cell proliferation* after 3 d incubation with OVAp (*C*); and frequency of *Foxp3$^+$ CD4$^+$ Treg cells* among OVA-specific CD45.1$^+$ cells (*D*). Data are presented as mean values ± SEM for six animals per group. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ versus PBS. Data are from one of two independent experiments giving similar results.

FIGURE 4. B cells from OVA/CTB-treated FcγRIIB$^{-/-}$ mice fail to suppress Teff cell response or induce Treg cells *in vitro*. CD19$^+$ B cells were isolated from pooled CLN and spleen cells of FcγRIIB$^{-/-}$ and WT mice that had previously received the standard s.l. treatment with OVA/CTB or PBS; the isolated cells were then co-cultured in vitro with freshly isolated CD4$^+$ OT-IIxLy5.1 cells in the presence of the OVAp. CD4$^+$ *T cell proliferation* (*A*), *IFN-γ production* in culture supernatants (*B*), and percentages of *Ag-specific Foxp3$^+$CD4$^+$ Treg cells* among CD4$^+$ OT-IIxLy5.1 cells (*C*) were determined. Data are presented as mean values ± SEM for six animals per group. **$p < 0.01$ and *$p < 0.05$ versus PBS. Data are from one of two independent experiments giving similar results.

FIGURE 5. Engraftment of FcγRIIB$^{-/-}$ mice with FcγRIIB-expressing B cells restores responsiveness to mucosal tolerization. WT mice, FcγRIIB$^{-/-}$ mice or FcγRIIB$^{-/-}$ mice that had adoptively received WT B cells were given the standard s.l. treatment with OVA/CTB or PBS followed by s.c. immunization with OVA/CFA. Two wk later DTH reactions were recorded by measuring ear thickness (A), and PLN cells were prepared and examined for Ag-specific T cell proliferation (B) and Frequency of Foxp3^{+} Treg cells among CD4^{+} OT-IIxLy5.1 cells (C). Data are presented as mean values ± SEM for six animals per group; $^{**}p < 0.01$ and $^{*}p < 0.05$ versus PBS. Data are from one of two independent experiments giving similar results.

FIGURE 6. B cells overexpressing FcγRIIB have enhanced capacity to promote mucosally induced T cell tolerance and Treg cells. CD19^{+} B cells were isolated from spleens of transgenic mice overexpressing FcγRIIB on B cells (B-TG), from littermate non-transgenic mice expressing normal WT levels of FcγRIIB (B-NTG), or from FcγRIIB$^{-/-}$ mice. These B cells were i.v. transferred into groups of B cell-deficient μMT mice together with purified CD4^{+} T cells from OT-IIxLy5.1 mice, whereafter the mice were given the standard s.l. treatment with OVA/CTB or PBS followed by s.c. immunization with OVA/CFA. Two wk later the mice were sacrificed and their PLN cells collected and analysed for *T cell proliferation* (*A*), percentages of OVA-specific *Teff cells* (Foxp3^{-}CD45.1^{+}) among the total CD4^{+} cells (*B*), and frequencies of *Treg cells* among OVAp-specific CD45.1^{+} cells (*C*), the frequency of *apoptotic Teff cells* (Annexin V^{+} 7AAD^{-} cells) among CD4^{+} OT-IIxLy5.1 cells (*D*) and *LAP/TGF-β*$^{+}$ *Treg cells* among CD4^{+} T cells (*E*). Data are presented as mean values ± SEM for six animals per group; $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ versus PBS. Data are from one of two independent experiments giving similar results.

FIGURE 7. IgG immune-complexed OVA/CTB exerts stronger tolerance induction. $CD19^+$ B cells were isolated from either naive WT or $Fc\gamma RIIB^{-/-}$ mice and were next *in vitro* pulsed for 1 h with PBS, OVA/CTB, or OVA/CTB pre-mixed with anti-OVA IgG. After thorough washing the differently treated B cells were then i.v. transferred into either WT mice (*A*) or B cell-deficient μMT mice (*B*) together with purified $CD4^+$ OT-IIxLy5.1 T cells. All the recipient mice were then s.c. immunized with OVA/CFA. PLN cells and serum were collected 2 wk after immunization and examined *in vitro* for OVA-specific T cell proliferation which is expressed as the percentage inhibition in each treatment group vs PBS controls. Data are presented as mean values ± SEM for six animals per group; $p < 0.05$ and $p < 0.01$ versus PBS. Also indicated is the statistical significance between treatment with OVA/CTB+IgG antibody (Ab) versus OVA/CTB: *, $p < 0.05$.

FIGURE 8. *DCs from OVA/CTB-treated WT but not $Fc\gamma RIIB^{-/-}$ mice increase Treg cells in vitro and engraftment with WT DCs can restore the defective tolerance in vivo of $Fc\gamma RIIB^{-/-}$ mice to mucosal tolerization. (A) DCs from OVA/CTB-treated WT but not $Fc\gamma RIIB^{-/-}$ mice suppress Teff cell response and increase Treg cells in vitro.* $CD11^+$ DCs were isolated from pooled CLN and spleen cells of $Fc\gamma RIIB^{-/-}$ and WT mice 3 d after completion of the standard s.l. treatment with OVA/CTB or PBS; the isolated cells were then co-cultured for 3d in vitro with freshly isolated $CD4^+$ OT-IIxLy5.1 cells in the presence of the OVAp. Data presented show: $CD4^+$ *T cell proliferation* (*left panel*); *IFN-γ production* in culture supernatants by ELISA (*middle panel*); and *percentage of Ag-specific $Foxp3^+CD4^+$ Treg cells* among $CD4^+$ OT-IIxLy5.1 cells (*right panel*). *(B) Engraftment of $Fc\gamma RIIB^{-/-}$ mice with $Fc\gamma RIIB$-expressing DCs restores responsiveness to mucosal tolerization but not the induction of $Foxp3^+$ Treg cells.* WT mice, $Fc\gamma RIIB^{-/-}$ mice that had adoptively received WT DCs were given the standard s.l. treatment with OVA/CTB or PBS followed by s.c. immunization with OVA/CFA.

Two wk later DTH reactions were recorded (as described in Fig. 5), and PLN cells prepared and examined for Ag-specific T cell proliferation and Treg cells. Left panel: *DTH response*; Middle panel: *T cell proliferation* after 3 d culture with OVAp; Right panel: *Frequency of* $Foxp3^+$ *Treg cells* among $CD4^+$ OT-IIxLy5.1 cells. Data presented are mean values ± SEM for 4 (*A*) or 6 (*B*) animals per group; $^{**}p < 0.01$ and $^{*}p < 0.05$ versus PBS.

Important role for Fcγ IIB receptor on B lymphocytes for mucosal antigen-induced tolerance and Foxp3^{+} regulatory T cells

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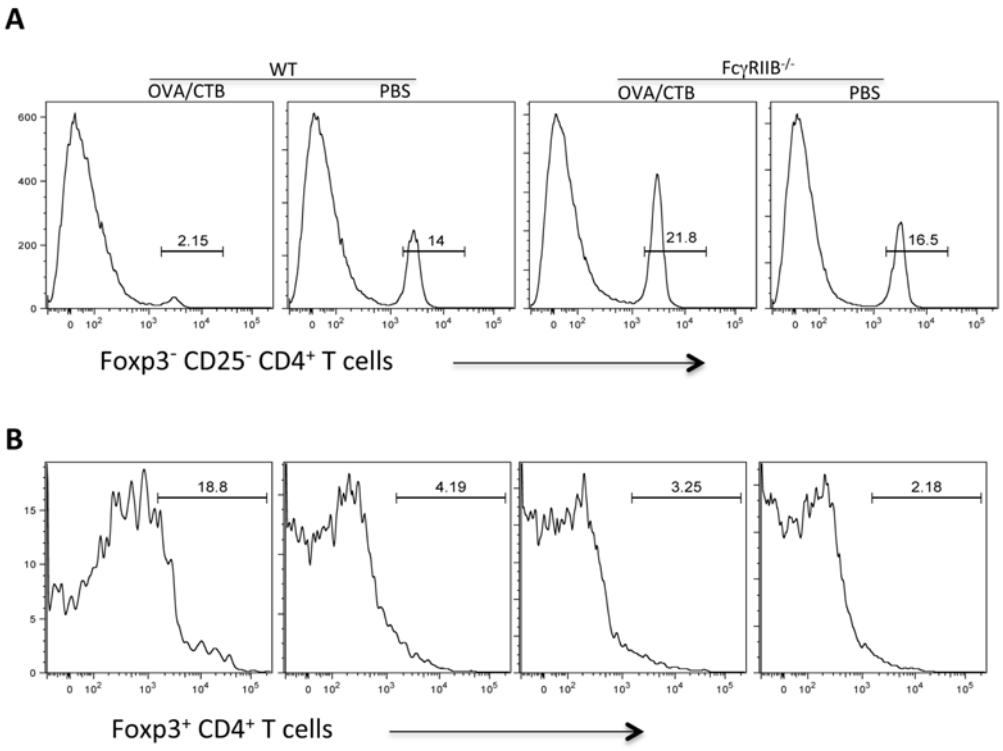
Supplemental figure legends

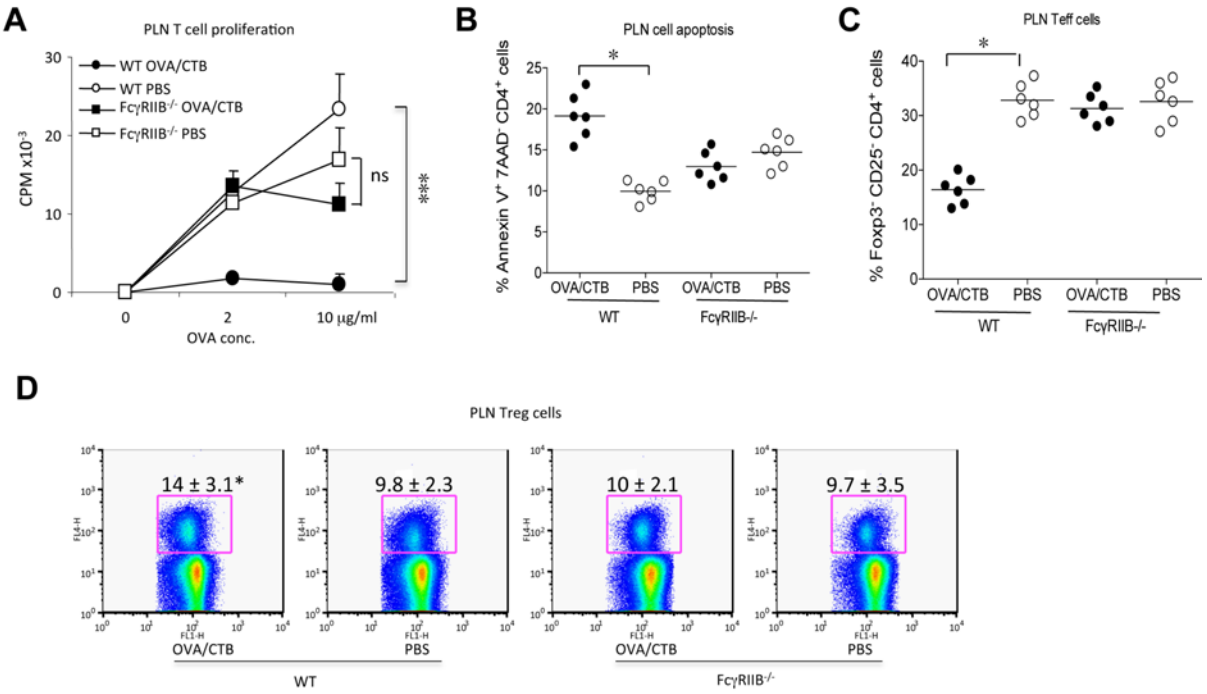
SUPPLEMENTAL FIGURE 1. Mucosal administration of OVA/CTB conjugate induces immunological tolerance in WT but not FcγRIIB$^{-/-}$ mice. WT or FcγRIIB$^{-/-}$ mice received three s.l. treatments every 2nd d with OVA/CTB or PBS followed by a s.c. immunization with OVA/CFA and 2 wk later the mice were challenged i.d. in one ear with OVA. Twenty four h after challenge by i.d. injection of OVA single cell suspensions from the challenged ears were prepared for examination of Teff and Treg cells. (*A*) *Teff* (Foxp3^{-}CD25^{-}CD4^{+}) cells among CD4^{+} T cells and (*B*) *Foxp3^{+}CD4^{+} Treg cells* among CD4^{+} T cells; representative flow histograms are shown (n=6).

Fig. S1.



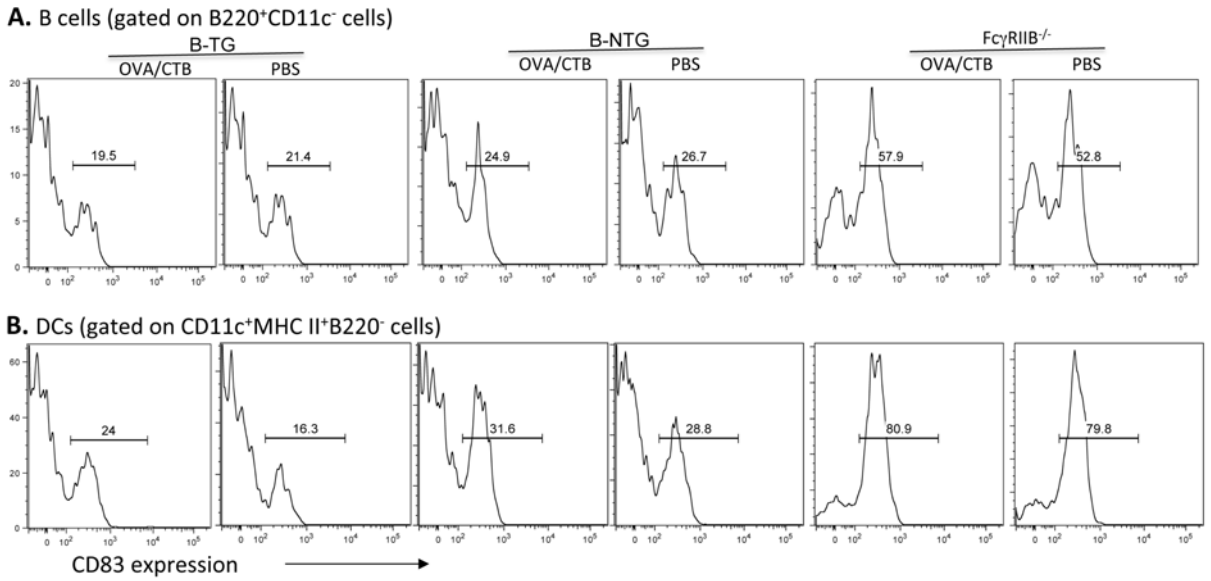
SUPPLEMENTAL FIGURE 2. Lack of tolerogenic effects on peripheral T cells in FcγRIIB$^{-/-}$ mice after s.l. administration of OVA/CTB. WT or FcγRIIB$^{-/-}$ mice received three s.l. treatments with OVA/CTB or PBS and were then immunized s.c. with OVA in CFA, and another 2 wk later PLNs were collected and single cell suspensions prepared and examined for *T cell proliferation* after incubation *in vitro* with OVA for 3 days (**A**), and by flow cytometry for *apoptosis* among CD4$^+$ T cells (**B**), frequency of Foxp3$^-$CD25$^-$ *Teff cells* among CD4$^+$ T cells (**C**) and frequency of *Foxp3$^+$ Treg cells* among CD4$^+$ T cells (**D**). Data are presented as mean ± SEM for six animals per group (A and D). Each dot refers to a measurement representing one mouse (B and C). A dot-plot graph was shown which represents six animals per experimental condition (D). *$p < 0.05$ and ***$p < 0.001$ versus PBS. Data are from one of two independent experiments giving similar results.

Fig. S2.



SUPPLEMENTAL FIGURE 3. DCs and B cells were activated in μMT mice which had received FcγRIIB$^{-/-}$ B cells and been s.c. immunized with OVA in CFA. μMT mice received B cells overexpressing FcγRIIB (B-TG) or B cells from non-transgenic mice expressing normal WT levels of FcγRIIB (B-NTG), or FcγRIIB$^{-/-}$ mice, and the recipient mice were given the standard s.l. treatment with OVA/CTB or PBS followed by s.c. immunization with OVA/CFA. Two wk later the mice were sacrificed and their PLN cells collected and treated as described in Fig. 7 and stained for expression of co-stimulating molecule CD83 on MHC class II^{+} and CD11c^{+} DCs (*A*) or B220^{+} B cells (*B*). Representative flow histograms are presented (n=6).

Fig. S3



Supplemental Table I.

Generation of serum IgG anti-OVA and anti-CTB antibodies in WT but not μMT recipients by adoptive cell transfer of differently pretreated B cells followed by s.c. immunization with OVA in CFA

Donor B cells	In vitro B cell pretreatment	Serum IgG antibodies (log10, mean ± SD)	
		Anti-OVA	Anti-CTB
WT recipients			
WT	PBS	4.12 ± 0.3	<0.7
WT	OVA/CTB	4.44 ± 0.3	2.65 ± 0.4
WT	OVA/CTB+Ab	3.55 ± 0.1[a]	2.50 ± 0.4
FcγRIIB$^{-/-}$	PBS	4.33 ± 0.2	<0.7
FcγRIIB$^{-/-}$	OVA/CTB	4.47 ± 0.1	3.68 ± 0.3
FcγRIIB$^{-/-}$	OVA/CTB+Ab	4.40 ± 0.1	3.25 ± 0.3
μMT recipients			
WT	PBS	<0.7	<0.7
WT	OVA/CTB	<0.7	<0.7
WT	OVA/CTB+Ab	<0.7	<0.7
FcγRIIB$^{-/-}$	PBS	<0.7	<0.7
FcγRIIB$^{-/-}$	OVA/CTB	<0.7	<0.7
FcγRIIB$^{-/-}$	OVA/CTB+Ab	<0.7	<0.7

CD19^{+} B cells were isolated from either naive WT or FcγRIIB$^{-/-}$ mice and *in vitro* pulsed with PBS, OVA/CTB, or OVA/CTB pre-mixed with anti-OVA IgG. After thorough washes, the differently treated B cells were then i.v. transferred into either WT or μMT mice together with purified CD4^{+} OT-IIxLy5.1 T cells and then all mice (n=6) were immunized s.c. with OVA in CFA. Upon sacrifice 2 w later, serum was collected and examined for anti-OVA and anti-CTB IgG antibodies by ELISA. [a] $p <$ 0.05 versus mice that had received B cells treated with OVA/CTB without Ab.