Jia-Bin Sun, Zou Xiang, Kenneth G. C. Smith and Jan Holmgren

*J Immunol* published online 13 September 2013
http://www.jimmunol.org/content/early/2013/09/13/jimmunol.1301324

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/09/13/jimmunol.1301324.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Important Role for FcγRIIB on B Lymphocytes for Mucosal Antigen-Induced Tolerance and Foxp3⁺ Regulatory T Cells

Jia-Bin Sun,*†,1 Zou Xiang,†,1 Kenneth G. C. Smith,‡ and Jan Holmgren*†

FcγRIIB, the only FcγR expressed on B cells, is important in the maintenance of immunological tolerance to self-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. Such mucosally induced tolerance (oral tolerance) associated with induction of Ag-specific Foxp3⁺ regulatory T cells was restored in FcγRIIB⁻/⁻ mice by adoptive transfer of either WT B cells or WT dendritic cells before the mucosal Ag/CTB treatment; it was even more pronounced in μMT mice that received FcγRIIB-overexpressing B cells before treatment. Furthermore, cell transfer in either WT or μMT mice of WT but not FcγRIIB⁻/⁻ B cells pretreated for 1 h 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 dendritic cells, is important for mucosal induction of Ag-specific immune tolerance. The Journal of Immunology, 2013, 191: 000–000.

Oral tolerance is known as a systemic immune unresponsiveness to a normally immunogenic parenteral injection of 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 Ags or allergens was found to induce peripheral tolerance that can prevent or ameliorate autoimmune and allergic inflammatory diseases; therefore, it has attracted attention as a possible means for Ag-specific immune therapy in such disorders (reviewed in Refs. 1, 2). Oral tolerance primarily affects effector T cells (Teffs), and Ab responses, in general, are not reduced significantly 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 cells (Tregs), both Foxp3⁺ and Foxp3⁻ cells, suppressing the development and function of Teffs (1, 2).

We (3–5) and other investigators (6–8) demonstrated that mucosal administration of various types of Ags coupled to cholera toxin B subunit (Ag/CTB) by either chemical conjugation or genetic fusion can more efficiently, and at >100-fold reduced dosages compared with Ag alone or Ag mixed with cholera toxin B subunit (CTB), induce peripheral T cell tolerance. Similar to other low-dose tolerance, this effect is associated with induction of Foxp3⁺, as well as Foxp3⁻, Tregs (9). The coupling of Ag to CTB, which binds to GM1 ganglioside receptors present on many cell types, including all known APCs, strongly potentiates the mucosal tolerizing effect (10) by facilitating Ag transport across the mucosal barrier and, more importantly, increasing Ag uptake and presentation by APCs, such as dendritic cells (DCs), B cells, and macrophages (11, 12).

Recent work identified a previously poorly recognized role for B cells in immunological tolerance, including oral tolerance. Although it is well known that B cells can be pathological in autoimmune diseases by producing autoantibodies, recent studies showed 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 mice compared with normal mice that can be corrected by adoptive transfer of B cells (15, 16). Studies showed that, under certain conditions, activated B cells can suppress Teff responses through providing signals, including IL-10 and TGF-β, which promote the development and expansion of Foxp3⁺ Tregs and/or Tr1 cells (16–20). Consistent with a regulatory role for B cells, B cell–deficient mice have a defective ability to develop oral tolerance (21), as well as Tregs, in response to mucosal Ag administration, including Ag/CTB, which also can be corrected by adoptive transfer of B cells (10). Indeed, our studies (10, 22) showed 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

*University of Gothenburg Vaccine Institute, SE-405 30 Göteborg, Sweden; †Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, SE-405 30 Göteborg, Sweden; and ‡Department of Medicine, Cambridge Institute for Medical Research, Cambridge CB2 0XY, United Kingdom

J.-B.S. and Z.X. contributed equally to this work.

Received for publication May 17, 2013. Accepted for publication August 5, 2013.

This work was supported by grants (to J.H.) from The Swedish Science Council (Medicine) (Project K2000-06X-03382) and funds from Sahlgrenska University Hospital.

Address correspondence and reprint requests to Dr. Jia-Bin Sun, Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, Box 435, SE-405 30 Göteborg, Sweden. E-mail address: jia-bin.sun@microbio.gu.se

The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; Ag/CTB, Ag (OVA) coupled to cholera toxin B subunit; B-TG mice, transgenic mice overexpressing FcγRIIB on B cells; B-NTG mice, littermate nontransgenic mice expressing normal wild-type levels of FcγRIIB on B cells; CLN, cervical lymph node; CTB, cholera toxin B subunit; DC, dendritic cell; DTH, delayed-type hypersensitivity; GM1, ganglioside-monosialic acid; i.d., intradermal; LAP, latency-associated peptide; LN, lymph node; OVA, peptide 323-339 fragment of OVA; PLN, popliteal inguinal lymph node; s.i., sublingual; Teff, effector T cell; Treg, regulatory T cell; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301324

Published September 13, 2013, doi:10.4049/jimmunol.1301324

The Journal of Immunology
regulatory APCs for any contacted Ag coupled to CTB, irrespective of their BCR specificity. In this study, we investigated the role of the IgG FcR FcγRIIB, and specifically its expression on B cells, in oral tolerance induced by mucosally administered Ag (OVA) conjugated to CTB or given alone. FcγRs constitute a family of surface molecules expressed on hematopoietic 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 was 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 coligation 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 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 were 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 demonstrat that FcγRIIB-expressing B cells are both efficient and sufficient for induction of functional tolerance, as well as development of Foxp3+ Tregs, although partial tolerization (but not any increase in Tregs) 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). C57BL/6 female mice of similar age were also used (B & K Universal, Stockholm, Sweden) together with OT-I/Ly5.1 (CD45.1+) mice on a C57BL/6 background (kind gift from Dr. Mary Jo Wick, University of 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 used 6–8-wk-old female B cell-deficient μMT mice (kind gift from Dr. Nils Lycke, University of Gothenburg) on a C57BL/6 background. Transgenic mice overexpressing FcγRIIB on B cells (B-TG mice) and littermate nontransgenic mice expressing normal wild-type (WT) levels of FcγRIIB on B cells (B-NTG mice) were generated as previously reported (25). Transgenic and gene-deficient mice were bred or kept under specific pathogen-free conditions at the animal experimental facility of the University of Gothenburg. The studies were approved by the University of Gothenburg Ethical Committee for Animal Experimentation.

Ags and conjugation of OVA to CTB

OVA protein (grade VII) was purchased from Sigma-Aldrich (St. Louis, MO), and OvAp (sequence ISQA VHAAHAEINEAGR) with >95% purity was obtained from TAG Copenhagen (Klampenborg, Denmark). Highly purified recombinant CTB was kindly provided by Crucell-Sweden (Stockholm, Sweden). OVA protein was chemically coupled to CTB using N-succinimidyl (3-[2-pyridyl]-dithio) propionate (Pierce Biotechnology, Rockford, IL) as a bifunctional coupling reagent, as described elsewhere (9). The OVA/CTB conjugate was purified by fast protein liquid chromatography gel filtration on a Superdex 200 16/60 column (Pharmacia Biotech) using the BioLogic Workstation FPLC System (Bio-Rad, Richmond, CA). The purified conjugate was analyzed by ganglioside-mono- sialic acid (GM1) ELISA and shown to have strong GM1-binding activity, as well as high reactivity with Abs 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 preincubation and coculture with polymylin, but it was completely inhibited by preincubation and coculture with highly purified GM1 (a gift from the late Prof. Lars Svennerholm).

Mucosal tolerization and s.c. immunization

Mucosal administration of CTB/OVA conjugate for tolerization was given by the sublingual (s.l.) route using a well-established regimen (10). In short, mice were given 10-μl s.l. doses of 40 μg OVA/CTB conjugate (or PBS as control) on three occasions at 2-d intervals. The extent of tolerization was tested by immunizing the mice, 2-d after the last s.l. dose, with s.c. injections in two dorsal positions on either side of the spine close to the tail with a total of 100 μg 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 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 tolerization was tested as described for s.l. tolerization by Ag/CTB.

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

Splena and indicated lymph nodes (LNs; cervical LNs [CLNs] or popliteal/ inguinal LNs [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; BD, Franklin Lakes, NJ). 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). Purified CD19+ B cells (purity >99%), at 106 cells/ml, were admistively transferred to mice (see below) or incubated in vitro with 0.1 μg/ml OVA/CTB conjugate or OVA/CTB premixed with anti-OVA IgG (2.5 μg/ml; Sigma-Aldrich), 1 μg/ml OVA, or PBS for 1 h at 37°C in 5% CO2, 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 and 400 U/ml DNase I (both from Roche Applied Science, Mannheim, Germany) at 37°C for 30 min, followed by filtration through a nylon net and centrifugation. CD11c+ DCs were isolated by positive selection using MACS MicroBeads coated with Ab against mouse CD11c (Miltenyi Biotec), according to the manufacturer’s recommendation, and the DCs (purity >90%) were 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 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) 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).

Adaptive transfer of cells

Purified CD19+ B cells (1 × 107 cells), purified CD4+ T cells (5 × 106 cells), or purified CD11c+ DCs (2 × 105 cells), all administered in 200 μl PBS/mouse, were adaptively 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 2 wk after the subsequent s.c. immunization, six mice in each group were given an intradermal (i.d.) injection in the left ear of 10 μg OVA in 20 μl 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 was measured, and single-cell suspensions of challenged ears were prepared by digesting minced ears in 25 μg/ml Liberase and 400 U/ml DNase I (both from Roche Applied Science).
Flow cytometry staining and analyses
For staining of surface markers, cells were incubated with FITC-, PE-, or allophycocyanin-labeled mAbs (BD Biosciences Pharmingen, San Jose, CA) 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), according to the manufacturer’s recommended protocol, followed by incubation with allophycocyanin-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 Biosciences, San Jose, CA). Apoptotic cells were detected by staining with annexin V-allophycocyanin and 7-aminoactinomycin D (7-AAD; BD Biosciences), according to the manufacturer’s protocol. For detection of cells expressing latency-associated peptide (LAP)/TGF-β, cells were incubated for 2 d in the presence of OVAp and then stained with biotinylated anti-LAP Ab (R&D Systems), 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 t tests (two-tailed) were used to calculate statistical differences between experimental and control groups.

Results
Mucosal administration of low-dose Ag/CTB conjugate or high-dose free Ag fails to induce oral tolerance in FcγRIIB<sup>−/−</sup> mice
Sublingual tolerization with OVA/CTB. Previous studies showed that intragastic, 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 Tregs (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<sup>−/−</sup> mice and WT mice after s.l. mucosal administration of OVA/CTB conjugate. DTH is a typical T cell–dependent inflammatory response that is readily established by s.c. immunization with Ag, together with CFA, followed 2 wk later by an i.d. challenge with the same Ag. As shown in Fig. 1A, 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 received three s.l. doses of OVA/CTB before a parenteral OVA/CFA immunization; in contrast, similarly treated FcγRIIB<sup>−/−</sup> mice showed no evidence of tolerance. Furthermore, infiltration of both total CD4<sup>+</sup> T cells (data not shown) and Foxp3<sup>+</sup> CD4<sup>+</sup> T cells (Fig. 1B, Supplemental Fig. 1A) was strongly reduced in challenged ears following s.l. administration of OVA/CTB to WT mice but not to FcγRIIB<sup>−/−</sup> mice. To determine whether the lack of mucosal tolerance in OVA/CTB-treated FcγRIIB<sup>−/−</sup> mice was associated with reduced development or infiltration of Tregs, we also examined Foxp3<sup>+</sup> CD4<sup>+</sup> Tregs 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<sup>−/−</sup>, mice (Fig. 1C, Supplemental Fig. 1B).

In parallel, we examined the PLN reaction of the mice following 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<sup>−/−</sup> mice (Supplemental Fig. 2A). The treatment also resulted in an increase in apoptotic PLN T cells from WT mice (Supplemental Fig. 2B), a reduction in Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> T cells (Supplemental Fig. 2C), and an increase in Foxp3<sup>+</sup> CD4<sup>+</sup> Tregs (Supplemental Fig. 2D), effects that were not seen in PLN cells from FcγRIIB<sup>−/−</sup> mice.

We also examined the levels of anti-OVA IgG Abs 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 Ab responses was evident in either the WT or FcγRIIB<sup>−/−</sup> mice after the s.l. dose 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<sup>−/−</sup> mice were given an intragastric 20-μg dose of OVA, and 1 wk later the mice were immunized s.c. with OVA in CFA. Two weeks later, the mice were sacrificed, and PLN cells were examined in vitro for OVA-specific T cell proliferation and frequencies of Teffs and Tregs. The proliferation of PLN T cells from OVA-treated WT mice was markedly suppressed (by 73 ± 1.3%) compared with cells from the PBS-treated controls, whereas the proliferation was increased in cells from similarly treated FcγRIIB<sup>−/−</sup> mice (Fig. 2A). Consistent with these findings, PLN cells from the OVA-treated WT mice, but not FcγRIIB<sup>−/−</sup> mice, showed a decrease in Teffs (Fig. 2B) and an increase in Foxp3<sup>+</sup> Tregs (Fig. 2C) compared with 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 response and tolerogenic Ag-presenting B cells in FcγRIIB<sup>−/−</sup> mice after mucosal treatment with Ag/CTB
Our previous work using WT mice showed that oral tolerance induced by s.l. treatment with Ag/CTB conjugates is associated with increased numbers of Foxp3<sup>+</sup> Tregs in the draining CLNs, as well as with Ag-presenting tolerogenic B cells that can induce Treg development and suppress Teff responses both in vitro and in vivo (10). We examined to what extent these effects depended on FcγRIIB. To this end, OVA-specific CD4<sup>+</sup> T cells from OT-IIxLy5.1 mice were adoptively transferred into WT or FcγRIIB<sup>−/−</sup> 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 second day with OVA/CTB conjugate or PBS. Three days later, CLNs and spleens were collected, and their CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells were isolated and examined as described in the following sections.

Defective Treg response in FcγRIIB<sup>−/−</sup> mice. Isolated CD4<sup>+</sup> cells from CLNs of the s.l.-treated WT and FcγRIIB<sup>−/−</sup> mice were examined for Ag-specific Tregs 3 d after the last s.l. treatment. As shown in Fig. 3A and 3B, the frequencies of both OVA-specific Foxp3<sup>+</sup> Tregs and regulatory LAP/TGF-β<sup>+</sup> T cells among CD4<sup>+</sup> 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 Treg populations was observed in the OVA/CTB-treated FcγRIIB<sup>−/−</sup> mice (Fig. 3A, 3B).

We also examined whether mucosal treatment with Ag/CTB in FcγRIIB<sup>−/−</sup> mice would generate CD4<sup>+</sup> T cells that, upon adop-
tive transfer into WT recipients, could develop into Tregs and suppress Teff responses to parenteral immunization. We isolated CD4+ T cells from pooled CLN and spleen cells of WT or FcγRIIB−/− mice after the standard s.i. treatments with CTB/OVA or PBS. These cells were adoptively transferred to WT recipients that were immunized s.c. 1 d later with OVA in CFA. Two weeks later, the immunized mice were sacrificed, their draining PLNs were collected, and the PLN cells were examined for OVA-specific T cell proliferation in vitro and frequency of Foxp3+ Tregs. The results show that T cell proliferation upon Ag restimulation was significantly suppressed in PLN cells from the mice that received FcγRIIB+ T cells isolated from the mucosally OV A/CTB-treated WT mice; in contrast, mice that received T cells from OV A/CTB-treated FcγRIIB−/− mice did not demonstrate any suppression (Fig. 3C). Consistent with this, OVA-specific Foxp3+ Tregs were increased in the PLNs of mice that received CD4+ T cells isolated from the mucosally OV A/CTB-treated WT, but not FcγRIIB−/−, mice (Fig. 3D).

B cells from mucosally OV A/CTB-treated FcγRIIB−/− mice fail to suppress T cell responses and to induce Tregs in vitro. We (10) and other investigators (1, 32) showed that B cells are important in the induction of oral tolerance and Treg development. Thus, we examined whether isolated B cells from mucosally OV A/CTB-treated FcγRIIB−/− mice could induce OVA-specific Tregs and suppress OVA-specific T cell proliferation when cocultured with OVA-stimulated T cells in vitro. To test this, we cultured CD19+ B cells isolated from pooled CLN and spleen cells of s.i.-treated WT or FcγRIIB−/− mice for 3 d, together with freshly isolated CD4+ T cells from naïve OT-IIxLy5.1 mice in the presence of OVAp, and examined OVA-specific T cell proliferation, IFN-γ production, and induction of Tregs in the cultures. As shown in Fig. 4A and 4B, cells from OV A/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. 4B) and OVA-specific Foxp3+ Tregs were increased (Fig. 4C) in the culture with B cells coming from OV A/CTB-treated WT, but not FcγRIIB−/−, mice. These results indicate that, during coculture with T cells in vitro, only B cells from Ag/CTB-treated mice that express FcγRIIB can induce the development of Ag-specific CD4+ Tregs and suppress Teff 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 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.i. treatment with OV A/CTB or PBS, followed by the initiation of DTH responses, as described above (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. 1A, FcγRIIB−/− mice that did not receive any WT B cells failed to respond to OV A/CTB-mediated tolerization and, thus, did not suppress either the Ag-specific DTH response (Fig. 5A) or proliferation of PLN T cells (Fig. 5B). In contrast, engrafting FcγRIIB−/− mice with WT B cells fully restored their capacity to suppress both of these responses after mucosal OV A/CTB treatment (Fig. 5A, 5B). Fur-
thermore, PLN cells from these chimeric mice, similar to cells from WT mice, contained more Tregs following tolerization and immunization (Fig. 5C). In addition, there was a marked decrease in the number of Ag-specific Teffs (Foxp3^2^CD25^2^CD4^+^) and an increase in apoptotic Ag-specific T cells (CD45.1^+^Annexin V^+^7-AAD^2^CD4^+) in the PLNs of the chimeric mice (data not shown).

**Increased tolerogenic effect of engraftment of B cell–deficient \( ^{\mu} \)MT mice with B cells overexpressing Fc\gammaRIIB compared with B cells expressing normal levels of Fc\gammaRIIB**

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

**FIGURE 3.** Poor Treg responses in Fc\gammaRIIB^−/− mice after mucosal treatment with OVA/CTB. (A and B) Defective development of Foxp3^+^ and LAP/TGF-\( \beta \)^+ Tregs in treated Fc\gammaRIIB^−/− mice. OVAp-specific transgenic CD4^+^ T cells from OT-I\( \times \)Ly5.1 mice were transferred i.v. into either WT or Fc\gammaRIIB^−/− mice; beginning 1 d later, the mice received three s.l. doses of OVA/CTB conjugate or PBS at 2-d intervals. Three days after the last s.l. treatment, the mice were sacrificed, single-cell suspensions were prepared from their CLNs, and the percentages of Foxp3^+^ (A) and LAP/TGF-\( \beta \)^+ (B) Tregs among CD4^+^ OT-I\( \times \)Ly5.1 cells were determined by FACS. (C and D) Adoptively transferred T cells from OVA/CTB-treated Fc\gammaRIIB^−/− mice fail to convey tolerance in vivo. CD4^+^ T cells from OT-I\( \times \)Ly5.1 mice were transferred i.v. into either WT or Fc\gammaRIIB^−/− mice, which were then subjected to the standard three-dose s.l. OVA/CTB or PBS treatment. Three days 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-I\( \times \)Ly5.1 cells, into naive C57BL/6 recipients, which were immunized s.c. with OVA/CFA. (C) Two weeks later, PLNs were collected from the immunized mice, and PLN cells were examined for in vitro T cell proliferation after a 3-d incubation with OVAp. (D) Frequency of Foxp3^+^CD4^+^ Tregs among OVA-specific CD45.1^+^ cells. Data are mean ± SEM for six animals/group. Data are from one of two independent experiments giving similar results. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
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), treated mice versus controls) than did the B cells from B-NTG mice (88.9 ± 3.7%, p < 0.01) (Fig. 6A).

A similar pattern was observed in the percentages of Foxp3+ Teffs in the PLNs (Fig. 6B). Again, not only did B cells from FcγRIIB−/− mice totally lack suppressive capacity, B cells from B-TG mice transferred a stronger suppressive effect compared with B cells from B-NTG mice (97.4 ± 2.5% and 87.4 ± 4.2%, treated mice versus controls) (Fig. 6B).

These observations were found to correlate with the generation of Tregs. Thus, as shown in Fig. 6C, engrafting B cells from B-TG, B-NTG, and FcγRIIB−/− mice provided a mean 240% (p < 0.01), 140% (p < 0.05), and 30% (p < 0.20) increase in Foxp3+ Tregs, respectively. We demonstrated previously that OVA/CTB tolerization induced Teff apoptosis and increased Foxp3+ (and LAP/TGF-β+) CD4+ Tregs in WT mice but not in FcγRIIB−/− mice (Fig. 3B, Supplemental Fig. 2B). In this study, we show that tolerization of the chimeric mice, in addition to increasing Foxp3+ Tregs (Fig. 6C), resulted in apoptosis of Teffs (Fig. 6D) and an increase in LAP/TGF-β+CD4+ Tregs in WT mice but not in FcγRIIB−/− mice (Fig. 3B, Supplemental Fig. 2B). We also investigated PLN cells from the recipient mice for their expression of the costimulatory/activation molecules CD80, CD83, and CD86 on B cells and DCs. Not surprisingly, PLN cells from mice that 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 apoptosis (Fig. 6D) or LAP/TGF-β+CD4+ Tregs (Fig. 6E) was seen in the mice that received cells from FcγRIIB−/− mice.

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 Tregs, and these effects are increased further 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 induction in the recipients, even without any in vivo mucosal tolerization treatment (22). To determine whether FcγRIIB is critical in this process, as well as to study the effect of IgG immune complexes interacting with FcγRIIB on B cells for the induction of immu-
nological tolerance, we isolated B cells from WT and Fc γRIIB\(^{2/2}\) mice and pulsed them in vitro with PBS, OVA/CTB, or OVA/CTB preincubated with anti-OVA IgG Ab. After washing, the differently treated B cells were transferred i.v., together with CD4\(^{+}\) OT-II x Ly5.1 T cells, to either WT or \(\mu\)MT mice, followed by s.c. immunization with OVA/CFA. Two weeks later, the mice were sacrificed, and their PLN cells were collected and analyzed for T cell proliferation (A), percentage of OVA-specific Teffs (Foxp3\(^{+}\) CD45\(^{+}\) cells) among total CD4\(^{+}\) cells (B), frequency of Tregs among OVA-specific CD45\(^{+}\) cells (C), frequency of apoptotic Teffs (Annexin V\(^{+}\)/7-AAD\(^{+}\) cells) among CD4\(^{+}\) OT-IIxLy5.1 cells (D), and frequency of LAP/TGF-β\(^{+}\) Tregs among CD4\(^{+}\) T cells (E). Data are mean ± SEM for six animals/group. Data are from one of two independent experiments giving similar results. *p < 0.05, **p < 0.01, ***p < 0.001.

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 Fc γRIIB\(^{2/2}\) mice and pulsed them in vitro with PBS, OVA/CTB, or OVA/CTB preincubated with anti-OVA IgG Ab. After washing, the differently treated B cells were transferred i.v., together with CD4\(^{+}\) OT-IIxLy5.1 T cells, to either WT or \(\mu\)MT mice, followed by s.c. immunization of the mice with OVA in CFA. Two weeks later, the mice were sacrificed, and their PLN cells were analyzed; in addition, serum was collected and examined for Abs 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 \(\mu\)MT mice that received WT B cells treated in vitro with OVA/CTB, and this effect was increased further when the donor B cells had been pretreated with OVA/CTB plus IgG anti-OVA Ab. The suppressive effects were more pronounced in WT mice compared with \(\mu\)MT mice as recipients (Fig. 7), possibly owing to the fact that the WT mice, in contrast to the B cell-deficient \(\mu\)MT mice, had developed serum anti-OVA IgG Abs 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 received B cells from Fc γRIIB\(^{2/2}\) mice, irrespective of whether these cells had been treated with OVA/CTB alone or with OVA/CTB plus anti-OVA IgG; in fact, the T cell proliferation was increased in \(\mu\)MT mice (Fig. 7). In addition, the serum levels of anti-OVA IgG were reduced in WT recipients that received WT B cells treated with OVA/CTB plus IgG anti-OVA Ab compared with controls, suggesting a partial tolerance of the OVA-specific Ab response as well (Supplemental Table I).

**DCs also require expression of FcγRIIB to induce T cell tolerance**

Given the important role of the expression of FcγRIIB on B cells for mucosal induction of Ag-specific T cell tolerance, as well as the report by Samsom et al. (30) suggesting a similar role for 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\(^{2/2}\) mice after the standard three-dose s.l. treatment with OVA/CTB to suppress OVA-specific Teffs and promote Tregs in vitro. The results in Fig. 8A show that Ag-specific T cell proliferation (left panel) and IFN-γ production (middle panel) were
**ROLE OF FcγRIIB ON B CELLS IN ORAL TOLERANCE**

**FIGURE 7.** IgG immune-complexed OVA/CTB exerts stronger tolerance induction. CD19+ B cells were isolated from either naive WT or FcγRIIB−/− mice and were in vitro pulsed for 1 h with PBS, OVA/CTB, or OVA/CTB premixed with anti-OVA IgG. After thorough washing, the differently treated B cells were transferred i.v. into either WT mice (A) or B cell-deficient μMT mice (B), together with purified CD4+ OT-II-Ly5.1 T cells. All recipient mice were 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 suppression of T cell proliferation in each treatment group versus PBS controls. Data are mean ± SEM for six animals/group. *p < 0.05, **p < 0.01, versus PBS. The statistical significance (*p < 0.05) for treatment with OVA/CTB+IgG Ab versus OVA/CTB is also shown.

suppressed using DCs from OVA/CTB-treated WT mice as APCs but were increased using DCs from similarly treated FcγRIIB−/− mice. Consistent with this, only the DCs from OVA/CTB-treated WT mice induced Foxp3+ Tregs 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-IhLy5.1 T cells, were transferred into FcγRIIB−/− mice, which were then given the standard three-dose s.i. 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-IhLy5.1 T cells and s.i.-treated WT mice served as controls. As shown in Fig. 8B, engrafting FcγRIIB−/− mice with FcγRIIB-expressing WT DCs, much as 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 in Tregs in Fig. 8B, right panel), which is in contrast with both OVA/CTB-treated WT mice (Fig. 8B, right panel) and FcγRIIB−/− mice engrafted with FcγRIIB-expressing B cells (Fig. 8C). 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+7-AAD− 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 Tregs inactivating Teffs has been identified as the main effector mechanism. Our recent work identified B cells as playing an important role as tolerogenic APCs in mucosally induced tolerance (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 found that the inhibitory IgG receptor FcγRIIB is critical for the induction of oral tolerance and that the expression of this receptor on Ag-presenting B cells is both important and sufficient for tolerance induction. Using OVA as a 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 it completely failed to do so in FcγRIIB-deficient FcγRIIB−/− mice. We confirmed a previous study (30) that found a contribution of FcγRIIB expression on DCs to the induction of mucosal tolerance. However, we extended the study further by demonstrating that FcγRIIB expression on B cells is sufficient for induction of Ag-specific immune tolerance and Foxp3+ Tregs after mucosal Ag/CTB administration. In contrast to other myeloid cells, including DCs, which express several types of both activating and inhibitory FcγRs, the inhibitory FcγRIIB is the only FcγR expressed on B cells. Both the tolerogenic effect and the induction of Ag-specific Foxp3+ Tregs 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 tolerance in WT mice, as well as in μMT mice, provided that the transferred Ag/CTB-treated B cells expressed FcγRIIB. 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 response unless adoptively receiving FcγRIIB-expressing B cells (21). At the same time, as first described by Gonnella et al. (32) and confirmed by us (10), μMT mice normally can develop a reduced, modified form of tolerance in response to mucosally administered Ag, being mediated by Ag-specific Foxp3+ LAP+ Tregs rather than Foxp3+ Tregs (10). It was reported that B cells can modulate the Ag presentation to T cells by DCs in μMT mice by promoting IL-4 production and decreasing IL-12 production, thus providing a more balanced Th1/Th2 response (35). 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 tolerance capacity in μMT mice (Figs. 6A, 7B).

Although 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 coculture of naive OVA-specific T cells with Ag-presenting WT DCs, but not FcγRIIB−/− DCs, promoted the induction of CD4+ Tregs. In support of a role for FcγRIIB-expressing DCs as well, we found that in WT mice, but not in FcγRIIB−/− mice, mucosal Ag/CTB treatment induced tolerogenic B cells, as well as DCs, which after isolation and subsequent in vitro culture with Ag and T cells, promoted the expansion of Foxp3+ Tregs and suppressed Ag-specific Teff 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 mice, although apparently to a lesser extent than seen after transfer of FcγRIIB-expressing B cells; also different from the B cells, the transferred DCs failed to induce Ag-specific Foxp3+ Tregs in vivo.

Normally, like other FcγRs, FcγRIIB is activated only after being cross-linked by IgG–Ag immune complexes (26, 36). However, our results suggest that FcγRIIB on B cells may be activated by exposure to Ag/CTB, even in the absence of any IgG Ab, although the FcγRIIB-expressing B cells became even more effective in inducing tolerizing and Treggs following treatment with IgG Ab immune-complexed Ag/CTB compared with 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 because 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 Abs 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 FcγRIIB on B cells in the absence of IgG Ab and render the B cells tolerogenic; studies are in progress to determine possible direct interactions 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. (37) found that the inhibitory function of FcγRIIB depends on its aggregation and integration into cell membrane lipid rafts, which also could be induced in vitro in the absence of IgG by treating FcγRIIB-expressing cells with PMA; it was found to result in colocalization of FcγRIIB with fluorescently labeled CTB, which is an established marker of lipid rafts.

APCs initiate either proinflammatory Teff or anti-inflammatory Treg responses, depending on their activation status, which is significantly controlled by autologous inhibitory signals generated via FcγRIIB. Samsom et al. (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 costimulatory 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 adoptively received FcγRIIB−/− B cells and were s.c. immunized with OVA in CFA contained 3–5-fold increased numbers of activated DCs and B cells expressing various costimulatory molecules (e.g., CD83; Supplemental Fig. 3) in their PLNs compared with similarly treated mice that 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 T effector, rather than Treg, responses. These results may also suggest that the refractoriness of 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 induced the strongest tolerizing effect and the highest levels of Foxp3+ and LAP/TGF-β+ Tregs after mucosal OVA/CTB treatment and, by themselves (after mucosal treatment only with PBS), induced an approximate doubling of both of these Treg populations in comparison with similar transfer of WT-like B-NTG B cells. These findings underline the important role of FcγRIIB expression on B cells for regulating tolerance and Treg numbers in response to specific mucosal Ag/CTB administration and suggest that the levels of FcγRIIB expression on B cells (and possibly other APCs) may have a more general impact for regulating Treg levels and LAP/TGF-β expression.

Finally, as mentioned above, although 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 increased further by incubating the cells with IgG–immune–complexed Ag/CTB conjugate compared with Ag/CTB conjugate alone. This finding could have direct implications as a means to maximize the efficacy of B cells in cell therapy against autoimmunity and related inflammatory diseases. We recently described, as was also evident in the current study, that a highly efficient way to generate tolerogenic B cells is by incubating naïve 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 irrespective of BCR, to take up and present the CTB-linked Ag and induce their expression of LAP/TGF-β and, after adoptive transfer, their in vivo production of IL-10 as well (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 oligoden-drocyte glycoprotein peptide conjugated to CTB prevented the development of experimental autoimmune encephalomyelitis and increased the number of Foxp3+ Tregs; similar effects were seen when B cells were given “therapeutically” to mice with early-stage experimental autoimmune encephalomyelitis (22). Our present findings suggest that the protective effect against autoimmune and related inflammatory and related inflammatory diseases (22). The authors have no financial conflicts of interest.


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

Revised manuscript: 13-01324-FL

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 (Foxp3CD25CD4+) cells among CD4+ T cells and (B) Foxp3+CD4+ Treg cells among CD4+ T cells; representative flow histograms are shown (n=6).

Fig. S1.

A

B
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− T effector cells among CD4+ T cells (C) and frequency of Foxp3+ T regulatory 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
A. B cells (gated on B220−CD11c+ cells)

B. DCs (gated on CD11c+MHC II+B220− cells)
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

<table>
<thead>
<tr>
<th>Donor B cells</th>
<th>In vitro B cell pretreatment</th>
<th>Serum IgG antibodies (log10, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-OVA</td>
</tr>
<tr>
<td><strong>WT recipients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>PBS</td>
<td>4.12 ± 0.3</td>
</tr>
<tr>
<td>WT</td>
<td>OVA/CTB</td>
<td>4.44 ± 0.3</td>
</tr>
<tr>
<td>WT</td>
<td>OVA/CTB+Ab</td>
<td>3.55 ± 0.1a</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>PBS</td>
<td>4.33 ± 0.2</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>OVA/CTB</td>
<td>4.47 ± 0.1</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>OVA/CTB+Ab</td>
<td>4.40 ± 0.1</td>
</tr>
<tr>
<td><strong>μMT recipients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>PBS</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>WT</td>
<td>OVA/CTB</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>WT</td>
<td>OVA/CTB+Ab</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>PBS</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>OVA/CTB</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>FcγRIIB−/−</td>
<td>OVA/CTB+Ab</td>
<td>&lt;0.7</td>
</tr>
</tbody>
</table>

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.