SCANDINAVIAN JOURNAL OF Immunology
AN INTERNATIONAL MONTHLY JOURNAL

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|---|---|
| Journal: | *Scandinavian Journal of Immunology* |
| Manuscript ID | SJI-16-300.R1 |
| Manuscript Type: | Regular Manuscript |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Sun, Jia-Bin; Biomedicine, Göteborg University, Microbiology & Immunology<br>Holmgren, Jan; University of Gothenburg, Microbiology and Immunology;<br>Cragg, Mark; University of Southampton Faculty of Medicine, Antibody and Vaccine Group, Cancer Sciences Unit<br>Xiang, Zou; University of Gothenburg, Microbiology and Immunology |
| Keywords: | Fc Receptors < Molecules, Mucosal Immunity < Processes, Vaccination < Processes, Experimental Animals < Subject |
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# Lack of Fc gamma receptor IIIA promotes rather than suppresses humoral and cellular immune responses after mucosal or parenteral immunization with antigen and adjuvants

Running title: Lack of FcγRIIIA promotes immune responses

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Total text word count: 3235

Abstract word count: 202

Scientific heading: Basic Immunology

**Abstract**

The Fcγ receptor IIIA (FcγRIIIA) has traditionally been known as a positive regulator of immune responses. Consistent with this, mice deficient in FcγRIIIA are protected from various inflammation-associated pathologies including several autoimmune diseases. In contrast to this accepted dogma, we show here that mice lacking FcγRIIIA developed increased rather than reduced both humoral and cellular immune responses to mucosal (sublingual) immunization with ovalbumin (OVA) given together with the strong mucosal adjuvant cholera toxin as well as to parenteral (subcutaneous) immunization with OVA in complete Freund's adjuvant. After either route of immunization, in comparison with concomitantly immunized wild-type (WT) mice, FcγRIIIA$^{-/-}$ mice had increased serum anti-OVA IgG (IgG1 but not IgG2) antibody responses as well as augmented cellular responses that included memory B cells and effector T cells. The increments in immune responses in FcγRIIIA$^{-/-}$ mice were similar to those seen in FcγRIIB$^{-/-}$ mice. Furthermore, OVA-pulsed FcγRIIIA$^{-/-}$ DCs, similarly to OVA-specific FcγRIIB$^{-/-}$ DCs, had enhanced capacity to activate OVA-specific OT-II T cells, which was even further pronounced when DCs were pulsed with IgG1-complexed OVA. Our data support an inhibitory-regulatory role of FcγRIIIA on vaccine/adjuvant-induced immune responses and demonstrate that lack of FcγRIIIA can promote rather than suppress both humoral and cellular immune responses.

## Introduction

The Fc gamma receptor (FcγR) family is widely expressed on many immune cells, where these receptors help to regulate both innate and adaptive immunity. In both mice and humans, the family consists of several activatory FcγRs, one of which is FcγRIIIA, and a single recognized inhibitory receptor, FcγRIIB. Both FcγRIIIA and FcγRIIB are expressed on all myeloid cells including mast cells, basophils, eosinophils, neutrophils, monocytes/macrophages, and dendritic cells; FcγRIIB is in addition also expressed on B cells [1].

FcγRIIIA and FcγRIIB have two 95% identical Ig-like extracellular domains that are capable of binding the Fc portion of IgG [2]. FcγRIIIA is composed of an IgG-binding α-chain and a signal-transducing adaptor molecule that contains a homodimer of the common FcRγ-chain. The FcRγ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM) that becomes phosphorylated following ligand engagement of the receptor, leading to docking and activation of protein tyrosine kinases such as spleen tyrosine kinase (Syk). In contrast, FcγRIIB is composed of a single α-chain which contains an intracytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) which negatively regulates cell activation through activation of phosphatases such as Src homology 2-containing inositol 5'-phosphatase (SHIP).

Consistent with a normally activating role of FcγRIIIA, defective NK cell-mediated antibody-dependent cytotoxicity, macrophage phagocytosis of IgG-coated particles, and mast cell degranulation triggered by IgG immune complexes have been observed following genetic deletion of FcγRIIIA [3]. Furthermore, both passive cutaneous and

passive systemic anaphylaxis induced by IgG immune complexes are compromised in FcγRIIIA$^{-/-}$ mice [3, 4]. FcγRIIIA$^{-/-}$ mice are also protected, to variable degrees, in experimental models of arthritis [5-7], atherosclerosis [8, 9], acute glomerular inflammation [10], antibody-dependent type II autoimmunity [11], experimental autoimmune encephalomyelitis [12], antierythrocyte IgG-mediated anemia [13], and nephrotoxic nephritis [14]. Further, FcγRIIIA has been found to be important for Th2-mediated airway inflammation [15] and anaphylatoxin-induced lung pathology [16].

However, in contrast to the classically defined activatory role of FcγRIIIA, new evidence indicates that ITAM-containing receptors can generate a mixture of activation and inhibition signals [17]. The ITAM that transduces inhibitory signals dampening immune responses is specifically designated as ITAMi [18]. FcγRIIIA engagement by antibodies of the IgG1 subclass may result in partial phosphorylation of ITAM tyrosine residues following the recruitment of Src homology 2 domain-containing phosphatase-1 (SHP-1) recruitment, thus favouring the ITAMi configuration [18, 19].

The effect of FcγRIIIA on antigen-specific cellular and humoral immune responses following immunization has however not been systematically investigated. In this study, we explored such responses in FcγRIIIA-deficient mice in comparison with wild-type (WT) and FcγRIIB-deficient mice after mucosal immunization with a model protein antigen (ovalbumin, OVA) together with the strong mucosal adjuvant cholera toxin (CT), as well as after parenteral immunization with OVA in complete Freund's adjuvant (CFA). Interestingly, in both systems, we observed strongly

enhanced rather than suppressed antigen-specific humoral and cellular immune responses in FcγRIIIA-deficient mice compared with WT mice, with the enhancement of these responses in the FcγRIIIA$^{-/-}$ mice being of similar magnitude as seen in concomitantly immunized FcγRIIB-deficient mice.

## Materials and Methods

### Mice

FcγRIIIA$^{-/-}$ mice on the C57BL/6 background were originally purchased from The Jackson Laboratory and bred in-house at the MIVAC breeding unit of the experimental animal facility of the University of Gothenburg as were FcγRIIB$^{-/-}$ mice on the C57BL/6 background kindly donated by Dr Ken Smith, Cambridge University [20]. Also OT-II mice, whose T cells have a transgenic T cell receptor specific for the 323–339 peptide of ovalbumin ($OVA_{323–339}$) were obtained from the MIVAC breeding unit. Six- to eight-week old female mice at the start of experiments were used; and all mice, including C57BL/6 WT control mice (purchased from B&K Universal AB, Stockholm, Sweden) were housed together under specific pathogen-free conditions at the experimental animal facility from at least two weeks before and for the duration of the experiments. The studies were approved by the University of Gothenburg Ethical Committee for Animal Experimentation.

### Immunization and collection of specimens

OVA (grade VII) purchased from Sigma (St. Louis, MO, USA) was used for immunization and analyses. For sublingual (s.l.) mucosal immunization, groups of

WT, FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice were given 200 μg OVA with or without 5 μg CT in a 10-μl droplet placed under the tongue on two occasions at an interval of 10 days unless otherwise stated. Ten days after the last s.l. immunization, sera and spleens were collected and analysed for total IgG anti-OVA as well as IgG1 and IgG2c subclass anti-OVA antibody levels by ELISA and for various cellular responses by flow cytometry. Alternatively, spleens were removed three days after the 2$^{nd}$ immunization to assess germinal center formation. In separate experiments, WT, FcγRIIIA$^{-/-}$ and FcγRIIB$^{-/-}$ mice were immunized subcutaneously (s.c.) in two dorsal positions on either side of the spine close to the tail with, in each site, a 100-μl emulsion containing 100 μg of OVA in CFA. Two weeks later, serum and popliteal lymph nodes (PLNs) were collected and examined for anti-OVA antibody and cellular responses.

**Enzyme-linked immunosorbent assay**

To determine serum OVA-specific total IgG as well as IgG1 and IgG2c antibody titers, enzyme-linked immunosorbent assays (ELISA) were performed as described previously [21]. Endpoint titers were expressed as the reciprocal $\log_{10}$ of the extrapolated dilution giving an absorbance at 450 nm of 0.4 above the background after a 20-min incubation with the enzyme substrate.

**Stimulation of T cells by co-cultured DCs**

Bone marrow-derived DCs were generated by incubating bone marrow cells isolated from naive WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice in the presence of 200 ng/ml Flt3-L (Purchased from R&D System) for 9 days. The DCs were then incubated for 2 h in

medium alone, in medium containing 20 μg/ml OVA or medium containing immune complexes generated by preincubation of 20 μg/ml OVA with 30 μg/ml anti-OVA IgG1 (Sigma), followed by rigorous cell washing. Next, the treated DCs ($2\times10^4$/well) were co-cultured with freshly isolated OT-II T cells ($1\times10^5$/well) for 3 or 4 days with [$^3$H]-thymidine being added during the last 6 h of co-culture. T cell proliferation was determined by a radioactive assay based on incorporation of [$^3$H]-thymidine and expansion of Foxp3$^-$CD25$^+$CD4$^+$ T effector cells was analyzed by flow cytometry.

**Flow cytometric staining and analyses**

Single cell suspensions from spleens or PLNs were prepared as described [22]. For staining of surface markers, cells were incubated with antibodies with appropriate fluorochrome conjugation (Supplementary Table 1) to mouse CD1d, CD4, CD5, CD25, CD103, CD69, CD138, B220, CD11c, CD19, CD80, CD62L, CD86, ICOS, CXCR5, PD-1, CD38, and GL7 (BD or eBioscience, San Diego, CA, USA or Biolegend, Nordic BioSite AB, Sweden). For detection of FcγRIIB expression, cells were stained with FITC-labelled anti-FcγRIIB mAb (AT130-2) [23]. For detection of cells expressing latency-associated peptide (LAP)/TGF-β, cells were stained with biotinylated anti-LAP Ab (R&D Systems), followed by streptavidin-allophycocyanin, in addition to staining with Abs against selected surface markers before flow cytometric analysis. For detection of intracellular Foxp3, cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the manufacturer's recommended protocol, followed by incubation with FcγR block for 15 min and thereafter an allophycocyanin-conjugated anti-Foxp3 antibody (Clone FLK-16; Nordic Biosite, Taby, Sweden) at 4°C for 30 min in the dark. Cells were analyzed by an LSR II flow cytometer (BD Pharmingen, San Jose, CA, USA). The

gating strategies are shown in Supplementary Fig. 1. Data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

**Statistics**

Unpaired Student's *t*-tests (two-tailed) were used to calculate statistical differences between experimental and control groups.

## Results

### Increased B cell and antibody responses in FcγRIIIA-deficient mice compared to WT mice after mucosal immunization with OVA and CT

To evaluate the importance, if any, of FcγRIIIA on the development of humoral and cellular immune responses after mucosal immunization, we immunized FcγRIIIA-deficient mice twice with OVA together with the strong mucosal adjuvant CT and compared the responses 10 days after the second dose with those seen in concomitantly immunized WT and FcγRIIB-deficient mice. In the WT mice this immunization regimen elicited a high-titered serum IgG anti-OVA antibody response which, as expected, was even further (15-fold) increased in similarly immunized FcγRIIB$^{-/-}$ mice. Against expectation, however, similarly increased levels of IgG antibodies were also found in the immunized FcγRIIIA$^{-/-}$ mice (Fig. 1A). Analyses of IgG1 and IgG2c subclasses demonstrated a similar pattern for IgG1 anti-OVA antibody levels, while IgG2c antibodies remained very low in both WT and FcγRIIIA$^{-/-}$ mice and were only marginally increased in the FcγRIIB$^{-/-}$ mice (Supplementary Fig. 2). While these results are consistent with previous literature demonstrating increased antibody responses in mice lacking the inhibitory receptor FcγRIIB [24], the similar increase in mice deficient in FcγRIIIA is novel and thus deemed to be worth further investigation.

To examine if the increased antibody response in FcγRIIIA$^{-/-}$ mice was associated with increased germinal center reactions, we examined splenic CD38$^{-}$ B cells for their expression of GL7, a marker for germinal center localization, after immunization. Both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice demonstrated higher levels of germinal center B cells after immunization than the WT mice (Fig. 1B). CD138$^{+}$ plasma B cells were

also significantly increased in especially FcγRIIB$^{-/-}$ but also FcγRIIIA$^{-/-}$ mice in comparison with WT mice after immunization (Fig. 1C). Frequencies of CD138$^{+}$ plasma B cells were also much increased in FcγRIIB$^{-/-}$ control mice immunized with OVA without CT compared with the WT mice (Fig. 1C). This is possibly associated with the role of FcγRIIB on induction of apoptosis of B cells [20, 25, 26], such that the lack of FcγRIIB might result in persistence of B cells under steady state conditions The levels of splenic T follicular helper (Tfh) cells, which contribute to class-switch recombination, somatic hypermutation, and memory B-cell development in the germinal center [27, 28], were similar in mice of all three genotypes (Fig. 1D).

**Effector T cell responses are enhanced in FcγRIIIA$^{-/-}$ mice after mucosal immunization**

The mucosal immunization with OVA plus CT induced OVA-specific spleen T cell proliferative responses at similar levels in FcγRIIIA$^{-/-}$ as in WT mice, but of lesser magnitudes than achieved in FcγRIIB$^{-/-}$ mice (Supplementary Fig. 3). In contrast, the frequencies of Foxp3$^{-}$CD25$^{+}$CD4$^{+}$ effector T cells were increased to significantly higher levels in both FcγRIIIA$^{-/-}$ and FcγRIIB$^{-/-}$ mice than in WT mice (Fig. 2A). As expected when CT is used as an adjuvant [29], immunization downregulated the production of Foxp3$^{+}$ Treg cells in WT and, to a lesser extent, in FcγRIIB$^{-/-}$ mice, whereas this effect on Treg cells was compromised in FcγRIIIA$^{-/-}$ mice (Fig. 2B). Similar to the Foxp3$^{+}$ Treg cell responses, TGF-β$^{+}$CD1d$^{+}$CD5$^{+}$ B cells which are also known to have an inhibitory regulatory function were reduced after immunization with OVA adjuvanted with CT in WT mice and to a lesser extent in FcγRIIB$^{-/-}$ mice,

while the frequency of these regulatory B cells was instead slightly increased in FcγRIIIA$^{-/-}$ mice after immunization (Fig. 2C).

**Activation of antigen presenting cells to mucosal immunization is elevated in FcγRIIIA$^{-/-}$ mice**

To test if the elevated antibody and effector T cell responses in FcγRIIIA$^{-/-}$ mice after mucosal immunization with OVA plus CT were associated with increased expression of co-stimulatory molecules on antigen presenting cells (APC), we examined the expression of CD86 on DCs and B cells. The results revealed an increased expression of CD86$^{+}$ splenic B cells in FcγRIIIA$^{-/-}$ mice compared with WT mice, which in turn had a slightly higher response than FcγRIIB$^{-/-}$ mice after mucosal immunization with OVA and CT (Fig. 2D). When CD86$^{+}$CD38$^{+}$ memory B cells were examined separately, they increased to higher levels in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice than in WT mice (Fig. 2E), whereas the increase in CD86$^{+}$ cells in DCs was similar in all the three strains of mice (Fig. 2F).

The modulation of the expression of one FcγR may affect that of other FcγRs [30]. To exclude the possibility that the increased responses to immunization in the FcγRIIIA$^{-/-}$ mice were due to alteration of FcγRIIB expression on their APCs, we measured the expression of FcγRIIB on DCs and B cells from mice of different genotypes by flow cytometry. As expected no FcγRIIB could be detected on either B cells or DCs in FcγRIIB-deficient mice. FcγRIIIA$^{-/-}$ mice had similar amounts of FcγRIIB on DCs compared with WT mice (mean fluorescence intensity: 447±120 and 440±61, respectively) and a slightly increased expression of FcγRIIB on their B cells than in the WT mice (mean fluorescence intensity: 629±53 and 442±75, respectively). Thus,

we conclude that the enhanced immune responses in FcγRIIIA$^{-/-}$ mice were not associated with decreased expression of FcγRIIB.

**Antibody and cellular responses are also increased in FcγIIIA$^{-/-}$ mice after parenteral immunization**

Despite the fact that the FcγRIIIA$^{-/-}$ mice were protected in an experimental autoimmune encephalomyelitis model [12], consistent with a proinflammatory effect of FcγRIIIA, these authors also observed augmented serum antibody responses in these mice against antigen (myelin oligodendrocyte glycoprotein) they used together with incomplete Freund's adjuvant to s.c. immunize the mice. We also analyzed the responses of FcγRIIIA$^{-/-}$ mice in comparison with WT and FcγRIIB$^{-/-}$ mice after s.c. immunization with OVA in CFA. Both FcγRIIB- and FcγRIIIA-deficient mice responded with markedly increased levels of serum anti-OVA IgG in comparison with WT mice (Fig. 3A). Similar to the findings after s.l. immunization, these antibodies were predominantly of the IgG1 subclass and IgG2c anti-OVA antibodies were in fact reduced rather than increased in the FcγRIIIA$^{-/-}$ mice compared to the WT mice (Fig. 3B), both in absolute terms and even more pronounced when comparing their levels relative to the IgG1 antibody titers. Thus, while the IgG2c titers were 20-fold lower than the IgG1 titers in the WT mice, they were 60-fold lower in the FcγRIIB$^{-/-}$ mice and as much as 500-fold less in the FcγRIIIA$^{-/-}$ mice (Fig. 3B). In the s.c. immunized FcγRIIIA$^{-/-}$ mice, similar to the findings after mucosal immunization, the overall enhanced humoral immune response was accompanied with increased expansion of Foxp3$^{-}$CD25$^{+}$CD4$^{+}$ effector T cells in draining lymph nodes (Fig. 3C). These

observations support a suppressive role of FcγRIIIA also in mice immunized by s.c. injection with OVA emulsified in CFA.

**FcγRIIIA expression on DCs contributes to Ag-specific T cell responses**

The mechanisms that may account for the enhanced immune responses in FcγRIIIA$^{-/-}$ mice described here remain to be determined. As neither B cells nor T cells express FcγRIIIA, the immune enhancing effects leading to augmented effector responses involving these cells (e.g. antigen-specific antibody production, B and T cell activation) in the FcγRIIIA$^{-/-}$ mice are most likely secondary to modulation of FcγRIIIA-expressing immune cells such as APCs. In order to evaluate if the loss of FcγRIIIA on DCs affects their *in vitro* induction of Ag-specific T cell responses, we pulsed BMDCs generated from WT, FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice with either OVA or OVA/anti-OVA IgG immune complexes followed (after extensive washing of the DCs) by co-culture of the treated DCs with freshly isolated CD4$^{+}$ T cells from OT-II mice. We observed an enhanced capacity of DCs from both FcγRIIB- and FcγRIIIA-deficient mice in comparison to DCs from WT mice to stimulate OVA-specific T cell activation with regard to both proliferation (Fig. 4A) and effector T cell expansion (Fig. 4B), which was most pronounced when DCs had been pulsed with the OVA/anti-OVA IgG immune complexes (Fig. 4A, 4B).

## Discussion

The strong enhancement of both antibody and cellular immune responses in FcγRIIIA$^{-/-}$ mice demonstrated in this study after both mucosal and parenteral immunization supports the paradigm that FcγRIIIA is not obligatorily an activating FcγR but may also or alternatively signal through ITAMi to suppress immune responses [17, 18]. In our immunization models using two strong adjuvants, CT for mucosal immunization and CFA for systemic immunization, the augmented antigen-specific immune responses in FcγRIIIA$^{-/-}$ mice may reflect a predominating abrogation of ITAMi signalling. In preliminary experiments, we have found that WT DCs treated *in vitro* with OVA plus CT reduced the inhibitory SHP-1 formation by 60 – 80% compared with DCs treated with OVA only, and instead increased the activating signalling SYK by 40 – 60% (unpublished data).

Consistent with the role of IgG1 in partial phosphorylation of ITAM tyrosine residues following the recruitment of SHP-1 that favours the ITAMi configuration [18], it has been reported in both human and mouse models that intravenously administered IgG (IVIg), with IgG1 as its most abundant subclass, may generate inhibitory signals to suppress inflammatory responses through targeting FcγRIIIA [19]. Mouse IgG1 is much less pathogenic compared with IgG2b in autoimmune hemolytic anemia mediated through FcγRIIIA [13]. FcγRIIIA$^{-/-}$ mice are resistant to *Leishmania mexicana* infection in which the initial antibody response to the parasite is predominantly IgG1 [31]. IgG1-mediated inhibition of osteoclastogenesis by FcγRIII has also been observed [32]. We have previously demonstrated that IgG1-containing immune complexes fail to activate mast cells in contrast to IgG2a and IgG2b complexes [33]. In this study, antibodies of the IgG1 subclass were

significantly increased in FcγRIIIA$^{-/-}$ mice compared to WT mice following either s.l. immunization with OVA plus CT or s.c. immunization with OVA in CFA. Interestingly and in sharp contrast to the findings for IgG1 antibodies, OVA-specific antibodies of the Th1-associated subclass IgG2c were significantly lower in FcγRIIIA$^{-/-}$ mice than in WT and FcγRIIB$^{-/-}$ mice following CFA-facilitated s.c. immunization. In contrast to WT mice that promote the adjuvant-stimulated induction of both Th1 and Th2 responses, FcγRIIIA$^{-/-}$ mice instead selectively induced a Th2-associated IgG1 antibody response with actual suppression of IgG2c antibody levels compared to the WT mice. Conceivably, as a hypothesis for further studies, FcγRIIIA may play a role as a functional Th1/Th2 switch that could lead to overall stimulation of Th1-dependent immune responses.

We also noted in confirmation of our previous work [22] that the s.l. treatment with mucosal adjuvant CT could strongly inhibit the development of Foxp3$^{+}$ regulatory T cells and TGF-β$^{+}$ regulatory B cells, but had no such effect in either FcγRIIB$^{-/-}$ mice or FcγRIIIA$^{-/-}$ mice. If we surmise an inhibitory role of these two regulatory T and B cell populations in adaptive immune responses, our findings regarding these cells in FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice after immunization with CT are not readily reconciled with the enhanced antibody responses. The explanation might be that the effect of these regulatory cells cannot compensate for the overall and stronger enhancement exerted by other critical components of the immune responses following the deletion of FcγRIIB or FcγRIIIA.

It is also notable that even though FcγRIIIA has usually been found to promote arthritis development [5-7], FcγRIIIA$^{-/-}$ mice were not protected against arthritis

induced by immunization with methylated bovine serum albumin adjuvanted with strong adjuvants including CFA and heat-killed *Bordetella pertussis*. In this model, increased antigen-specific antibody responses and joint inflammation were seen in mice lacking FcγRIIIA, suggesting a possible immune modulatory-suppressive role of this receptor [34]. Our current findings of markedly enhanced immune responses in FcγRIIIA$^{-/-}$ mice after both mucosal and parenteral immunization are consistent with this observation. However, in previous work we observed reduced immune responses to intranasal immunization adjuvanted with IgG-complexed cholera toxin A1 subunit (CTA1) coupled to two Ig-binding domains (DD) derived from *Staphylococcus aureus* protein A (CTA1-DD) in FcγRIIIA$^{-/-}$ mice [35]. We believe that the immune modulatory roles of FcγRIIIA are complex and that FcγRIIIA may differentially impact the immune responses depending on the experimental system; the outcome of the immune effector function appears to depend on the delicate balance between the inhibitory and activating signals mediated through the engagement of this receptor.

**Acknowledgments**

The study was supported by grants from The Swedish Research Council, The EU Aditec Program and the Swedish Strategic Research Foundation.

We thank Annelie Ekman, Madeleine Löfstrand, Frida Jeverstam and Margareta Blomquist for their skillful technical assistance.

**Disclosure**

No competing financial interests exist.

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# Legends

**Figure 1** Mucosal immunization with OVA plus CT enhances serum levels of anti-OVA IgG, germinal center B cell expansion and follicular helper T cell (Tfh) expansion in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice as compared to WT mice. WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were sublingually (s.l.) immunized with 200 μg OVA with or without 5 μg CT on two occasions at an interval of 10 days. Sera and spleens were collected 10 days after the last s.l. treatment. (A) Anti-OVA IgG antibodies in serum were measured by ELISA. (B-D) Spleens were examined for the frequencies of cells associated with germinal center formation including GL7$^{+}$ B cells (B), CD138$^{+}$ plasma cells (C) and CXCR5$^{+}$PD1$^{+}$ T follicular helper cells (D) by flow cytometry. Dead cells were excluded by Live/Dead Aqua. The gating strategies are shown in Supplementary Figure 1. Each symbol represents data from one mouse and the bars indicate the means of each group. The data are representative of three separate experiments with similar results.

**Figure 2** Mucosal immunization with OVA plus CT expands effector T and B cells in spleen in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice to a larger extent than in WT mice. WT, FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice were sublingually (s.l.) treated with 200 μg OVA mixed with or without 5 μg CT on two occasions at an interval of 10 days. Spleens were collected 3 days after the last s.l. treatment. Splenic cell phenotypes including CD25$^{+}$CD4$^{+}$ effector T cells (A), Foxp3$^{+}$ cells (B), LAP/TGF-β$^{+}$CD1d$^{+}$CD5$^{+}$ B cells (C), CD86$^{+}$B220$^{+}$ B cells (D), CD86$^{+}$CD38$^{+}$B220$^{+}$ memory B cells (E) and CD86$^{+}$MHC-II$^{+}$CD11c$^{+}$ DCs (F) were examined by flow cytometry. Each symbol represents data from one mouse and the bars show the mean values of each group. Dead cells were excluded by Live/Dead Aqua. CD4$^{+}$Foxp3$^{-}$CD11c$^{-}$B220$^{-}$ cells were

gated for analyzing $CD25^+CD4^+$ Teff cells. $CD4^+CD62L^+$ cells were gated for analyzing $Foxp3^+$ Treg cells; $CD19^+CD1d^+CD4^-CD11c^-$ cells were gated for analyzing TGF-$\beta^+CD1d^+CD5^+$ cells. $CD4^-B220^+$MHC-$II^+CD38^+$ cells were gated for analyzing CD86 expression on B cells, and $CD4^-CD11c^+$MHC-$II^+CD69^+$ cells were gated for analyzing $CD86^+$ DCs.

**Figure 3** Subcutaneous immunization with OVA in complete Freund's adjuvant (CFA) induces anti-OVA IgG responses in serum and popliteal lymph node (PLN) T effector cell expansion in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice to a larger extent than in WT mice. WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were subcutaneously immunized with 100 μg OVA emulsified in 100 μl CFA (Imm) or left unimmunized (Nil controls). Serum and PLN cells were collected two weeks after the immunization. Anti-OVA IgG (A), and anti-OVA IgG1 (solid symbols) and IgG2c (empty symbols) (B) antibodies in serum were measured by ELISA. (C) PLN cells were examined for the frequencies of $CD25^+CD4^+$ effector T cells among $Foxp3^-$ cells by flow cytometry. Each symbol represents data from one mouse and the bars show the means of each group. Data are from one of two independent experiments showing similar results.

**Figure 4** Bone marrow derived, antigen-pulsed DCs generated from FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice demonstrate enhanced capacity to activate antigen-specific T cells. Bone marrow dendritic cells (BMDCs) were prepared by incubating bone marrow cells isolated from naive WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice in the presence of 200 ng/ml Flit3-L for 9 days. The BMDCs were then in vitro pulsed with medium alone or

10 μg/ml OVA or IgG-complexed OVA for 2 h. After thorough washing, 2 x $10^4$ BMDCs were then co-cultured with 1x$10^5$ freshly isolated OT-II x Ly5.1 $CD4^+$ T cells for 3 days. T cell proliferation was measured by [$^3$H]-thymidine incorporation (A), and cells were also tested for the proportions of $Foxp3^-CD25^+CD4^+$ effector T cells by flow cytometry (B). Data are from one of three experiments showing similar results and are expressed as mean + SEM for three mice per group.

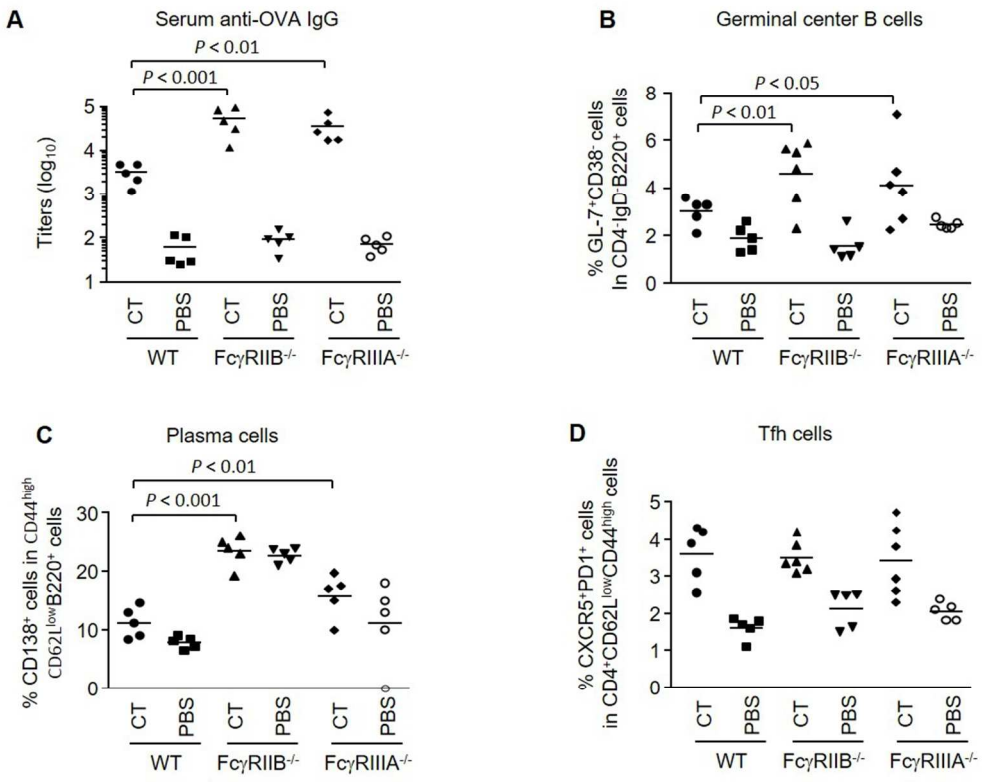


Figure 1 Mucosal immunization with OVA plus CT enhances serum levels of anti-OVA IgG, germinal center B cell expansion and follicular helper T cell (Tfh) expansion in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice as compared to WT mice. WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were sublingually (s.l.) immunized with 200 μg OVA with or without 5 μg CT on two occasions at an interval of 10 days. Sera and spleens were collected 10 days after the last s.l. treatment. (A) Anti-OVA IgG antibodies in serum were measured by ELISA. (B-D) Spleens were examined for the frequencies of cells associated with germinal center formation including GL7$^{+}$ B cells (B), CD138$^{+}$ plasma cells (C) and CXCR5$^{+}$PD1$^{+}$ T follicular helper cells (D) by flow cytometry. Dead cells were excluded by Live/Dead Aqua. The gating strategies are shown in Supplementary Figure 1. Each symbol represents data from one mouse and the bars indicate the means of each group. The data are representative of three separate experiments with similar results.

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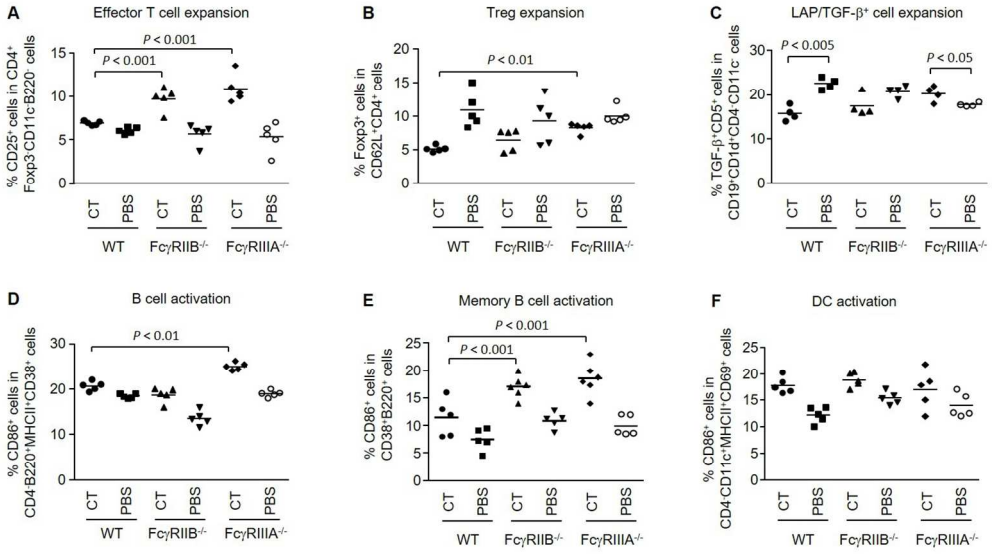


Figure 2 Mucosal immunization with OVA plus CT expands effector T and B cells in spleen in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice to a larger extent than in WT mice. WT, FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice were sublingually (s.l.) treated with 200 μg OVA mixed with or without 5 μg CT on two occasions at an interval of 10 days. Spleens were collected 3 days after the last s.l. treatment. Splenic cell phenotypes including CD25$^{+}$CD4$^{+}$ effector T cells (A), Foxp3$^{+}$ cells (B), LAP/TGF-β$^{+}$CD1d$^{+}$CD5$^{+}$ B cells (C), CD86$^{+}$B220$^{+}$ B cells (D), CD86$^{+}$CD38$^{+}$B220$^{+}$ memory B cells (E) and CD86$^{+}$MHC-II$^{+}$CD11c$^{+}$ DCs (F) were examined by flow cytometry. Each symbol represents data from one mouse and the bars show the mean values of each group. Dead cells were excluded by Live/Dead Aqua. CD4$^{+}$Foxp3$^{-}$CD11c$^{-}$B220$^{-}$ cells were gated for analyzing CD25$^{+}$CD4$^{+}$ Teff cells. CD4$^{+}$CD62L$^{+}$ cells were gated for analyzing Foxp3$^{+}$ Treg cells; CD19$^{+}$CD1d$^{+}$CD4$^{-}$ CD11c$^{-}$ cells were gated for analyzing TGF-β$^{+}$CD1d$^{+}$CD5$^{+}$ cells. CD4$^{-}$B220$^{+}$MHC-II$^{+}$CD38$^{+}$ cells were gated for analyzing CD86 expression on B cells, and CD4$^{-}$CD11c$^{+}$MHC-II$^{+}$CD69$^{+}$ cells were gated for analyzing CD86$^{+}$ DCs.

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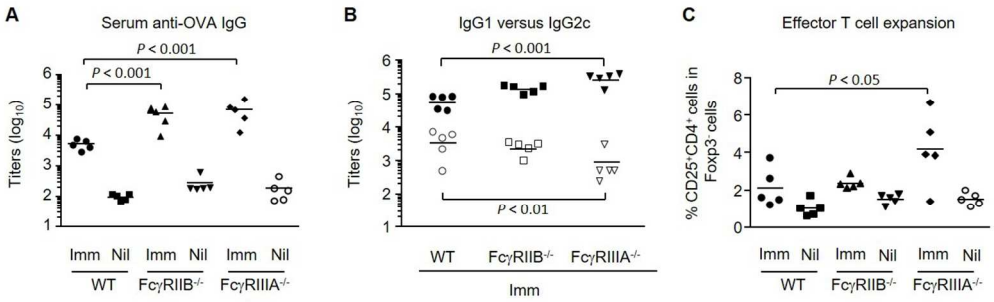


Figure 3 Subcutaneous immunization with OVA in complete Freund's adjuvant (CFA) induces anti-OVA IgG responses in serum and popliteal lymph node (PLN) T effector cell expansion in both FcγRIIB$^{-/-}$ and FcγRIIIA$^{-/-}$ mice to a larger extent than in WT mice. WT, FcγRIIB$^{-/-}$ or FcγRIIIA$^{-/-}$ mice were subcutaneously immunized with 100 μg OVA emulsified in 100 μl CFA (Imm) or left unimmunized (Nil controls). Serum and PLN cells were collected two weeks after the immunization. Anti-OVA IgG (A), and anti-OVA IgG1 (solid symbols) and IgG2c (empty symbols) (B) antibodies in serum were measured by ELISA. (C) PLN cells were examined for the frequencies of CD25$^{+}$CD4$^{+}$ effector T cells among Foxp3$^{-}$ cells by flow cytometry. Each symbol represents data from one mouse and the bars show the means of each group. Data are from one of two independent experiments showing similar results.

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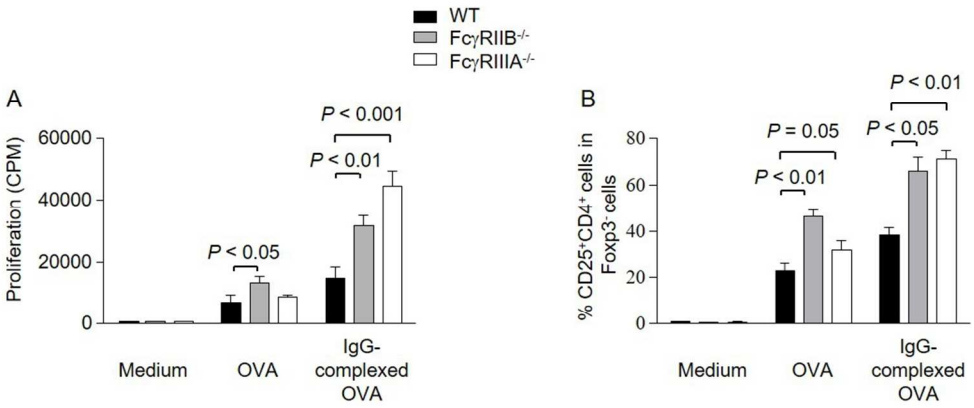


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