Increased IL-12 inhibits B cells’ differentiation to germinal center cells and promotes differentiation to short-lived plasmablasts

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B cells activated by antigen in T cell–dependent immune responses can become short-lived plasma cells, which remain in the spleen, or germinal center–derived memory or plasma cells, which show evidence of affinity maturation and, in the case of plasma cells, migrate to the bone marrow. We show that this cell fate decision can be governed by the cytokine environment engendered by activated dendritic cells (DCs). DCs from mice lacking the Fc receptor γ chain exhibited an activated phenotype in vitro. They secreted more of the proinflammatory cytokine IL-12, which led to the preferential generation of short-lived splenic plasma cells, with ensuing low affinity antibodies and a diminished recall response. Understanding the factors that regulate antigen–activated B cell differentiation and memory cell formation has implications for both antibody-mediated autoimmune disease and protective antibody responses.
NZW mice uncouples immune complex formation from the inflammatory response in the kidney, implying a pathogenic role of FcRγ chain in autoimmune disease (4).

There are several factors involved in determining the phenotype of antigen-experienced B cells, including BCR signal strength (5), expression of transcription factors (6), and costimulatory influences (7–9). In the course of studying renal pathology in peptide-immunized mice deficient in the FcRγ chain (Fcγ−/−), we made the surprising observation that Fcγ−/− mice preferentially develop short-lived plasma cells and fail to develop a germinal center response. We show that this altered B cell response is a consequence of increased IL-12 production by DCs.

RESULTS

***Fcγ−/− mice display a distinct humoral response to antigen***

Immunization of BALB/c mice with a peptide mimotope of dsDNA, DWEEYSVLWSLN, octamerized on a polylysine backbone (multiple antigenic peptide [MAP] peptide) induces antibodies that cross-react with peptide and dsDNA (1). We immunized Fcγ−/− and WT mice i.p. with MAP peptide in CFA and boosted with antigen in IFA on days 7 and 14. By day 28, Fcγ−/− mice generated significantly higher serum titers of both antipeptide (approximately fivefold) and anti-dsDNA antibody (approximately sevenfold; Fig. 1 a). The difference in serum antibody reactivity did not reflect a difference in the kinetics of the response, as both strains exhibited a similar timing of antibody production (Fig. 1 a). An antigen-specific IgM response was induced in WT and Fcγ−/− mice. Consistent with the IgG response, there was a higher IgM response in Fcγ−/− mice at week 2, but this declined to the basal level by week 4 in both strains (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1).

One explanation for the greater IgG serum reactivity in Fcγ−/− mice would be an increased affinity of antipeptide antibodies. We, therefore, performed inhibition ELISAs with soluble peptide and calculated apparent affinities by determining the molar concentration of soluble antigen that led to 50% inhibition of the antibody reactivity (10). Contrary to our initial expectation, antipeptide antibodies from Fcγ−/− mice had a log lower apparent affinity than antipeptide antibodies from WT mice (1.4 × 105 vs. 2.2 × 106; P ≤ 0.01), demonstrating that the increased titers resulted from the production of more antigen-specific antibody.

Furthermore, although IgG1 was the predominant isotype observed in both the antipeptide and anti-dsDNA response in WT mice, Fcγ−/− mice produced both IgG1 and IgG2a antipeptide (Fig. 1 b) and anti-dsDNA antibodies (not depicted). This did not reflect an intrinsic bias toward increased IgG2a, as the isotype distribution for total serum IgG was similar in both mouse strains (not depicted).

Because the difference in antibody titer between strains was ~5–10-fold, we believed it was important to ask whether this increase in serum antibody titer might be of pathological significance. Kidneys from MAP peptide–immunized Fcγ−/− mice, analyzed 13 wk after the initial antigen challenge, had significantly more glomerular IgG deposition than WT kidneys, and the mice displayed increased proteinuria (Fig. 1 c). Thus, the antibodies from Fcγ−/− mice, although of lower affinity than the antibodies in WT mice, were still of sufficient affinity to cause glomerular dysfunction. This observation is

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**Figure 1. Fcγ−/− mice display an increased production of antigen-specific antibodies in their serum and increased renal antibody deposition.**

(a) Fcγ−/− (gray triangles) and WT (black squares) mice (n = 5 in each group) were immunized with 100 μg MAP peptide and boosted twice (arrows indicate time of boosts). Serum was collected on days 0, 14, 28, 42, and 56, and antipeptide and anti-dsDNA IgG ELISAs were performed. P ≤ 0.05 in both graphs. (b) Mice were immunized as described in a. 7-wk serum was assayed for isotype-specific antipeptide antibody ELISAs. Titration were twofold and began at the dilution indicated on the x-axis. Gray triangles show Fcγ−/− mice and black squares show WT mice. IgG1, P = 0.02; IgG2a, P = 0.048. n = 5. (c) Kidneys were taken at day 91, and sections were stained for IgG deposition. Sections shown above are representative of five mice in each group. Sections were magnified 100x. Bars, 50 μm. 50 glomeruli were counted in each kidney section, and the percent positive for IgG deposition was graphed. *P ≤ 0.05. Proteinuria was determined by measuring the amount of protein in the urine of mice at day 91 after immunization. (d) Both WT and Fcγ−/− mice were immunized with 100 μg MAP peptide in CFA on day 0 and boosted on day 7 with MAP peptide in IFA. On day 56, mice were challenged with 100 μg MAP peptide in IFA, and serum was collected 5 d after challenge. Antipeptide ELISAs were performed. P ≤ 0.05. (e) WT and Fcγ−/− mice (five in each group) were immunized with 100 μg 10–2–BSA and boosted twice. Day-0, -14, -28, and -42 serum was assayed by ELISA for anti-10–2 IgG. P ≤ 0.05. Data are presented as mean ± SD.
consistent with clinical experience where a four- to eightfold increase in autoantibody titer may correlate with the onset of disease or with increased disease activity. For example, an antinuclear antibody titer of 1:40 is normal, whereas an eightfold increase to 1:320 is highly significant for disease. Likewise, a four to eightfold increase in anti-DNA antibody titer in a patient with SLE will often precede a clinical flare (11).

High affinity antibody responses are generated by a germinal center reaction in which B cells undergo somatic hypermutation and there is selection for survival of B cells making the highest affinity antibodies. These cells mature to become long-lived plasma cells and memory cells. To determine if the lack of a high affinity antibody response reflected a failure of B cells to undergo germinal center maturation, we questioned whether Fcγ−/− mice developed a memory response. WT and Fcγ−/− mice were immunized and boosted with MAP peptide. 8 wk later, mice were challenged with 100 μg MAP peptide. 5 d later, blood was collected to measure the antipeptide response in serum. Fig. 1 d shows that WT mice have a rapid increase in the serum antipeptide response, which was not observed in Fcγ−/− mice. These data suggest a significant reduction in the population of memory B cells in Fcγ−/− mice.

To determine whether Fcγ−/− mice immunized with a nonself antigen would also develop an enhanced serum response, we examined the response of Fcγ−/− mice to 10−2, a peptide mimotope of phosphorylcholine, conjugated to BSA (12). As seen in Fig. 1 e, Fcγ−/− mice made significantly more anti-10−2 antibodies (an approximately threefold increase in titer) than WT mice. In addition, although WT mice made predominantly IgG1 anti-10−2 antibodies, Fcγ−/− mice also produced antigen-specific IgG2a antibodies (unpublished data). Thus, the strain-specific difference in antibody response was not limited to an autoreactive response.

Differential humoral response in Fcγ−/− mice is not determined by B cells

The increased antipeptide and anti-DNA response in Fcγ−/− mice might reflect either a B cell–intrinsic phenotype or, alternatively, a different response to immunization in non-B cells. To test whether the difference was B cell intrinsic, splenic B cells from WT or Fcγ−/− mice were transferred to B cell–deficient BALB/c mice (muMT). Both WT B cell–reconstituted and Fcγ−/− B cell–reconstituted muMT mice were immunized with MAP peptide. As shown in Fig. 2, there was no statistically significant difference in serum antipeptide response between WT and Fcγ−/− B cell–reconstituted mice. Moreover, analysis of splenic B cell subsets (mature vs. immature and transitional vs. follicular vs. marginal zone) confirmed that there was no difference in B cell differentiation between Fcγ−/− and WT BALB/c B cells. The expression of several cell surface markers (CD22, CD40, CD43, CD79b, CD27, MHC II, Fas, CD80, and CD86) also was not different between Fcγ−/− and WT BALB/c B cells. B cell function, assayed by IgM-mediated apoptosis and B cell proliferation to anti-IgM plus anti–CD40 antibody or anti–CD40 antibody plus IL-4, was also similar between both strains (unpublished data). Thus, the phenotype and function of naive B cells of both strains were indistinguishable.

Fcγ−/− B cells preferentially differentiate into short-lived plasma cells

Because the antibody response in Fcγ−/− mice was of low affinity and the memory response was impaired, we hypothesized that there was a defect in germinal center maturation, which generates both memory B cells and long-lived plasma cells, and a preferential generation of short-lived plasma cells (13–16). Short-lived plasma cells develop in T cell–dependent responses but do not display affinity maturation and are not associated with a clonally related memory B cell population (17, 18). After activation with antigen, short-lived plasma cells reside primarily in the extrafollicular foci of the spleen, whereas long-lived plasma cells preferentially migrate to the BM (16–19). First, we determined the number of antibody-secreting cells (including plasmablasts, short-lived plasma cells, and long-lived plasma cells) in the spleen. There was a higher percentage of B220+ and CD138+ cells in the B cell population in MAP peptide–immunized Fcγ−/− mice compared with WT mice on day 8 (Fig. 3 a). To determine the location of B220+ and CD138+ cells within the spleen, immunohistochemistry was performed on spleens of immunized mice. The majority of CD138+ cells were positioned outside the B cell follicles where short-lived plasma cells reside (Fig. 3 b). CD138+ cells colocalized with kappa chain positivity but not with CD4 positivity (unpublished data). To confirm this observation, we also performed peptide-specific ELISPOT assays of splenic B cells. Fig. 3 c shows that 2.5-fold more cells secreting specific antibody were present in spleens of Fcγ−/− mice than in WT spleens. By day 28, splenic plasma cells from both Fcγ−/− and WT mice declined in number, presumably because of apoptosis of short-lived plasma cells.
Long-lived plasma cells migrate to the BM, where they secrete antibody. To determine whether long-lived plasma cells were being generated in Fcγ<sup>-/-</sup> mice, we enumerated plasma cells by peptide-specific ELISPOT assays in the BM. Plasma cells were detectable in the BM between days 21 and 35 after the initial immunization. Fcγ<sup>-/-</sup> mice had 5.3-fold fewer peptide-specific plasma cells in the BM on day 35 (Fig. 3 d).

These results were consistent with the preferential generation of short-lived plasma cells in Fcγ<sup>-/-</sup> mice in contrast to the predominance of long-lived plasma cells present in the BM of WT mice.

Because the antipeptide antibody response was previously shown to be a T cell–dependent response in WT mice (2), we questioned whether the antibody response in Fcγ<sup>-/-</sup> mice was also T cell dependent, as it displayed less evidence of germinal center formation. Fig. 4 a shows that antibody generation is abolished in CD4<sup>+</sup> T cell–depleted mice. Thus, the diminished germinal center response is not caused by an absent T cell response.

Antigen-specific T cells are activated by MAP peptide immunization in Fcγ<sup>-/-</sup> mice. To demonstrate T cell activation in Fcγ<sup>-/-</sup> mice, we assessed antigen-specific proliferation of T cells from MAP peptide–immunized mice. T cells from MAP peptide–immunized Fcγ<sup>-/-</sup> and WT mice proliferated in response to antigen (Fig. 4 b). In fact, the response of T cells from Fcγ<sup>-/-</sup> T cells was greater than that of WT T cells. To ascertain whether this enhanced T cell response reflected a change in T cell repertoire, we assessed Vβ usage. Fig. 4 c shows that there was no significant difference in TCR Vβ chain usage between WT and Fcγ<sup>-/-</sup> mice, either before or after MAP peptide immunization. Furthermore, antigen-specific T cells from both strains produced IL-2 and IFN-γ (unpublished data). These data demonstrate that T cells in Fcγ<sup>-/-</sup> mice were activated to at least the same extent as T cells from WT mice after MAP peptide immunization. Thus, the altered antibody response is not a consequence of a failure of T cell priming.

Fcγ<sup>-/-</sup> mice display a diminished germinal center response

To demonstrate directly that Fcγ<sup>-/-</sup> mice display reduced germinal center maturation of B cells, we asked whether the reduced affinity of serum antibodies in Fcγ<sup>-/-</sup> mice reflected a relative lack of somatic hypermutation occurring in the Fcγ<sup>-/-</sup> mice, as somatic hypermutation is a feature of the germinal center response and a critical contribution to affinity maturation. To address this question, we generated antigen-specific hybridomas from WT and Fcγ<sup>-/-</sup> mice immunized with MAP peptide. We examined the number of mutations in the region downstream of the rearranged J<sub>H</sub> in B cells making antipeptide antibody. We chose to enumerate mutations in this region because it has been shown to undergo hypermutation. Because it is not a coding region, it is not subject to the pressures of selection. As seen in Fig. 5 a, hybridomas from Fcγ<sup>-/-</sup> mice had many fewer mutations. Splenic histology displayed fewer germinal centers in Fcγ<sup>-/-</sup> mice after immunization (Fig. 5 b). This was confirmed by quantification of B220<sup>+</sup> PNA<sup>+</sup> cells in the spleen (Table S1, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1). There were very few PNA<sup>+</sup> cells in the B220<sup>+</sup> population in Fcγ<sup>-/-</sup> mice on days 8 and 16 after immunization compared with WT mice (Fig. 5 c). We also measured activation-induced cytidine deaminase (AID) induction in germinal center B lymphocytes because expression
of AID is suppressed in mature B cells and only expressed in germinal center B cells (20). WT mice display a significant induction of AID in germinal center B cells. Fcγ−/− mice, however, did not show any significant induction of AID, which is consistent with a diminished germinal center reaction in these mice (Fig. 5d). These studies all support a reduced germinal center response in Fcγ−/− mice.

Fcγ−/− DCs have increased IL-12 production and an increased ability to cause B cell proliferation in an IL-12-dependent manner

Vogel et al. (21) showed that IL-12 can bind directly to B cells and induce plasma cell formation, and Dubois et al. (22, 23)
have shown that DCs can induce naive B cells to proliferate in an IL-12-dependent manner. We therefore examined whether BM-derived DCs (BMDCs) from Fcγ−/− mice were more potent producers of IL-12 and stimulators of B cell proliferation. IL-12 was measured in immature and LPS-matured BMDCs by intracellular flow cytometry and cytokine ELISA. Flow cytometry, which measured IL-12 p40, demonstrated a twofold increase in the number of DCs producing IL-12 in Fcγ−/− mice (Fig. 6 a). A cytokine ELISA for IL-12 p40/70 detected IL-12 production only by LPS-stimulated BMDCs and confirmed a two- to threefold increase in production by Fcγ−/− DCs (Fig. 6 b). Real-time PCR also demonstrated increased induction of IL-12p35 and p40 in LPS-stimulated BMDC from Fcγ−/− mice (Fig. 6 c). Fcγ−/− BMDCs also displayed significantly higher expression of MHC II, CD80, and CD86 than WT BMDCs (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1). Finally, we measured IL-12 expression in splenic DCs after MAP peptide immunization in vivo. Both WT and Fcγ−/− mice were immunized. On days 1 and 3, total splenic DCs were purified and further cultured for 16 h to permit cytokine secretion. Fig. 6 d demonstrates that splenic DCs from Fcγ−/− mice expressed about twofold more IL-12 than DCs from WT mice by day 3.

Because there are other cell types that can produce IL-12 upon activation, we measured production of IL-12 by macrophages and B cells. As seen in previous studies, LPS stimulation strongly induced production of IL-12 from peritoneal macrophages, but there was no difference between WT and Fcγ−/− macrophages. B cells from WT and Fcγ−/− mice also produced equal amounts of IL-12 p40 after upon LPS and LPS plus anti-CD40 antibody stimulation. (Fig. 6 e).

To confirm that the increased IL-12 could lead to an expanded B cell population, naive WT B cells were cultured with anti-CD40 antibody and irradiated BMDCs from either Fcγ−/− or WT mice. After 5 d in culture, B cells proliferated in the presence of Fcγ−/− BMDCs, whereas little or no BMDC-dependent proliferation was seen in the presence of WT BMDCs (Fig. 7 a). Furthermore, when a neutralizing anti-IL-12 antibody was added at the beginning of the culture, B cell proliferation driven by Fcγ−/− BMDCs decreased by as much as 80% (Fig. 7 b). Because anti-IL-12 antibody recognizes both the p35 and the p40 subunits of IL-12 and p40 is also a subunit of IL-23, there is a possibility of inhibition of both cytokines by the anti-IL-12 antibody. To address this problem, IL-12a (p35)-specific siRNA was used. Transfection efficiency was determined and specificity of IL-12a siRNA was confirmed using control siRNA and analyzing gene expression of IL-12b (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1). Consistent with the results using anti-IL-12 antibody, IL-12a siRNA transfection of BMDCs abrogated the proliferation of B cells (Fig. 7 c). The inhibition was specific, as transfection with control siRNA showed no significant reduction in B cell proliferation. Therefore, Fcγ−/− DCs are potent stimulators of B cell proliferation, and their stimulatory function depends largely on the production of IL-12.
IL-12 secretion influences the differentiation of plasma cells in vivo

To confirm that the increased IL-12 production by DCs contributed to the altered antibody response observed in vivo in Fcγ−/− mice, we determined if WT mice given supplemental IL-12 would produce more plasma cells in spleen. WT mice were injected i.p. with 1 μg of either soluble IL-12 or saline on days −1, 0, and +1 of the immunization schedule. Antigen boosts were given with no additional IL-12. 11 d after the last boost, WT mice treated with IL-12 had approximately threefold more splenic antibody-secreting cells than untreated mice, which is similar to the difference observed in Fcγ−/− mice (Fig. 8 a). IL-12–treated WT mice also experienced an ~35% decrease in BM plasma cells, which is also similar to Fcγ−/− mice (Fig. 8 b). Thus, exogenous IL-12 caused WT mice to acquire a phenotype of antigen-specific B cells similar to that of Fcγ−/− mice. We confirmed this observation in a study of DCs, as it is altered when IL-12 production is diminished.

DISCUSSION

Understanding mechanisms underlying the commitment to a particular B cell fate has clinical importance. Memory cells can proliferate extensively upon secondary challenge, and long-lived plasma cells can secrete antibody for long periods of time without antigen contact. Therefore, the prevalence of autoreactivity in the memory population may result in a chronic antibody-mediated disease. In contrast, short-lived plasma cells may generate large amounts of a pathogenic...
antibody that, as shown in this study, does not necessarily need to be of the highest affinity to cause organ damage.

Through the analysis of Fcγ−/− mice, we have demonstrated that DCs participate in determining B cell differentiation in vivo. Fcγ−/− B cells became predominantly splenic plasma cells upon activation instead of forming the more heterogeneous population of short-lived and long-lived plasma cells and memory cells seen in WT mice. Current data suggest that the majority of splenic plasma cells are short lived, whereas plasma cells in the BM can be either short lived or long lived (15, 18, 19, 24). In our study, Fcγ−/− mice had a transient surge of plasma cells in the spleen, while displaying a diminished rise in plasma cells in the BM and a reduced memory B cell response. In contrast, WT mice had a smaller population of splenic plasma cells and an increase in BM plasma cells after immunization and demonstrated B cell memory upon secondary challenge with antigen. Fcγ−/− mice produced significantly higher serum titers of specific antibody than WT mice and displayed class switching to IgG2a as well as IgG1. The increase in serum antipeptide and anti-DNA response appears to be an Fcγ-dependent phenomenon because FcγR1−/− immunized with MAP peptide showed a similar phenotype (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1). These features of the response may account for the increased glomerular dysfunction. Interestingly, antibodies from Fcγ−/− mice displayed less affinity maturation. This serologic data were confirmed by the demonstration of reduced GL-7+positive cells in splenic B cells of Fcγ−/− mice (unpublished data) and less somatic mutation in antigen-specific hybridomas. Because some studies suggest that short-lived and long-lived plasma cells arise from completely separate precursors (25), we are currently studying the B cells responding to antigen in WT and Fcγ−/− mice to determine if the same clones are activated or whether the plasma cell precursors differ between the strains.

DCs have previously been shown to mediate a critical function in B cell activation by transporting antigen from the periphery to T and B cells (26). The altered B cell phenotype we observed in Fcγ−/− could also be traced to the influence of Fcγ−/− DCs and IL-12. Fcγ−/− DCs had a more mature phenotype and produced at least twofold more IL-12 than WT DCs. Several studies have established a role for IL-12 in B cell differentiation and function. IL-12 can induce plasma cell formation and B cell heavy chain class switching to IgG1 and IgG2a. The switch to IgG2a is IFN-γ-dependent, whereas the promotion of differentiation to plasma cells and the switch to IgG1 does not require IFN-γ (27). Studies in human primary B cells have also shown that IL-12 up-regulates IL-12Rβ2 expression and IFN-γ production by B cells, possibly allowing them to function as B effector cells and promote the activation of Th1 cells (28). Notably, although IL-12 promotes short-lived plasma cells, perhaps in synergy with CD40L (27), memory B cell differentiation occurs independently of IL-12 (22, 23). In our study, when IL-12 was administered to WT mice, the mice responded to antigen immunization with the generation of more splenic plasma cells and fewer BM plasma cells, a pattern mimicking that seen in immunized Fcγ−/− mice. Therefore, IL-12 produced by DCs seems to be highly influential in directing B cell differentiation. It is possible that this difference in differentiation pathway reflects a change in the environment in which the B cell resides rather than a direct effect on the B cell. We do not favor this explanation, as chemokine expression in the spleen is similar in both strains (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20070731/DC1). Furthermore, IL-12 has been shown by others to act directly on B cells (21). Interestingly, CR2 (CD21/CD35) has also been reported to diminish the development of long-lived plasma cells while leaving intact the generation of short-lived plasmablasts, germinal center, and memory B cells. This effect is mediated through the regulation of Blimp-1 and XBP-1, which are critical transcription factors in plasma cell differentiation (29). CR2, like FcR, appears to function through a B cell–intrinsic pathway that specifically arrests the differentiation of long-lived plasma cells. We should note, however, that antigen-specific T cell proliferation was enhanced in Fcγ−/− mice. The enhanced number of antigen-specific T cells expressing CD40L and available to interact with antigen-specific B cells may also contribute to the preferential generation of short-lived plasma cells. Thus, both the direct action of DCs on B cells as well as the action of T cells in Fcγ−/− mice may contribute to skewing the B cell response.

Although the differences in IL-12 production between the mouse strains may seem small, we have previously shown in studies of hormonal effects on B cells that a 20% change in expression of CD22 can affect B cell receptor signaling and alter negative selection (30). Recently, McGaha et al. (31) have shown that a 50% increase in FcγRIIB is required in B cells to prevent disease in lupus-prone mice. Thus, it should not be surprising that a twofold increase in IL-12 production by DCs can affect B cell differentiation. In fact, a study of experimental myasthenia gravis in mice also demonstrated that exogenous IL-12 given in a similar protocol led to increased serum antibody (27).

A previous study reported no alteration in humoral response to the hapten NP in Fcγ−/− mice. This study was performed on C57BL/6 mice deficient in the FcR γ chain and may reveal a strain–dependent difference in FcR γ chain function. It is also possible, however, that the force of selection for high-affinity antibodies in the NP model is so strong that differences in B cell differentiation were obscured. Some effects were seen that were similar to those we report. For example, in the reported study that Fcγ−/− mice had fewer splenic germinal centers after immunization.

We do not yet fully understand why the absence of the γ chain leads to the observed DC phenotype. Studies on γ chain–deficient mice have focused on alterations in expression and function of FcR1, FcRIII, and FcRIV (32). To confirm that the serological changes were mediated through FcR engagement, we have immunized FcRIII-deficient mice with MAP peptide. These mice show increased serum antipeptide antibodies (fivefold increase) and an activated phenotype of BMDCs. Similarly, DCs derived from BM cells under
serum-free conditions also display an activated phenotype, which is, again, consistent with Fc receptor engagement modulating DC function (26). The activated phenotype was abrogated by addition of purified IgG to the culture. Although y chain–containing Fc receptors have been demonstrated to be activating receptors, we speculated that engagement of these receptors may diminish the response to other activating receptors. Such reciprocal regulation of toll-like receptor 9 and IgE receptor signaling has already been demonstrated. Production of type I IFN by plasmacytoid DC is inhibited by IgE receptor signaling has already been demonstrated. Pro-ceptors. Such reciprocal regulation of toll-like receptor 9 and activating receptors, we speculated that engagement of these receptors may diminish the response to other activating receptors.

**MATERIALS AND METHODS**

**Mice.** 5–10-wk-old female Fcγ−/− mice (Fcε1g(BALB/cBy)) on the BALB/c background and WT BALB/c mice were purchased from Taconic. The mice were housed in a specific pathogen-free barrier facility. IL-12−/− mice (C.B17-Scid/Scid/HSD) were purchased from Jackson ImmunoResearch Laboratories. IL-12−/− mice were bred to FcγRII- and FcγRIII-deficient mice (B6-H2−/−H2−/−mice). These studies were approved by the Institutional Animal Care and Use Committees of the Albert Einstein College of Medicine, Columbia University Medical Center, and Feinstein Institute for Medical Research.

**Antigens.** The peptides DWEYSVWLSN, linked to an eight-branched poly-lysine backbone (MAP peptide), and ADGSGGRDEMQASMW5 were purchased from both Invitrogen and AnaSpec, Inc. The peptide ADGSGGRDEMQASMW5 (10–2) was conjugated to BSA with glutaraldehyde as previously described (12).

**Immunizations.** Mice were immunized i.p. with 100 μg MAP peptide or ADGSGGRDEMQASMW5-BSA in CFA (Becton Dickinson) on day 0 and then boost in IFA (Becton Dickinson) on days 7 and 14. For the recall assay, mice were immunized i.p. with 100 μg MAP peptide in CFA on day 0 and boosted with antigen in IFA on day 56.

**Adaptive transfer of B cells to muMT mice.** 6–8-wk-old WT and Fcγ−/− mice were killed and total splenic B cells were purified. Recipient μMT mice were injected with 2 × 10^7 cells intravenously. 2 wk after transfer, both WT B cell–reconstituted and Fcγ−/− B cell–reconstituted muMT mice were immunized with MAP peptide as described. The antipeptide response from both groups was measured every 2 wk until 6 wk after immunization.

**ELISAs.** ELISA/RIA high binding 96-well plates (Costar; Corning) were coated with 15 μg/ml of the relevant peptide. For dsDNA ELISAs, plates were coated with 100 μg/ml of sonicated calf thymus DNA (Sigma-Aldrich) that was filtered through a 0.45-μm nitrocellulose Millex-HA syringe filter (Millipore) to produce dsDNA. Plates were then blocked with 3% FCS or 5% milk. Alkaline phosphatase–conjugated anti-mouse total IgG or anti–mouse isotype-specific antibodies (SouthernBiotech) were used as secondary antibodies, and plates were developed with p-nitrophenyl phosphate (Sigma-Aldrich). OD was monitored at 405 nm.

ELISAs determining the apparent affinity of peptide-specific antibodies were performed with the following modification: sera were preincubated with varying concentrations of the relevant peptide before being loaded onto the antigen-coated plates. The K_d was calculated as the reciprocal of the molar concentration of peptide inhibiting 50% of the binding (10).

**Memory response.** WT and Fcγ−/− mice were immunized and boosted as described above. Mice were bled every 2 wk to measure the antipeptide response in serum. After 8 wk, mice were challenged with 100 μg MAP peptide in IFA, and blood was drawn 5 d later to analyze the antipeptide antibody response.

**Immunohistochemistry of kidneys.** To determine glomerular IgG deposition, kidneys were obtained 13 wk after the initial immunization, fixed in 10% paraformaldehyde, and embedded in paraffin. Sections were blocked with 2% BSA and stained with biotinylated goat anti-mouse IgG. Alkaline phosphatase–labeled ABC reagent from the VECTASTAIN ABC kit (Vector Laboratories) was added. Sections were developed with 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and nitroblue tetrazolium chloride substrate (Invitrogen). Proteinuria was determined by color comparison using Multistix (Sigma-Aldrich).

**Purification of B cells.** For B cell purification, single-cell suspensions were prepared from splenocytes. After red blood cell lysis, cells were incubated with biotinylated anti-CD3, anti-CD11b, and anti-CD11c mAb (BD Biosciences), and B cells were isolated by negative selection with magnetic streptavidin–linked Dynabeads (Invitrogen). After depletion, >90% of the remaining cells were B220+ by flow cytometry. All flow cytometry was performed on a FACSCalibur (Becton Dickinson) and analyzed with FlowJo Software (Tree Star, Inc.).

**Purification of T cells.** Mice were immunized in the right front and hind footpads with 100 μg MAP peptide in 100 μl of 1:1 PBS/CFA and in the left front and hind footpads with 100 μl of 1:1 PBS/CFA alone (50 μl/footpad). 1 wk later, left and right popliteal, axillary, and brachial lymph nodes were harvested, and a single cell suspension was prepared. Red blood cells were lysed, and cells at 2 × 10^7 cells/ml were incubated at 4°C for 30 min with supernatants from the following ATCC cell lines: TIB-120 (anti-αβ整合) and anti-β2 (BD Biosciences). Excess antibody was washed away and the cells were incubated with sheep anti–rat IgG Dynal/Dynal beads at 4°C for 30 min. T cells isolated by magnetic bead depletion (Invitrogen) and were demonstrated by flow cytometry to be >95% pure.

**Peritoneal macrophages.** To collect peritoneal macrophages, WT and Fcγ−/− mice were injected with 2 ml of 3% aged thioglycollate i.p. On day 4, mice were killed and macrophages were harvested in Hank’s balanced salt solution. The purity of the cell population was assessed by staining with F480 antibody. All samples were >85% F480 positive.
Purification of DCs. BMDCs were generated according to a previously described protocol (36). In brief, BM was harvested and red blood cells were lysed. T cells and B cells were depleted by incubating the cells with the ATCC supernatants TIB-120, TIB-211 (anti-CD8), TIB 207 (anti-CD4), and TIB-146 (anti-B220) in the presence of rabbit complement (Pel-Freeze Biologicals). The remaining cells were cultured in RPMI 1640 with 5% FCS and GM-CSF-containing supernatant from J558L cells (gift of the laboratory of R. Steinman, The Rockefeller University, New York, NY). Cells were grown fresh medium on days 2 and 4. On day 6, the nonadherent cells were collected and were incubated overnight in medium with or without 50 ng/ml LPS from Escherichia coli serotype 055:B5 (Sigma-Aldrich). To further deplete monocytes, the cells were plated for 2 h, and nonadherent cells were collected. The depleted cells were >85% CD11c+ by flow cytometry.

To purify splenic DCs, a modification of the original method of Steenman et al. (37) was used. In brief, spleen cell suspensions were prepared by digestion with 400 U/ml collagenase D at 37°C for 20 min, followed by 0.5 M EDTA. Total splenocytes were washed and resuspended in 30% BSA. Ice-cold PBS was loaded on top of the mixture. DCs were collected from the interface after centrifugation at 2,200 rpm for 30 min. To increase the purity of DCs, we used negative selection methods with a mouse DC enrichment set (BD Biosciences). After two steps of purification, the purity was measured by flow cytometry and ~75–80% of cells were CD11c+.

Enumeration of plasma cells. Antigen-specific plasma cells were enumerated in the spleen and BM of femurs by performing peptide-specific ELISPOT assays. For plasma cell enumeration, MAP peptide–immunized mice were killed and splenocytes were harvested. Red blood cells were lysed, and the splenocytes were added to plates coated with 10 μg/ml MAP peptide and blocked with 10% FCS. The plates were centrifuged at 1,000 rpm for 5 min and incubated overnight at 37°C. The next day, splenocytes were washed stringently and biotinylated goat anti–mouse IgG (Southern-Biotech) was added for 2 h at 37°C. Washed plates were incubated with streptavidin–alkaline phosphatase (SouthernBiotech) for 2 h and developed with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma-Aldrich). Spots were counted manually using a dissecting microscope. To enumerate plasma cells in the BM, CD138+ cells from WT and Fcγ−/− mice were sorted on a FACSArta and plated for MAP peptide–specific ELISPOT analysis.

Spleen histology. Spleens were harvested on days 0, 4, 8, 12, and 16 after immunization and snap frozen in Tissue-Tek O.C.T. compound (Sakura Finetek). Sections were incubated with anti-mouse B220-FITC and CD138-PE, and were visualized using a fluorescent microscope (AxioCam II; Carl Zeiss, Inc.).

AID expression in germinatal center B cells. Expression of AID gene was measured in purified germinat center B cells. WT and Fcγ−/− mice were immunized with 100 μg MAP peptide in CFA on day 0. On day 10, mice were killed and splenic B cells were purified by depletion of non-B cells: splenocytes were incubated with biotinylated anti-CD43, anti-CD11c, and anti-CD90.2 (BD Biosciences) and bound cells were removed by streptavidin–alkaline phosphatase (SouthernBiotech) for 2 h and developed with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma-Aldrich). Spots were counted manually using a dissecting microscope. To enumerate plasma cells in the spleen, CD138+ cells were identified by flow cytometry, and CD138+ cells from WT and Fcγ−/− mice were sorted on a FACSArta and plated for MAP peptide–specific ELISPOT analysis.

Analysis of DC phenotype and cytokine secretion. For cytokine secretion, intracellular flow cytometry was performed. LPS-treated or untreated BMDCs were cultured at 5 × 10^5/ml with 10 μg/ml brefeldin A (Sigma-Aldrich) for 5 h. The cells were then stained with anti-CD11c PE (BD Biosciences) and fixed in 2% paraformaldehyde for 10 min before staining with anti–IL-12 APC or anti–IL-10 APC in 0.3% saponin for 30 min. For cytokine analysis by ELISA, 10^7 BMDCs were cultured for an additional 24 h after LPS treatment, and cytokines were assayed in the culture supernatant using anti–mouse cytokine ELISA kits. Cytokine concentrations were determined based on standard curves for real-time PCR, total RNA was prepared from BMDCs either unstimulated or LPS-stimulated for 4 h using TRIzol Reagent (Invitrogen). 1 μg DNA-free RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis system for RT-PCR. (Invitrogen). Real-time PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) on an iCycler iQ instrument (Bio-Rad Laboratories). Amplification conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers for 18s ribosomal RNA, IL-12a, IL-12b, and AID were purchased from Applied Biosystems. Relative expression was determined using the following formula: relative expression = 2^{ΔΔCT}, where ΔΔCT = [Ct (gene of interest sample) - Ct(18s rRNA sample)] - [Ct(gene of interest calibrator) - Ct(18s rRNA calibrator)]. [See the ΔΔ CT methods, Taqman Bulletin 2; Applied Biosystems.] Data are presented as the mean ± SD of three experiments.

In vivo cytokine production by splenic DCs. BALB/c WT or Fcγ−/− mice were immunized with 100 μg MAP peptide in CFA i.p. Each group of mice was killed on either day 1 or 3 after immunization. Splenic DCs were purified as described in Materials and methods and cultured in RPMI 1640 supplemented with 10% FCS for 16 h. Supernatant was collected and cytokine production was analyzed by ELISA.

BMDC and B cell coculture assays. BMDCs were stained with anti–CD11c PE (BD Biosciences) and sorted on a MoFlo Cell Sorter (Dako). More than 95% of the sorted cells were CD11c+. Mature BMDCs, irradiated with 1,500 rads, were cultured with 5 × 10^4 B cells in triplicate in RPMI with 10% FCS in the presence of 20 μg/ml of purified anti–CD40 antibody (BD Biosciences). In some assays, 10 μg/ml of purified anti–IL-12 antibody (BD Biosciences) was added to the culture. 5 d later, 1 μCi of 3H-thymidine was added to each well for 8 h, and the incorporated radioactivity was determined. To block the secretion of IL-12 during the coculture, siRNA (siRNA ID#: 102773; Ambion) targeting the IL-12a gene was transfected to BMDC according to the manufacturer's protocol. In brief, irradiated BMDCs cultured from both WT and Fcγ−/− mice were transfected with 1 and 10 μM of anti–IL-12a specific or negative control siRNA by siPORT NeoFX (Ambion). The next day, siRNA/transfection agents were removed and 5 × 10^6 splenic B cells were added to the culture.

T cell proliferation. T cells were purified from lymph nodes of MAP peptide–primed WT or Fcγ−/− mice as described in Materials and methods. BMDCs were prepared from nonimmunized WT and Fcγ−/− mice and pulsed with MAP peptide on day 6 of culture. On day 7, 1–3 × 10^5 primed T cells were cocultured in triplicate for 72 h with 5 × 10^4 irradiated DCs. Cells were pulsed with 0.5 μCi of 3H-thymidine for the last 8 h. Cells were harvested, and the incorporated radioactivity was counted.

Analysis of Vβ usage and cytokine ELISAs. Cells from the draining lymph nodes of naive mice or mice immunized 7 d earlier with MAP peptide in CFA or CFA alone were harvested and plated at 3 × 10^5 cells/ml in IMDM supplemented with 10% FCS, 2 mM glutamine, 100 μU/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol in the presence of 50 μg/ml MAP peptide. 7 d later, the cultures were supplemented with 50 μU/ml of recombinant human IL-2 (Invitrogen) and 3 × 10^5 RBC-depleted irradiated (1,500 rad) splenocytes/ml along with fresh medium. 14 d after the initial culture, cells were collected. 2 × 10^6 cells were stained with 100 μl of 1 μg/ml CD4-PE in 2% FCS/PBS for 30 min on ice and then stained for the various Vβ chains using FITC-labeled antibodies. All labeled antibodies were obtained from BD Biosciences and data were acquired using a FACSCan (Becton Dickinson). Peptide-specific T cells were identified by comparison of lymph node cultures from mice immunized with MAP peptide in CFA to those from mice immunized with CFA alone. Little proliferation was detected in the latter with 3–4 times as many live cells found in the peptide-specific cultures compared with the CFA controls.
Generation of hybridomas and sequencing J\(\mu\)2-J\(\mu\)4 region. BALB/c WT and Fc\(^{-}\) mice were enumerated as described above. Generation of hybridomas and sequencing J\(\mu\)2-J\(\mu\)4 region. BALB/c WT and Fc\(^{-}\) mice were enumerated as described above.

In vivo IL-12 treatment. Mice were injected i.p. with 1 \(\mu\)g rIL-12 or saline on days -1, 0, and 1 after immunization with MAP peptide in CFA. Mice were then boosted as described in Materials and methods with no further administration of IL-12. On day 25, mice were killed, and a peptide-specific ELISPOT assay was performed on splenocytes. BM cells were harvested, and plasma cells were enumerated by flow cytometry as described above.

The J\(\mu\)2-J\(\mu\)4 region was amplified using Pfu turbo (Stratagene) following previously described protocol. PCR was performed with the following primers: 5’ primer, 5’-GCCACCATCCTCACTCTCTTCTAGG-3’; and 3’ primer, 5’-TGAGACGAGGCTAGATGCC-3’. PCR conditions were the following: 95°C for 30 s, 60°C for 30 s, and 72°C for 15 min for 35 cycles. PCR products were purified and sequenced at the DNA sequencing facility at the Columbia University Medical Center.

Online supplemental material. Fig. S1 shows serum anti–MAP peptide IgM titer. Fig. S2 shows the increased surface activation markers in BMDCs by FACS analysis. Fig. S3 shows the specific inhibition of IL-12a expression after siRNA treatment in BMDCs. Fig. S4 shows the relative expression of IL-12a in splenic DCs from WT, Fc\(^{-}\), and Fc\(^{-}\)-IL-12a\(^{-}\) mice. Fig. S5 shows titer of anti–MAP peptide and anti-dsDNA antibody in serum from WT, Fc\(^{-}\), and Flt3L mice. Fig. S6 is for the expression of chemokine/chemokine receptor in splenic B cells and DCs. Table S1 shows the number of germinal centers in MAP peptide–immunized WT and Fc\(^{-}\)- mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070731/DC1.

We want to thank Sylvia Jones for help in preparation of the manuscript. This work was supported by a grant from the National Institutes of Health. S.J. Kim is a recipient of a SLE Foundation Fellowship. The authors have no conflicting financial interests.

Submitted: 10 April 2007
Accepted: 29 August 2008

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