European Journal of Immunology Innate immunity

Short Communication:

Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRγ

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The inhibitory effect of DAP12 on macrophages has been revealed by examining myeloid cells from DAP12-deficient mice. In this report, we demonstrate that both DAP12 and the FcεRIγ-chain (FcRγ) are required for negative regulation of TLR responses in bone marrow-derived dendritic cells (DC). Loss of both DAP12 and FcRγ enhanced the pro-inflammatory cytokine production and maturation of DC after TLR stimulation, resulting in a greater percentage of DC that produced IL-12 p40, TNF, and IL-6, and expressed high levels of MHC class II, CD80, and CD86. Whereas DC lacking only DAP12 showed some increased TLR responses, those lacking only FcRγ had a greater enhancement of maturation and cytokine production, though to a lesser extent than DC lacking both DAP12 and FcRγ. Additionally, antigen-specific T cell proliferation was enhanced by DAP12$^{-/-}$FcRγ$^{-/-}$ DC relative to wild-type DC after maturation. Similar to DAP12$^{-/-}$FcRγ$^{-/-}$ DC, Syk-deficient DC also had increased inflammatory cytokine production, maturation, and antigen presentation. These results confirm the inhibitory effect of immunoreceptor tyrosine-based activation motif (ITAM) signaling in myeloid cells and show that DC and macrophages differ in their dependence on the ITAM-containing adapters DAP12 and FcRγ for negative regulation of TLR signaling.

Received 21/6/07
Revised 26/9/07
Accepted 7/11/07

[DOI 10.1002/eji.200737600]

Key words: Antigen presentation · Dendritic cell · TLR

Supporting Information for this article is available at http://www.wiley-vch.de/contents/jc_2040/2007/37600_s.pdf

Introduction

Dendritic cells (DC) are potent antigen-presenting cells, which capture and process antigens in peripheral tissues, and then migrate to secondary lymphoid organs where they activate T cells to initiate adaptive immune responses [1]. Exposure of DC to pathogen products or pro-inflammatory stimuli induces DC maturation, which dramatically enhances the ability of DC to activate antigen-specific T cells [2]. Toll-like receptors (TLR) are one family of receptors expressed by DC and other myeloid cells that detect pathogen-associated molecules

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Abbreviations: BMDC: bone marrow-derived DC · **FcRγ:** FcεRI γ-chain

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[3]. TLR ligation causes the activation of NF-κB and MAP kinase pathways, resulting in DC maturation, which is characterized by up-regulation of MHC class II and costimulatory molecule expression as well as cytokine production [4].

Immunoreceptor tyrosine-based activation motifs (ITAM) play a key role in signal transduction for many activating receptors on hematopoietic cells [5–7]. The ITAM-containing adapter DAP12 has recently also been reported to negatively regulate TLR signaling in macrophages [8–10] and plasmacytoid DC [11–13]. However, DAP12-deficient bone marrow-derived dendritic cells (BMDC), in contrast to DAP12-deficient macrophages, did not show increased cytokine production after TLR triggering [8]. This suggested that molecules other than DAP12 might negatively regulate TLR signaling in DC. Here, we demonstrate that another ITAM-containing adapter, FcεRIγ (FcRγ), functions with DAP12 to inhibit TLR signaling in DC.

Results and discussion

Enhanced maturation in DAP12$^{-/-}$FcRγ$^{-/-}$ DC

Recent studies demonstrate that ITAM-containing "activating" adapters can mediate inhibitory signals in myeloid cells (summarized in [14–17]). We have shown enhanced TLR-induced pro-inflammatory cytokine responses in DAP12-deficient macrophages but not in BMDC [8]. This finding suggests that macrophages and DC may use ITAM-containing adapters differently or that other molecules compensate for the DAP12 deficiency in DC, but not in macrophages. To test this hypothesis, we generated BMDC from WT, DAP12$^{-/-}$, FcRγ$^{-/-}$, and DAP12$^{-/-}$FcRγ$^{-/-}$ C57BL/6 mice and treated them with the TLR stimuli LPS (which stimulates via TLR4), CpG DNA (TLR9), flagellin (TLR5), poly (I:C) (TLR3), and zymosan (TLR2 and TLR6). Development of appropriate DC function is dependent on cell maturation following exposure to antigens and innate immune stimuli [2]. Thus, we examined the maturation status of DC following TLR stimulation. After 16 h of TLR stimulation, DAP12$^{-/-}$FcRγ$^{-/-}$ DC showed significantly enhanced maturation in comparison with WT DC, with an increased percentage of cells having a MHC class II^{high}, CD86^{high}, and CD80^{high} phenotype (Fig. 1A–D). We used doses of TLR agonists that induce low levels of maturation in WT DC because this allowed us to see the large increases in maturation present in DAP12$^{-/-}$FcRγ$^{-/-}$ DC. We found similar dose dependence in the ability of DAP12 to inhibit macrophage TLR responses [8]. Increased maturation of DAP12$^{-/-}$FcRγ$^{-/-}$ DC in comparison with WT DC was also seen at higher doses of TLR agonists that resulted in more substantial maturation of the WT DC, but this difference between WT and mutant DC was less pronounced than at the low doses shown in Fig. 1 (data not shown).

DAP12-deficient DC treated with CpG DNA or poly (I:C) had similar percentages of mature phenotype cells compared to WT DC. After stimulation with LPS or flagellin, DAP12-deficient DC had slightly increased percentages of mature cells that were statistically significant (Fig. 1D). Zymosan treatment resulted in significantly increased percentages of CD80^{high} DAP12$^{-/-}$ DC, but showed no significant increase in MHC class II^{high} or CD86^{high} cells. FcRγ-deficient DC exhibited a phenotype that was intermediate between WT and DAP12$^{-/-}$FcRγ$^{-/-}$ DC–they had significantly increased percentages of MHC class II^{high}, CD86^{high}, and CD80^{high} cells compared to WT DC with all stimuli tested, but not to the same extent as the DC lacking both DAP12 and FcRγ (Fig. 1A–D). Whereas we saw increases in the percentages of cells expressing high levels of MHC class II, CD86, and CD80 in FcRγ$^{-/-}$ and DAP12$^{-/-}$FcRγ$^{-/-}$ DC, the expression of CD40 was comparable between all sources of DC tested after stimulation (data not shown). Interestingly, unstimulated FcRγ$^{-/-}$ and DAP12$^{-/-}$FcRγ$^{-/-}$ DC had significantly higher levels of surface MHC class II and CD80 than those generated from WT, DAP12$^{-/-}$, and FcRγ$^{-/-}$ mice (Fig. 1A–C, thin lines, and Fig. 1D). These data suggest that whereas both DAP12 and FcRγ regulate the resting and TLR-stimulated maturation state of DC, that FcRγ plays a dominant role. It is also interesting that DAP12 deficiency only seemed to play a significant role in with some TLR agonists (such as LPS and flagellin), but not others [(poly (I:C) and CpG DNA]. In addition, with zymosan, FcRγ deficiency seemed to be dominant as there was little difference in maturation between FcRγ$^{-/-}$ and DAP12$^{-/-}$FcRγ$^{-/-}$ DC. This may reflect differences in the ability of the DAP12 and FcRγ ITAM to affect signaling through TLR recognizing the different stimuli (*i.e.* that use different TIR domain-containing adapters) or the use of additional receptors by some stimuli (*e.g.* Dectin-1 for zymosan) [18].

Increased cytokine production by DAP12$^{-/-}$FcRγ$^{-/-}$ DC

DC secrete pro-inflammatory cytokines after TLR stimulation. To determine whether the lack of DAP12 and FcRγ affects DC pro-inflammatory cytokine production, intracellular cytokines were measured by flow cytometry after TLR stimulation. As with DC maturation, we used sub-optimal doses of the TLR agonists LPS, CpG DNA and zymosan to allow the ability to see larger relative differences between WT and mutant DC. The production of IL-12 p40, IL-6, and TNF as measured by intracellular cytokine staining was increased in

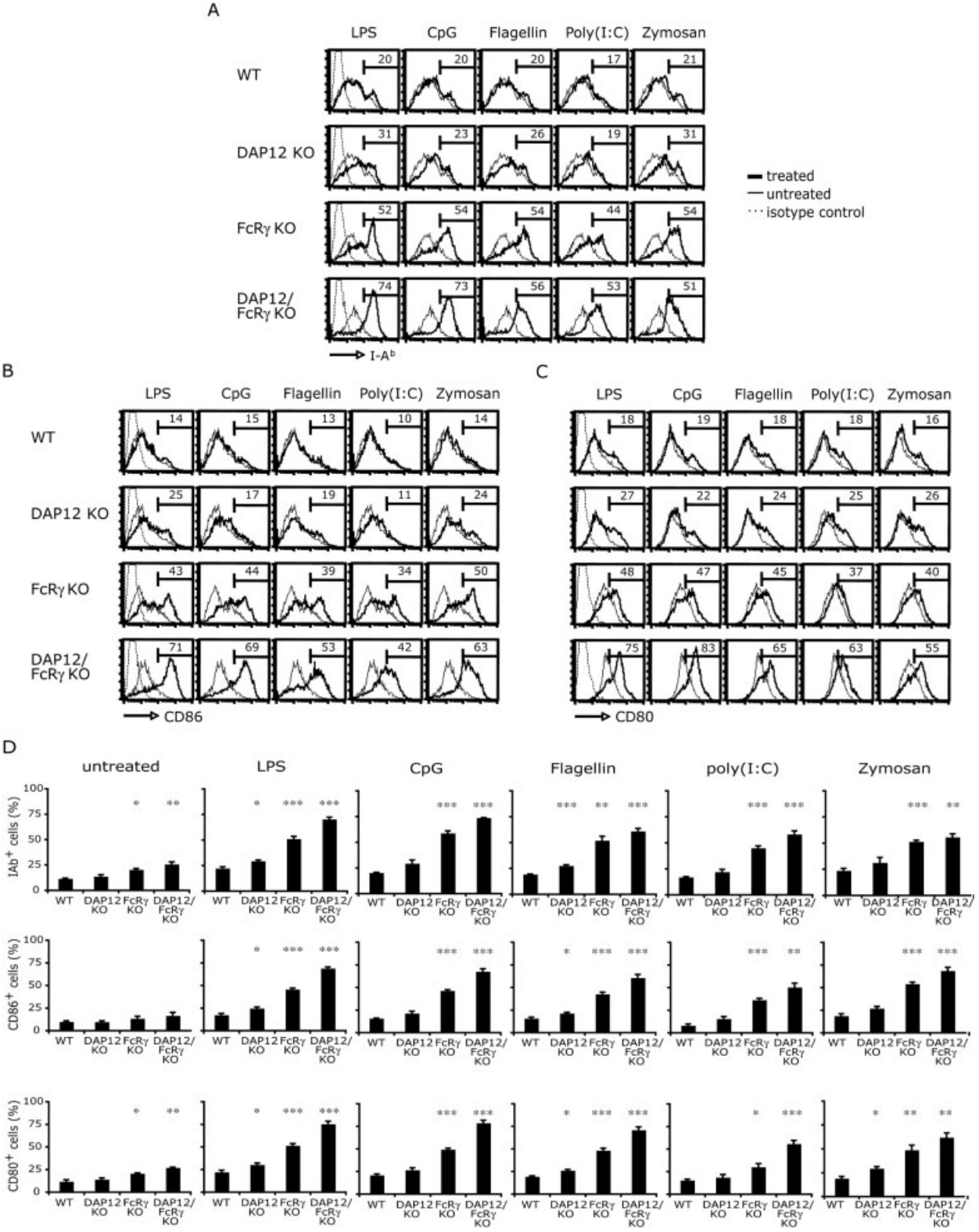

Figure 1. Enhanced maturation of DAP12$^{-/-}$FcRγ$^{-/-}$ DC after TLR stimulation. BMDC from WT, DAP12$^{-/-}$ (DAP12 KO), FcRγ$^{-/-}$ (FcRγ KO), and DAP12$^{-/-}$FcRγ$^{-/-}$ (DAP12/ FcRγ KO) mice were incubated with LPS (5 ng/mL), CpG DNA (100 nM), flagellin (25 ng/mL), poly (I:C) (5 μg/mL), or zymosan (20 μg/mL) (thick line) or were untreated (thin line) for 16 h. Dotted line represents staining with an isotype-matched control antibody. DC maturation was determined by flow cytometry after staining with mAb specific for (A) I-A^b, (B) CD86, and (C) CD80. All data shown are gated on CD11c$^+$ cells. The percentages of MHC class II^{high}, CD86^{high}, and CD80^{high} cells are shown above the regional markers for the treated cells. Data are representative of three independent experiments. (D) The percentages of MHC class II^{high}, CD86^{high}, and CD80^{high} DC are shown as the average and SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT DC using the Student's *t*-test.

DAP12$^{-/-}$FcR$\gamma^{-/-}$ DC in response to LPS, CpG DNA, and zymosan compared to WT DC (Fig. 2A) and this increase was significant in all cases except LPS- and CpG-induced TNF production. In some cases, such as with LPS- and zymosan-induced TNF production, only a small increase in the percentage of cells producing cytokine was observed in the DAP12$^{-/-}$FcR$\gamma^{-/-}$ DC. This may be because a very high percentage of WT cells were producing cytokine, not allowing for much of an increase in the deficient DC; however, we also did not see a large enhancement of TNF secretion by DAP12$^{-/-}$FcR$\gamma^{-/-}$ DC with lower doses of LPS or zymosan (data not shown).

Similar to what was seen with TLR-induced maturation, FcR$\gamma^{-/-}$ DC had an intermediate phenotype between WT and the DAP12$^{-/-}$FcR$\gamma^{-/-}$ DC for all stimuli and cytokines tested and had significantly higher cytokine production than WT DC for CpG DNA or zymosan-treated cells as determined by intracellular cytokine staining (Fig. 2A). The cytokine production by

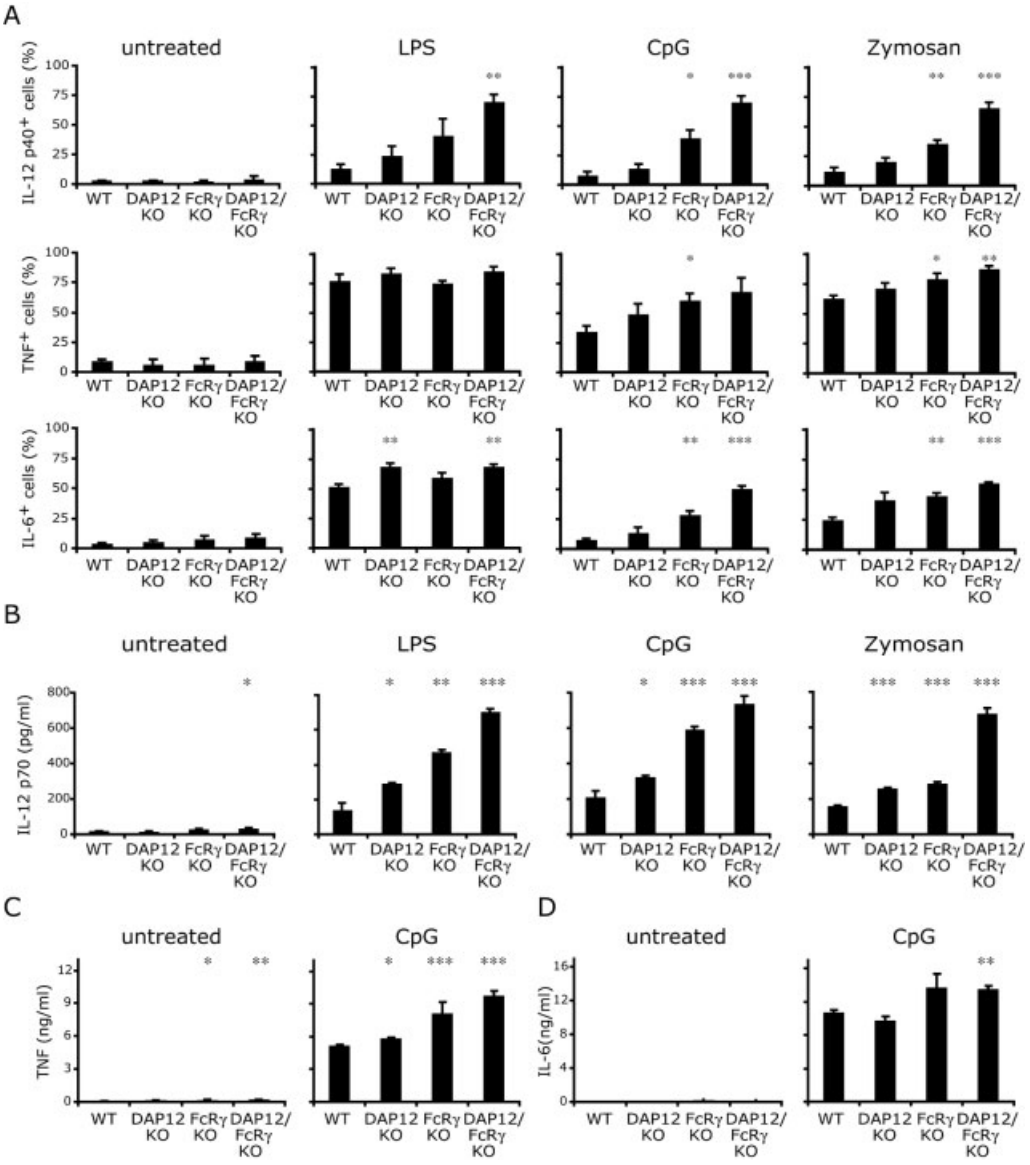

Figure 2. DAP12$^{-/-}$FcR$\gamma^{-/-}$DC produce increased amounts of pro-inflammatory cytokines in response to TLR stimulation. (A) BMDC from WT, DAP12$^{-/-}$, FcR$\gamma^{-/-}$, and DAP12$^{-/-}$FcR$\gamma^{-/-}$mice were incubated alone or with LPS (5 ng/mL), CpG DNA (200 nM), or zymosan (20 μg/mL) for 6 h in the presence of Brefeldin A for the last 4 h. The percentage of IL-12 p40 (top panels), TNF (middle panels), and IL-6 –producing (bottom panels) CD11c$^+$ cells was determined by flow cytometry. (B) The secretion of IL-12 p70 was measured by using ELISA for cells stimulated with the same doses of LPS, CpG DNA or zymosan as in (A) for 16 h. The secretion of TNF (C) and IL-6 (D) from DC treated with CpG DNA for 4 (TNF) or 16 h (IL-6). Data are represented as the mean + SEM of three independent experiments. * p <0.05, ** p <0.01, ***p <0.001 compared to WT DC using the Student's t-test.

DAP12$^{-/-}$ DC was similar to WT DC, though small increases were observed in some experiments, they were less than the FcRγ$^{-/-}$ DC except for LPS induced TNF and IL-6 production.

In addition to intracellular staining, we assessed cytokine secretion by DC using ELISA of cell supernatants. DAP12$^{-/-}$FcRγ$^{-/-}$ DC secreted significantly more IL-12 p70 (the bioactive form of IL-12 composed of the IL-12 p40/p35 heterodimer) in comparison with WT DC (Fig. 2B). Consistent with the trend seen with IL-12 p40 production as assessed by intracellular cytokine staining, IL-12 p70 secretion was increased to a greater extent in the DAP12$^{-/-}$FcRγ$^{-/-}$ DC in comparison with either the DAP12$^{-/-}$ DC or FcRγ$^{-/-}$ DC and the FcRγ$^{-/-}$ DC were intermediate between the DAP12$^{-/-}$ DC and the DAP12$^{-/-}$FcRγ$^{-/-}$ DC. In contrast, TNF and IL-6 production by these cells was significantly enhanced only following CpG DNA treatment (Fig. 2B–D and data not shown). These data suggest that DC hyperrespond to TLR-induced stimulation in the absence of both DAP12 and FcRγ, distinct from what is seen in macrophages, where the absence of DAP12 alone causes increased responses.

We also examined cytokine production by untreated DC. Unlike what was seen with DC maturation, DC deficient in DAP12, FcRγ or both did not show spontaneous activation as measured by intracellular cytokine staining for IL-12 p40, TNF and IL-6 or by ELISA for secreted IL-6 (Fig. 2A and D). In contrast, when measuring IL-12 p70 and TNF secreted from untreated DC, we saw very small amounts of these cytokines secreted from untreated DAP12$^{-/-}$FcRγ$^{-/-}$ DC that were significantly higher than that secreted from untreated WT DC (Fig. 2B and C). This supports that there may be a low level of activation of untreated DC generated from the DAP12$^{-/-}$FcRγ$^{-/-}$ mice.

Enhancement of maturation and function in Syk$^{-/-}$ DC

Syk tyrosine kinase is recruited to the phosphorylated ITAM after ITAM-associated receptor stimulation and is required for ITAM-mediated signaling in myeloid cells [14]. Syk is also required for the DAP12-dependent inhibitory signaling in macrophages [8]. We therefore generated BMDC from Syk$^{-/-}$ fetal liver chimeric mice (because Syk$^{-/-}$ mice are non-viable) and compared their TLR responses with those of DAP12$^{-/-}$FcRγ$^{-/-}$ BMDC. As shown in Fig. 3A and B, the enhancement of maturation and cytokine production of Syk$^{-/-}$ DC was similar to those of DAP12$^{-/-}$FcRγ$^{-/-}$ DC after CpG DNA treatment. As was seen with DAP12$^{-/-}$FcRγ$^{-/-}$ DC, unstimulated Syk$^{-/-}$ DC also had higher levels of MHC class II and CD86 in comparison with WT DC (Fig. 3C). Whereas unstimulated Syk$^{-/-}$ and DAP12$^{-/-}$FcRγ$^{-/-}$ DC had similar CD86 levels, Syk-deficient DC had even higher resting levels of MHC class II than DC lacking both DAP12 and FcRγ (Fig. 3C). As was seen with the DAP12$^{-/-}$FcRγ$^{-/-}$ DC in Fig. 2A, untreated Syk-deficient DC did not show increased percentages of cells producing TNF or IL-12 p40 as measured by intracellular cytokine staining (data not shown).

The primary function of mature DC is to induce antigen-specific T cell proliferation. We isolated CD4$^+$ T cells from OT II T cell antigen-receptor (TCR)-transgenic mice and cells were cocultured with CpG DNA-treated, $OVA_{323–339}$ peptide-pulsed DC for 72 h, following which T cell proliferation was determined by [^{3}H]thymidine incorporation. As shown in Fig. 3D, DAP12$^{-/-}$FcRγ$^{-/-}$and Syk$^{-/-}$ DC induced an elevated level of OT II T cell proliferation when compared with WT DC at two concentrations of OVA peptide. The enhanced ability of DAP12$^{-/-}$FcRγ$^{-/-}$and Syk$^{-/-}$ DC to activate antigen-specific T cells in comparison with WT DC was consistent with a higher percentage of cells expressing high levels of MHC class II and CD86 (Fig. 1 and 3B), and supports the conclusion that deficiency of DAP12 and FcRγ or Syk causes a relative enhancement in DC maturation. We also compared the ability of WT, DAP12$^{-/-}$, FcRγ$^{-/-}$ and DAP12$^{-/-}$FcRγ$^{-/-}$DC to present antigen to OT-II T cells after CpG DNA-induced maturation. DAP12-deficient and FcRγ-deficient DC did not have increased antigen presentation (Supporting Information Fig. 1), even though FcRγ$^{-/-}$ DC had increased maturation after CpG DNA treatment (Fig. 1). These data suggest that solely having the elevated percentages of mature DC seen with CpG DNA-treated FcRγ$^{-/-}$ DC is not enough to lead to increased antigen-specific T cell proliferation.

Increasingly, there is evidence for the inhibitory effects of ITAM signaling in myeloid cells [17]. This conclusion is best documented in studies of DAP12, but FcRγ has also been reported to mediate negative regulation [19–21]. We demonstrate here that both DAP12 and FcRγ are required for inhibition of immune responses in BMDC. Most studies of ITAM in these myeloid DC have focused on the activation role of these molecules [22–27], though there have been some reports of inhibitory function [28]. Our results reveal different requirements of ITAM-containing adapters for negative regulation in macrophages and DC. The inhibitory effect of Syk on TLR signaling is similar in macrophages and DC, consistent with the fact that both DAP12 and FcRγ use this kinase for downstream intracellular signaling. We also show that FcRγ most likely plays a more important role than DAP12 in ITAM-mediated regulation of TLR responses in these DC because FcRγ-deficient DC also showed increased TLR responses compared with WT DC, whereas DAP12-deficient DC were more similar to WT DC in their

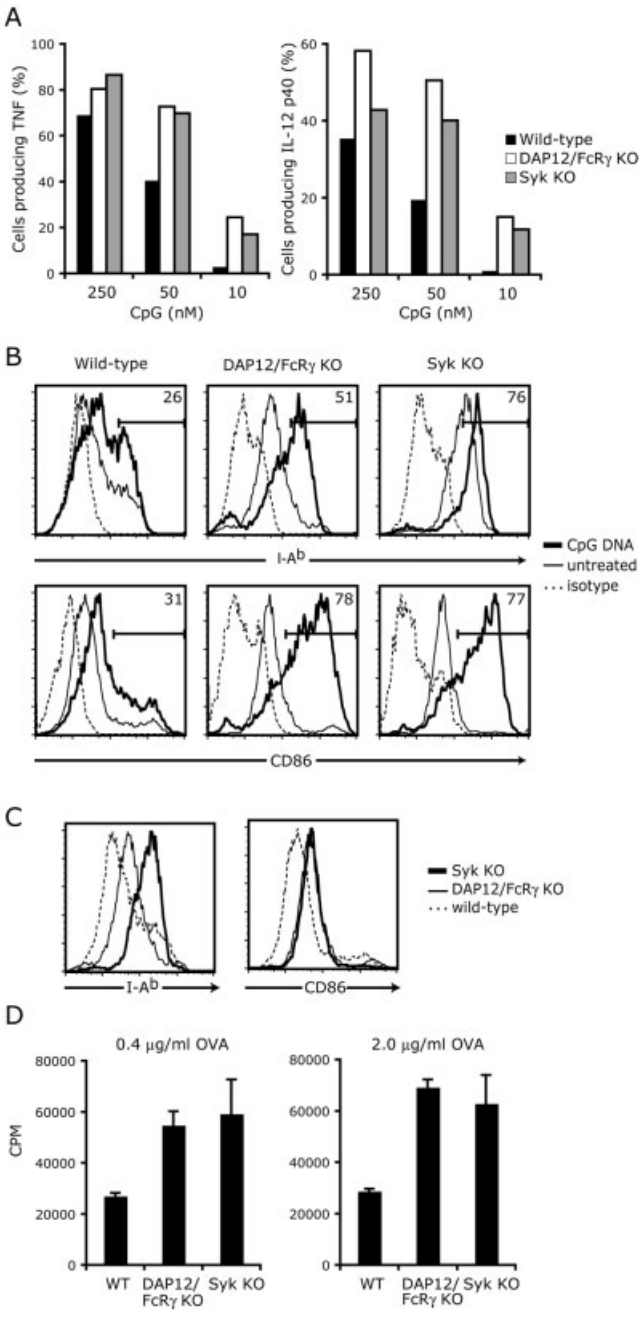

Figure 3. Enhanced cytokine production, maturation and specific antigen presentation of DAP12$^{-/-}$FcRγ$^{-/-}$ and Syk$^{-/-}$ DC after TLR9 stimulation. (A) BMDC from WT mice, DAP12$^{-/-}$FcRγ$^{-/-}$ mice, and Syk$^{-/-}$ chimeras were incubated with the indicated concentrations of CpG DNA for 4 h in the presence of Brefeldin A. The percent of CD11c^{+} TNF- and IL-12 p40- producing cells was determined by flow cytometry. Data are representative of four independent experiments. (B) BMDC from WT mice, DAP12$^{-/-}$FcRγ$^{-/-}$mice, and Syk$^{-/-}$ chimeras were incubated with CpG DNA (100 nM) for 16 h. DC maturation was measured as in Fig. 1. Thick lines represent CpG DNA-treated cells, thin lines represent untreated cells and dotted lines represent staining with isotype control antibody. (C) MHC class II and CD86 levels on unstimulated CD11c^{+} BMDC. Thick lines represent Syk$^{-/-}$ cells, thin lines represent DAP12$^{-/-}$FcRγ$^{-/-}$cells and dotted lines represent wild-type cells. (D) CD4^{+} T cells were isolated from OT II TCR- transgenic mice and cocultured with CpG DNA (35 nM)-activated DC in the presence of the indicated concentrations of OVA peptide for 72 h. T cell proliferation was determined by [^{3}H]thymidine incorporation. Error bars indicate SD of triplicate samples. Data shown (B, C and D) are representative of three independent experiments.

responses, though this is somewhat dependent upon which TLR is being ligated and the particular response measured. However, both molecules have some redundant function in this inhibitory regulation because the DAP12$^{-/-}$FcRγ$^{-/-}$ cells showed the most dramatic hyperresponsive phenotype.

How do these ITAM-containing adapters mediate the inhibition of TLR responses in DC? The requirement for both DAP12 and FcRγ for maximal inhibition of DC TLR responses suggests that either one receptor can pair with both DAP12 and FcRγ [29] to send inhibitory signals or that there is at least one DAP12-associated receptor and one FcRγ-associated receptor that are together required for inhibition. The DAP12-associated receptors expressed in myeloid cells include the TREM family receptors, MDL-1, the CD200 family receptors, the CD300 family receptors, PILRβ, and the SIRPβ receptors; the FcRγ-associated receptors on myeloid cells include the FcR for IgG and IgA, PIRα, Dectin-2, DCAR, and Oscar (summarized in [27]). We have shown that TREM-2 is involved in the inhibition of TLR and FcR-induced responses in mouse macrophages [9]. TREM-2 associates with DAP12, but not FcRγ [30], and in mice, TREM-2 is expressed on some macrophage populations, but not on DC [10]. The mechanism whereby DAP12-associated TREM-2 inhibits macrophage activation has not been defined. The suppression mediated by the FcRγ-associated IgA receptor on myeloid cells, FcαRI, has been attributed to recruitment of SHP-1 to the receptor complex when monomeric IgA is bound to the IgA receptor [19]. Further studies are needed to identify which DAP12 and FcRγ-associated receptors are required for the inhibition of DC activation and the mechanism of action.

Concluding remarks

In conclusion, we show here that both DAP12 and FcRγ are required for the inhibitory effect of ITAM signaling in conventional DC after TLR stimulation. TLR signaling is a key regulator of DC function in immune responses. TLR agonists are being developed for the treatment of cancer, allergies and viral infections, and as adjuvants for potent new vaccines to prevent or treat cancer and infectious diseases [31–34]. Thus, preferential blocking of the ITAM-mediated inhibition of TLR signaling

mediated through specific DAP12 or FcRγ-associated receptors might enhance immunity and potentially could be applied to vaccine development and DC-based cancer therapy.

Materials and methods

Mice and DC cultures

BMDC were prepared as described [35] from C57BL/6 (Charles River Laboratories, Wilmington, MA or National Laboratory Animal Center, Taipei, Taiwan), DAP12$^{-/-}$, FcRγ$^{-/-}$, and DAP12$^{-/-}$FcRγ$^{-/-}$mice [36–38]. As Syk$^{-/-}$ mice are embryonic lethal, we generated BMDC from Syk$^{-/-}$ fetal liver chimeras [39]. All mice were housed in the barrier facility in UCSF, NHRI (Taiwan) or the Benaroya Research Institute under an Institutional Animal Care and Use Committee-approved protocol.

DC maturation and cytokine production

For DC maturation, 1 ×x 10^6 DC (at day 6 of culture) were cultured in 24-well plates and treated with LPS, zymosan (Sigma-Aldrich, St. Louis, MO), CpG DNA (ODN1826), flagellin, or poly (I:C) (InvivoGen, San Diego, CA) at the indicated concentration. After 16 h, the cells were blocked with anti-CD16 and CD32 mAb 2.4G2, stained with mAb against CD11c, CD40, CD80, CD86, and I-A^b (BD PharMingen, San Diego, CA), and analyzed by flow cytometry. For intracellular cytokine production, 1 × 10^6 DC (at day 7 of culture) were cultured in 24-well plates and treated with LPS, CpG DNA, or zymosan for 4–6 h and 10 μg/mL Brefeldin A (BD PharMingen) was included in last 4 h. Cells were then blocked with 2.4G2 mAb, stained with anti-CD11c, fixed, permeabilized, and then stained with mAb to TNF, IL-6, and IL-12 p40 (BD PharMingen), and analyzed by flow cytometry. For cytokine production by ELISA, 1 × 10^6 DC (at day 6 of culture) were cultured in 24-well plates and treated with LPS, CpG DNA, or zymosan for 4 (for TNF) or 16 (for IL-6 and IL-12 p70) hours. An ELISA kit (R&D systems, Minneapolis, MN) was used to determine the cytokine production.

Antigen-presentation assay

Antigen presentation by DC was determined by using an OVA-specific T cell proliferation assay, as described previously [35]. Briefly, DC were purified by using an EasySep Positive Selection Kit (StemCell Technology, Vancouver, Canada), seeded in 96-well flat-bottom plates (Costar Corning, Cambridge, MA) with CpG DNA and $OVA_{323–339}$ peptide (a gift from Dr. Lawrence Fong, UCSF, CA), and incubated for 3 h. $CD4^+$ T cells were isolated from OT II TCR transgenic mice with an EasySep Positive Selection Kit (StemCell Technology) and added to BMDC cultures. Cells were incubated for 72 h, [^{3}H]thymidine (1 μCi/well) was added during the last 16 h of culture, and the incorporation was measured by scintillation counting.

Statistics

Significance of maturation or cytokine production of each source of DC in comparison with WT DC was determined by using a Student's *t*-test with two-sample equal variance with a two-tailed distribution.

Acknowledgements: We thank Yongmei Hu (UCSF, CA) for technical contributions. This work was supported by NSC grant NSC952320B400007 and NHRI grant VC095PP06 of Taiwan (C.L.C.), NIH grant AI068150 (C.A.L.), NIH grant AI068129 (L.L.L.), NIH grant AI073441 (J.A.H.), an Arthritis Foundation Investigator Award and Cancer Research Institute Investigator Award (J.A.H). L.L.L. is an American Cancer Society Research Professor.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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