Fc receptor γ-chain, a constitutive component of the interleukin 3 receptor, is required for interleukin 3-induced interleukin 4 production in basophils

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ABSTRACT

The Fc receptor common γ-chain (FcRγ) is a widely expressed adaptor bearing an immunoreceptor tyrosine-based activation motif (ITAM) that transduces activation signals from various immunoreceptors. We found here that basophils lacking FcRγ developed normally and proliferated efficiently in response to interleukin 3 (IL-3), but were severely impaired in IL-3-induced IL-4 production and in supporting T helper 2 cell differentiation. Through the transmembrane portion, FcRγ associated constitutively with the common β-chain of the IL-3 receptor and signaled via recruiting the kinase Syk. Retrovirus-mediated complementation revealed the essential role for the ITAM of FcRγ in IL-3 signal transduction. These results uncover a novel mechanism for FcRγ function to 'route' selective cytokine-triggered signals into the ITAM-mediated IL-4 production pathway.

INTRODUCTION

Cytokines are the molecules critical for numerous biological processes including immune responses. It is of note that not only multiple cytokines often induce a common biological activity (redundancy) but also a single cytokine can be responsible for multiple cellular responses (pleiotropy)[1-4]. A typical example of such unique nature of the cytokine signaling can be seen in the IL-3–IL-5–granulocyte macrophage colony stimulating factor (GM-CSF) system, in which the signal transducing β subunit, called common β (βc) (or Csf2rb; A001261), was shared by the receptors for those cytokines and provided the molecular basis for the functional redundancy of these cytokines[5, 6]. On the other hand, IL-5 could expand eosinophils, support generation of B1 cells and augment IgA class switching, while GM-CSF played roles in functional maturation of NKT cells as well as in proliferation and differentiation of macrophages, granulocytes and dendritic cells (DCs)[7, 8]. Likewise, IL-3 expands a variety of hematopoietic progenitor cells, activates phagocytosis and cytotoxicity in eosinophils and induces IL-4 and IL-6 production by basophils[9, 10]. Such a pleiotropy of the actions of those 'βc cytokines' has been considered to rely on the molecules that route the signals initiated at the receptors into multiple 'downstream' pathways.

The signal transduction pathways 'downstream' of the IL-3 receptor (IL-3R) have been clarified mainly for functions such as hematopoietic progenitor cell proliferation and survival, through studies using cytokine-dependent cell lines; for other effector functions in mature cells, the pathways remain largely uncharacterized[11]. Given that several signal transducing molecules associated directly and indirectly with distinct regions of βc, βc appeared to serve as a platform from which 'downstream' signaling pathways diverged[6]. The best established intracellular signaling pathway for βc signals is the one initiated by activating Jak protein kinases followed by tyrosine phosphorylation and activation of the transcription factors STAT5s that induce transcription of a variety of cytokine-inducible genes[11]. It is also known that βc cytokines activate various other signaling molecules including the kinase Syk (A000040)[12-14]. Syk and its related kinase Zap70 (A002396) were, on the other hand, well known to be critical for signal transduction by various immunoreceptors, such as lymphocyte antigen receptors and Fc receptors for IgE (FcεRI) and IgG (FcγRs)[15]. Those immunoreceptors indirectly recruit Syk and Zap70 via transmembrane adaptor molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs) such as CD3ζ, Ig-α, Ig-β, Fc receptor common γ-chain (FcRγ) and DAP12[16]. Upon phosphorylation of their tyrosine residues, ITAMs serve as the docking sites for the Src homology 2 domains of these kinases. However, βc does not have canonical ITAMs and hence it is not fully clear how βc recruits Syk[12, 17]. It also remains elusive which of many βc cytokine-inducible events are mediated by Syk[14].

Recently, the importance of basophils as a regulator of T helper type 2 (T_H2) cell responses has become increasingly recognized, based on the observations that these cells produce IL-4 in response to various stimuli including IL-3, parasite constituents, dietary allergens as well as crosslinkage of FcεRI[18]. We and others have shown indeed

that basophils could play indispensable roles in the generation of T_H2 cells in an IL-3-dependent manner *in vitro*, and acceleration of T_H2 differentiation resulted upon expansion of basophils *in vivo* by continuous exposure to IL-3[19] or in the absence of the transcription factor interferon regulatory factor-2 (IRF-2)[20]. For initiation of T_H2 responses, IL-3 is of particular interests among those molecularly heterogeneous basophils stimulants because this cytokine can directly induce IL-4 production in resting basophils[20], while other stimuli, such as FcεRI crosslinkage and the allergen protease papain, seemingly require prior activation of basophils for IL-4 induction[21-23]. As mentioned above, however, information is severely limited on the IL-3-triggered signaling pathway for IL-4 production, an important effector function of basophils.

We found here that FcRγ was a constitutive component of the IL-3R, and that it played an essential role, through its ITAM, in IL-3-induced IL-4 production and in supporting T_H2 differentiation by basophils. The involvement of FcRγ in IL-4 production appeared to be selective, as another function of IL-3, in promoting basophil proliferation, was not affected by FcRγ deficiency. We further found that the mechanism for the association of FcRγ with βc via their transmembrane portions was distinct from those between FcRγ and other immunoreceptors, including FcεRI and FcαRI. The current study identifies a hitherto undescribed function of FcRγ to 'channel' selective IL-3 signals into the ITAM-dependent pathway leading to IL-4 production. This represents an additional mechanism underlying the pleiotropy of the βc-dependent cytokine IL-3, and further widens the spectrum of biological events in which ITAM-bearing adaptors are involved.

RESULTS

Basophils lacking FcRγ develop normally

Although murine basophils were defined usually as FcεRI^{+}c-kit^{-} cells in bone marrow (BM) and spleen, basophils in mice lacking FcRγ (*Fcer1g*$^{-/-}$ mice)[24] did not express surface FcεRI and could not be identified in that way. However, additional markers such as IL-3Rα (CD123) and DX5 (CD49b)[19, 25, 26] made us able to unequivocally identify basophils even in *Fcer1g*$^{-/-}$ mice (**Fig. 1a**). Thus, in control mice, basophils (IL-3Rα^{+}DX5^{+}) expressed FcεRI but not c-kit, while FcεRI was absent on FcRγ-deficient basophils as expected (**Fig. 1a**). Basophils were present in BM and spleen in *Fcer1g*$^{-/-}$ mice at equivalent frequencies (**Fig. 1b**), morphologically indistinguishable (**Fig. 1c**) and expressing a more or less similar array of surface molecules in comparison to those in control mice (**Supplementary Fig. 1** online). Furthermore, infection by the nematode *Trichinella spiralis* induced basophil expansion in spleen and BM equally in wild-type and *Fcer1g*$^{-/-}$ mice (**Fig. 1d**). These observations together indicated that FcRγ was not required for the development, maturation, expansion and migration of basophils *in vivo*. In addition, FcRγ-deficient basophils proliferated as efficiently as control basophils in response to IL-3 *in vitro* (**Fig. 1e**). The amounts of cell surface expressed IL-3Rα and βc were equivalent in wild-type and FcRγ-deficient basophils (**Fig. 1f**), and STAT5 phosphorylation, an immediate event induced by IL-3, was also unaffected by FcRγ-deficiency (**Fig. 1g**). These results indicated that FcRγ was dispensable at least for IL-3R expression and 'downstream' signals for proliferation in basophils.

IL-3-induced IL-4 production in FcRγ-deficient basophils

Despite the normal proliferation of FcRγ-deficient basophils, we found that basophil-enriched BM cells prepared from *Fcer1g*$^{-/-}$ mice failed to produce IL-4 when stimulated *in vitro* with IL-3, under the condition where substantial amounts of IL-4 could be produced by those from control mice (**Fig. 2a**); in contrast, the amounts of IL-4 produced in response to ionomycin were comparable to those by control (**Fig. 2b**). BM basophils enriched from RAG1-deficient (*Rag1*$^{-/-}$) mice, lacking both T and B cells, could produce as much IL-4 as did wild-type BM cells (**Fig. 2a**), and IL-4 was present mainly in DX5^{+} cells within IL-3-stimulated BM cells from control but not *Fcer1g*$^{-/-}$ mice (**Fig. 2c**), excluding the involvement of other contaminating cells in IL-4 production by basophil-enriched BM cells.

The failure to produce IL-4 by FcRγ-deficient basophils might be due to their lack of surface FcεRI and FcγRs, which could possibly induce IL-4 production cooperatively with IL-3 signals. We therefore examined IL-4 production by spleen cells from *Rag1*$^{-/-}$ mice born to *Rag1*$^{-/-}$ mothers; basophils in these preparations produced substantial amounts of IL-4 even when cultured in medium containing Ig-deficient serum from *Rag1*$^{-/-}$ mice (**Fig. 2d**). In addition, basophils derived from *Stat6*$^{-/-}$ mice, which lacked serum IgE[27], also produced IL-4 in response to IL-3 (**Supplementary Fig. 2** online). Thus, FcRγ-deficient basophils were defective in IL-3-induced IL-4 production independently of the lack of signals through FcεRI or FcγRs. Involvement of

autocrine and/or paracrine actions of other cytokines induced by IL-3 was also unlikely because IL-4 mRNA could be induced normally by IL-3 even in the absence of *de novo* protein synthesis (**Fig. 2e**).

When cultured *in vitro* in the presence of IL-3, basophils expanded from BM cells[28], and could be purified by removing c-kit$^+$ mast cells (**Supplementary Fig. 3a** online). We found that wild-type BM-derived basophils, prepared as above, produced substantial amounts of IL-4 in response to IL-3 when they were left in the absence of IL-3 ('starvation') for several hours (**Fig. 2f**); the cells could be stained with anti-FcεRIα but not anti-IgE (**Supplementary Fig. 3b** online), confirming the IgE-independence of IL-4 production by IL-3 stimulation. In contrast, similarly prepared basophils from *Fcer1g*$^{-/-}$ mice failed to produce IL-4 (**Fig. 2f**), even though they expressed IL-3Rα and βc normally (**Fig. 2g**). These results, together with the data shown in **Fig. 1**, indicated that IL-3 signals diverge into at least two distinct pathways in basophils; one for proliferation and one for IgE- and IgG-independent IL-4 production; only the latter pathway required FcRγ. We also observed that IL-3-induced production of IL-6, another basophil cytokine implicated in T_H2 responses[29], was impaired, if not completely abolished, in basophil-enriched BM cells from *Fcer1g*$^{-/-}$ mice (**Supplementary Fig. 4** on line), indicating that FcRγ was involved in the IL-3 signaling pathway leading to IL-6 as well as IL-4 production.

Importantly, basophil-enriched spleen cells from mice lacking another ITAM-containing adaptor DAP12 showed no impairment in IL-3-induced IL-4 (**Fig. 2h**). In addition, DAP12 appeared to be functionally expressed in FcRγ-deficient BM-derived basophils as IL-4 production was normally induced upon CD200R3 crosslinkage (**Fig. 2i**), which has been shown to require DAP12[30]. Thus, IL-3 signals leading to IL-4 production requires FcRγ specifically, and DAP12 could not functionally replace FcRγ.

FcRγ is a constitutive component of IL-3R

Notably, we found that FcRγ associated with βc, as demonstrated by co-immunoprecipitation of FcRγ with βc from 'starved' (incubated in the absence of IL-3, as above) BM-derived basophils prepared from wild-type but not *Fcer1g*$^{-/-}$ mice (**Fig. 3a**). Restimulation of 'starved' BM-derived basophils with IL-3 did not affect the amounts of FcRγ co-precipitated with βc, even though STAT5 phosphorylation was readily induced (**Fig. 3a**). These results indicated that FcRγ was a constitutive, ligand-independent component of the IL-3R in BM-derived basophils. We also observed that both endogenous and exogenously introduced FcRγ associated with βc in Y16, an IL-3–IL-5-dependent pro-B cell line[31] (**Supplementary Fig. 5** online), suggesting that the association was not confined in basophils.

We also observed that IL-3 stimulation of basophil-enriched BM cells induced Syk recruitment to FcRγ (**Fig. 3b**) and elevated the phosphorylation of Syk in a manner dependent on FcRγ (**Fig. 3c**). Furthermore, expression of a dominant negative Syk mutant (DN-Syk, **Supplementary Fig. 6a,b** online) or short hairpin RNA for Syk

(Syk-shRNA, **Supplementary Fig. 6c,d** online) abolished IL-3-induced IL-4 production by BM-derived basophils (**Fig. 3d**,**e**) without affecting surface expression of either IL-3R or FcεRI (**Supplementary Fig. 6e** online), as did the Syk inhibitor Piceatannol (**Supplementary Fig. 7** online). In addition, IL-3-induced activation of mitogen-activate protein kinases (MAPK), in particular Erk and Jnk, was reduced in the absence of FcRγ significantly (**Supplementary Fig. 8** online). These results indicated that the FcRγ-Syk pathway was functionally involved in IL-3-induced IL-4 production in basophils. It was notable, in addition, that Syk and MAPKs seemed to be phosphorylated to some extent even in unstimulated basophils, independently of FcRγ (see **Fig. 3c** and **Supplementary Fig. 8** online). Such 'background signals' might potentiate overall IL-4 production by basophils through as-yet-unknown signaling pathways than the IL-3-FcRγ pathway, since ionomycin-induced IL-4 production was reduced with Syk-shRNA and DN-Syk, albeit much less prominently compared with IL-3-induced IL-4 production (see **Fig. 3d,e**).

IL-4 production requires the FcRγ ITAM

In immunoreceptor signaling, Syk is recruited to the ITAM of FcRγ. In order to examine the role of FcRγ ITAM for IL-4 production in basophils, we constructed retroviral vectors for complementation of FcRγ-deficient BM-derived basophils with wild-type FcRγ and a mutant with the disrupted ITAM (FcRγ-ΔITAM, **Fig. 4a**). The ITAM of FcRγ is known to be dispensable for the transport of FcεRI to the cell surface in mast cells[32]. Consistently, when introduced retrovirally into FcRγ-deficient BM-derived basophils, both wild-type FcRγ and FcRγ-ΔITAM restored the expression of FcεRI on the cell surface to the similar levels, mirroring the comparable levels of wild-type and the mutant FcRγ (**Fig. 4b**). In contrast, wild-type FcRγ, but not FcRγ-ΔITAM, restored IL-3-induced IL-4 production by FcRγ-deficient BM-derived basophils (**Fig. 4c**), demonstrating the essential role of FcRγ-ITAM in IL-3 signals leading to IL-4 production in basophils. It was also apparent that FcRγ-deficient basophils were otherwise fully competent in the machinery required for IL-3 signal transduction for IL-4 production.

Structural requirements for FcRγ-βc association

Even though DAP12 was unable to replace FcRγ in IL-3 signaling (see **Fig. 2h,i**), the cytoplasmic portion of DAP12 could transduce IL-3 signals for IL-4 production when fused with FcRγ extracellular (EC) and transmembrane (TM) portions (**Supplementary Fig. 9** online), suggesting that the inability of DAP12 to replace FcRγ was due solely to the failure of its EC and TM portions to associate with βc. Given that the TM portion of FcRγ is important in association with immunoreceptors, it is tempting to consider that the same portion is also involved in the association with βc. We transduced FcRγ-deficient BM-derived basophils with an FcRγ mutant containing an alanine residue in place of leucine at position 21 within the TM portion (FcRγ-L21A, **Fig. 5a** and **Supplementary Fig. 10a** online), a mutant known to be unable to bind to FcεRIα or FcαRI[33]. BM-derived basophils expressing FcRγ-L21A indeed failed to express FcεRI on the surface even though FcRγ-L21A and wild-type FcRγ proteins were expressed at comparable levels (**Fig. 5b**). Notably, despite their inability to express

FcεRI on the cell surface, 'starved' BM-derived basophils harboring FcRγ-L21A produced IL-4 in response to IL-3 even more efficiently in comparison to those expressing wild-type FcRγ (**Fig. 5c**), indicating that the mode of the βc-FcRγ association was distinct from that between FcεRI and FcRγ. The relatively augmented IL-4 production by FcRγ-L21A-harboring BM-derived basophils might be due to the elevated availability of FcRγ-L21A molecules for associating with βc, as FcεRI would not be able to compete with βc for this mutant. This observation further supported the FcεRI independence of IL-3-induced IL-4 production by basophils. On the other hand, another mutant FcRγ (FcRγ-D11A, **Fig. 5a**) carrying an alanine residue at position 11 also within the TM, rescued neither surface FcεRI expression (**Fig. 5b**) nor IL-3-induced IL-4 production (**Fig. 5c**). Thus the aspartic acid residue 11 of the TM was essential for FcRγ not only to associate with FcεRI but also to transduce IL-3 signals in basophils.

In order to directly examine the physical association of wild-type and mutant FcRγs with βc, we retrovirally introduced Y16 cells with Flag-tagged wild-type and mutant FcRγs. Both wild-type FcRγ and FcRγ-ΔITAM could associate with βc in Y16 cells, as could FcRγ-L21A; however, FcRγ-D11A could not associate with βc (**Fig. 6**). While we found the FcRγ-D11A migrated slowly during SDS-PAGE, the protein appeared to be translated correctly, since both the FcRγ antibody and the Flag antibody—specific for the Flag tag appended at the C terminus—could bind it (**Fig. 6**). The above results indicated that for the association of FcRγ with βc, the ITAM and TM L21 were dispensable, while TM D11 was essential. The differential requirement of L21 indicated that physical and functional association between FcRγ and βc were distinct from those between FcRγ and FcεRI.

Fcer1g$^{-/-}$ basophils failed to support T_H2 generation

We observed that, in contrast to spleen cells prepared from wild-type mice transgenic for OT-II T cell receptor (OT-II TCR-tg mice), those from OT-II TCR-tg *Fcer1g*$^{-/-}$ mice could not differentiate into T_H2 cells under neutral conditions as indicated by the severely impaired IL-4 production by differentiated T_H cells (**Fig. 7a**). We further observed that OT-II TCR-tg CD4$^+$ T cells purified from *Fcer1g*$^{-/-}$ mice differentiated readily into T_H2 cells when cultured in the presence of T, B, NK and erythroid cell-depleted wild-type spleen cells, which we refer to as 'antigen presenting cells' (APCs), in which CD11c^{hi} (DCs) and IL-3Rα$^+$DX5$^+$ (basophils) occupied 10-12% and 3-4%, respectively (**Fig. 7b**), while conversely even wild-type OT-II TCR-tg CD4$^+$ T cells were unable to generate T_H2 cells when stimulated in the presence of similar APCs but from *Fcer1g*$^{-/-}$ mice (**Fig. 7c**). Notably, basophil-enriched cell preparations from wild-type but not *Fcer1g*$^{-/-}$ mice compensated the failure of OT-II TCR-tg FcRγ-deficient spleen cells to generate T_H2 cells, as did exogenously supplemented IL-4 (**Fig. 7d**). Although these basophil-enriched populations still contained unidentified cells other than DCs, basophils appeared to be responsible for the compensation, since depletion of DX5$^+$ cells made those preparations unable to restore T_H2 cell generation (**Fig. 7d, '**+ Control ΔBs', see also **Supplementary Fig. 11** online). These observations together indicated that FcRγ expression was required not in CD4$^+$ T cells and DCs but

in basophils for efficient T_H2 cell generation. We showed previously that T_H2 differentiation in this setting was completely dependent on IL-3 and IL-4[20]. Indeed, substantial amounts of IL-3 were produced in the *in vitro* cultures independently of basophils and irrespective of the *Fcer1g* genotype (**Fig. 7e**). Such IL-3 induced control basophils, but not FcRγ-deficient basophils, to produce 'early' IL-4 in those cultures, as IL-4 produced within the initial 2 days disappeared when basophils were depleted from wild-type cell cultures (**Fig. 7f**). Such 'early' IL-4 production was not seen in FcRγ-deficient cell cultures at all (**Fig. 7f**). Thus, the inability of FcRγ-deficient spleen cells to support T_H2 differentiation appeared to be due to the failure of basophils to produce 'early' IL-4 in response to endogenously produced IL-3.

These *in vitro* observations raised an intriguing possibility that *Fcer1g*$^{-/-}$ mice were defective in T_H2 responses. In agreement with this prediction, two reports have already shown that *Fcer1g*$^{-/-}$ mice failed to mount efficient T_H2 responses *in vivo*[34, 35]. We also observed less efficient production of serum IL-5, a T_H2 cytokine, in *Fcer1g*$^{-/-}$ mice than in control mice upon infection with the T_H2-indicing nematode *T. spiralis*[36] (**Supplementary Fig. 12a** online), yet basophil expansion in spleen and BM occurred normally (see **Fig. 1d**). Furthermore, $CD62L^{lo}CD4^+$ T cells expressing ST2, the receptor specifically expressed on T_H2 cells for the pro-T_H2 cytokine IL-33[37], were less frequent in mesenteric lymph nodes in *Fcer1g*$^{-/-}$ mice than in wild-type mice on day 10 of infection, and the levels of ST2 on $CD62L^{lo}CD4^+$ T cells were lower in *Fcer1g*$^{-/-}$ mice than in control (mean fluorescent intensity = 20.8 $\pm$ 5.3 vs. 11.5 $\pm$ 2.6; **Supplementary Fig. 12b** online). Although we could not detect serum IL-4 in both uninfected and infected mice (less than 15 pg/ml), these results were in accordance with the *in vitro* observations (see **Fig. 7a,f**), and suggested that *Fcer1g*$^{-/-}$ mice could not mount efficient T_H2 responses *in vivo* upon *T.spiralis* infection.

DISCUSSION

In the IL-3–IL-5–GM-CSF system, the βc component of the receptors for these cytokines constitutes the 'platform' where various non-receptor type kinases and adaptors are recruited and activated to initiate multiple 'downstream' signals, ensuring the functional pleiotropy of these cytokines. In this study, we found an unexpected role of the ITAM-bearing adaptor FcRγ in IgE-independent IL-3 signal transduction for IL-4 production but not proliferation or survival of basophils. With respect to the function of ITAM-bearing adaptors in non-immunoreceptor signaling, both DAP12 and FcRγ were reported recently to be critical in priming of macrophages by IFN-γ for enhanced IFN-α responses[38] and for the outside-in signaling through macrophage integrins[39]. In these cases, while Syk activation was essential, it remained to be determined if IFN-α receptors (IFNAR) or integrins associated with FcRγ or DAP12, and an alternative possibility could not be excluded that other unidentified FcRγ- and DAP12-associated receptors played roles in coupling IFNARs or integrins functionally to the ITAM-mediated pathways. In this regard, this study is the first to demonstrate the incorporation of FcRγ into non-immunoreceptors. While DAP12 and FcRγ were redundant in the enhancement of IFNAR and integrin signal transduction[38, 39], FcRγ but not DAP12 was specifically required for IL-3 signals leading to IL-4 production in basophils, underscoring the uniqueness of this specific FcRγ function in IL-3 signal transduction.

It has been well established that FcRγ associates with various cell surface receptors through the intramembrane interaction between the transmembrane portions on both partners. In a group of FcRγ-associated receptors including FcαRI (also termed CD89), NKp46, the platelet collagen receptor glycoprotein VI and the paired Ig-like receptor A, an arginine residue in their transmembrane regions is required for interaction with the negatively charged aspartic acid residue in FcRγ [40]. In contrast, receptors such as FcεRI, FcγRI and FcγRIII lack the canonical transmembrane arginine but are nevertheless able to associate with FcRγ through mechanisms still not fully understood[33]. This same arginine is also absent in the transmembrane region of βc. In addition, we showed here that leucine-21 in the transmembrane region of FcRγ, a residue required for both arginine-dependent and independent associations[33], is dispensable for physical and functional association of βc with FcRγ, suggesting that this association occurs via a mode distinct from those previously known for FcRγ.

In contrast to various immunoreceptors, IL-3Rs could be expressed normally even in the absence of FcRγ and functionally competent in signaling for other events such as proliferation in basophils. Participating in selective 'downstream' events evoked by IL-3, FcRγ functions something like an auxiliary signaling module that 'appends' additional outcomes of the IL-3R signaling pathway by 'channeling' IL-3 signals into the ITAM-Syk pathway. A similar auxiliary function of FcRγ was seen for FcαRI that could be expressed on the surface and mediate phagocytosis in neutrophils and macrophages, but did not support other functions such as superoxide production and bacterial killing in the absence of FcRγ[41, 42]. Integrins and IFNARs could also be

expressed independently of FcRγ and DAP12, and are competent in other functions even in the absence of these adaptors[38, 39]. Thus, the auxiliary function of FcRγ in IL-3 signal transduction is not peculiar to this cytokine system.

IL-3 and its related cytokines IL-5 and GM-CSF were shown previously to induce Syk recruitment to βc in a myeloid cell line and eosinophils[12, 17]. In eosinophils, Syk was considered to be recruited directly to βc possibly through ITAM-like YxxL motifs in the cytoplasmic region[12]. Although Syk could potentially be recruited directly to βc also in IL-3-stimulated basophils, independently of FcRγ, such recruitment and subsequent activation, if any, of Syk appears to be functionally irrelevant to IL-3 signal transduction for IL-4 production. In this regard, it is known that mice but not humans have an additional βc-like molecule termed $\beta_{IL\text{-}3}$ that can be a component of the receptors for IL-3 but not GM-CSF and IL-5[43]. A speculation would hence be that only $\beta_{IL\text{-}3}$ might contribute to the IL-3 signaling pathway leading to IL-4 production by recruiting Syk indirectly via FcRγ. However, since the structure of the TM portions was almost indistinguishable between βc and $\beta_{IL\text{-}3}$, having identical sequence but substitutions only at two positions, we do not consider it highly likely that only one of these two β subunits associated selectively with FcRγ. Further studies are needed to explore the precise molecular mechanism for FcRγ-βc association and to directly test these possibilities. Nevertheless, if both βc and $\beta_{IL\text{-}3}$ associated with FcRγ, some but perhaps not all cellular responses to the other βc cytokines IL-5 and GM-CSF in B cells, macrophages and DCs as well as basophils might also involve FcRγ.

Failure of FcRγ-deficient basophils to produce IL-4 in response to IL-3 resulted in the inability to support T_H2 differentiation *in vitro* and conceivably *in vivo* in mice infected with T_H2-inducing nematode *T. spiralis*, substantiating further the idea that basophils are the cells responsible for early IL-4 production leading to the initiation of T_H2 responses in allergy and helminth infection[18, 21, 44]. We also observed that IL-3-induced production of IL-6 was impaired in FcRγ-deficient basophils. Although basophil-derived IL-4 was sufficient for T_H2 generation in our *in vitro* culture system[20], FcRγ might contribute to T_H2 responses *in vivo* by mediating IL-3 signals for IL-6, in addition to IL-4, production by basophils. The role of FcRγ in T_H2 responses *in vivo* has also been reported earlier in mice where *Fcer1g*$^{-/-}$ mice showed inefficient T_H2 differentiation in lymph nodes upon infection with *Leishmania major*[34] and exhibited milder air way inflammation than control mice[35]. The involvement of basophils was not examined in these studies, and rather such impaired type 2 immune responses were originally attributed to the defect in DCs[34, 35].

Contrary to such conclusions, DCs lacking FcRγ did not show any defect in T_H2 induction, at least in our *in vitro* analyses. A possible explanation for this apparent discrepancy may be that the cell types required for efficient T_H2 responses were different according to the means to induce T_H2 responses, or alternatively, basophils and DCs functioned in different timing, for example at the initiation and propagation phases, respectively, even though both of them were important for efficient T_H2 responses *in vivo*. Because FcRγ is expressed so widely and can serve as a signal transducing adaptor

for various, functionally diverse receptors[39], multiple, but not a single, cell species involved in T_H2 responses might be affected by FcRγ deficiency under the *in vivo* situation. Furthermore, since basophils could also produce IL-4 in response to apparently FcRγ-independent stimulation via CD200R3, Toll-like receptor 2, IL-18 receptor and other as-yet-unidentified 'receptors' for the proteases from house dust mites or a parasite[28, 30, 45, 46], the defect in FcRγ signals alone in basophils might not necessarily result in severely impaired T_H2 responses. Nevertheless, indispensable in both IL-3R- and FcεRI-mediated IL-4 production in basophils, FcRγ may play more important roles in type 2 immune responses than considered before.

In conclusion, the crosstalk uncovered here between the signaling pathways mediated by ITAM and those 'downstream' of the receptor for a βc cytokine not only provided a new mechanism to account for the functional pleiotropy of some cytokines but it also extends further the functional spectrum of ITAM-bearing adaptors beyond immunoreceptor signaling.

METHODS

Mice. All mice used in this study were maintained in the animal facility in Shinshu University under strictly controlled specific pathogen-free conditions, with regular monitoring of infection with agents including *Pasturella pneumotropica*, ectoparasites, intestinal protozoa and pinworms, and used at 8-12 weeks of age. *Fcer1g*$^{-/-}$ mice on the C57BL/6 background were described previously[24]. *Stat6*$^{-/-}$[27], *Rag1*$^{-/-}$ or *Tyrobp*$^{-/-}$ (lacking DAP12)[47] mice were backcrossed at least 10 times with C57BL/6 mice. C57BL/6 mice were from SLC (Shizuoka, Japan). Transgenic mice expressing OT-II TCR for an ovalbumin peptide, described previously[20], were crossed with *Fcer1g*$^{-/-}$ mice to generate OT-II tg *Fcer1g*$^{-/-}$ mice. Control mice for *Stat6*$^{-/-}$, *Fcer1g*$^{-/-}$ and *Tyrobp*$^{-/-}$ mice were in most cases littermates heterozygous for the genes that gave results undistinguishable from those obtained using wild-type mice. All animal experiments were approved by the Committee for Animal Experimentation and Care of Shinshu University and conducted according to the guideline.

Antibodies. Antibodies to CD3 (145-2C11), CD28 (37.51), CD11b (M1/70), CD117 (c-kit, 2B8), CD123 (IL-3Rα, 5B11), CD49b (DX5), CD90.2 (53-2.1), F4/80 (BM8), FcεRIα (MAR-1), NKG2D (CX5), NK1.1 (PK136), TCRβ (H57-597), B220 (RA3-6B2), Sca-1 (D7), Gr-1 (RB6-8C5), TER119 (TER119) and Ly-6C (AL-21) as well as isotype-matched control antibodies, conjugated with biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC), were from e-Bioscience. Antibodies to CD4 (RM4-5), CD62L (MEL-14), CD11c (HL3), CD131 (βc, JORO50), IL-4 (BVD4-1D11) and IFN-γ (XMG1.2), conjugated as above, were from BD Biosciences. FITC-anti-ST2 (DJ8), biotin-anti-rat CD2 (rCD2, OX34), and Alexa647-anti-rabbit IgG were from MD Biosciences, Cedarlane and Molecular Probes, respectively. These antibodies were used for flowcytometry and/or cell separation. Ba91 anti-CD200R3 was described previously[30] and used at 1 μg/ml for stimulating BM-derived basophils. Polyclonal rabbit antibodies to Syk, βc–$\beta_{IL\text{-}3}$ (both from Santa Cruz Biotechnology) and FcRγ (recognizing a C-terminal epitope, Upstate Biotechnology) were used for detecting intracellular FcRγ, immunoprecipitation and blotting. Also used in blotting were monoclonal antibodies to STAT5 (clone 89) and phosohorylated STAT5 (clone 47) (BD Biosciences), and anti-Flag (M2, Sigma-Aldrich). Monoclonal antibodies to native and phosphorylated forms of p38 (clones 27 and 30) and Erk (MK12 and 20A) were obtained from BD Bioscience, and polyclonal anti-Jnk was from Cell Signaling Technology.

Flowcytometry. After red blood cells were lysed with lysis buffer, cells were incubated with biotin-, FITC-, PE- or APC- conjugated antibodies. Fc-mediated non-specific staining was blocked with anti-CD16/32 (2.4G2 hybridoma culture supernatant). Biotin-conjugated antibodies were developed using PE-Cy7-streptavidin (BD Biosciences). Analyses of stained cells were carried out on the Cytomics FC500 flowcytometer (Beckman-Coulter) with the RXP software (Beckman-Coulter). FcRγ was stained in cells fixed and permiabilized using Cytofix-Cytoperm Plus (BD Biosciences) with rabbit anti-FcRγ antibody followed by development with Alexa647-anti-rabbit IgG antibody. Intracellular staining for phosphorylated STAT5

were carried out on basophil-enriched BM cells (see below) stimulated with IL-3 (5 ng/ml) for 15 minutes using Alexa 647-conjugated antibodies purchased from BD Biosciences according to the protocol provided by the supplier with slight modification that fixed cells were treated with 90% methanol only for 20 minutes.

Basophil preparation and culture. Enrichment of BM and splenic basophils was carried out as described[20] through depletion of T, B, NK, NKT, mast and erythroid cells, DCs and granulocytes using antibodies against TCRβ, B220, NKG2D, Sca-1, Gr-1, TER119, CD11c, c-kit and Ly-6C with IMag beads (BD Biosciences). BM-derived basophils were obtained as described[28] with slight modification, followed by enrichment of c-kit$^-$ cells and 'starvation' (see **Supplementary Methods** online for detail). The purities of fresh spleen, BM and 'starved' BM-derived basophils subjected to stimulation were described in figure legends for each experiment. For examination of morphology, basophils were enriched from BM as above and cells positive for DX5 were further purified by sorting with AutoMACS (Myltenyi Biotech). Cytospins prepared with these sorted cells were stained with Wright's stain.

Stimulation of basophils for IL-4 production. Fresh basophils or 'starved' BM-derived basophils (1 x 10^5 cells) were stimulated with IL-3 (5 or 20 ng/ml) for 24 hours, and the supernatants were collected to measure IL-4 proteins using Mouse IL-4 ELISA kit (e-Bioscience). Chemical inhibitor of Syk (Piceatannol) purchased from Calbiochem was added at concentrations indicated in **Supplementary Fig. 7** online. The calcium ionophore ionomycin was from WAKO chemicals (Japan) and used at 0.1 or 0.5 μg/ml. Intracellular IL-4 was revealed by flowcytometry in basophils stimulated with IL-3 for 18 hours with the last 6 hours treated with GolgiStop (BD Biosciences). Cells were surface-stained, fixed, permeabilized and stained with PE-conjugated anti-IL-4 antibody (BD Biosciences). Total RNA was prepared from basophils stimulated with IL-3 (5ng/ml) for 6 hours, and converted to cDNA with ImProm-II Reverse Transcription System (Promega). Quantitative PCR analysis was conducted on the Thermal Cycler Dice Real-Time System (Takara Bio) using a SYBR Premix Ex Taq kit (Takara Bio) according to the manufacturer's instruction. The amount of IL-4 mRNA was calculated in each sample according to that of β-actin determined in parallel amplification. For the sequence of PCR primers used, see **Supplementary Methods** online.

DNA construction. The cDNA for DN-Syk mutant lacking the kinase domain was constructed by amplifying a part of murine Syk cDNA (**Supplementary Fig. 6a** online), corresponding to amino acid position 1-260 (1-261 in human Syk[48]), and inserted into pMX-IRES-rCD2 vector (modified from the original pMX-IRES-GFP vector provided by Prof. Toshio Kitamura). A sequence in Syk (the 'target sequence' in **Supplementary Fig. 6c** online) and a control sequence (5'-TCTTAATCGCGTATAAGGC-3') were used as target sequences together with their respective antisense sequences to construct the retrovirus vectors expressing shRNA based on pSINsi-mU6 that contained a neomycin-resistant cassette (TAKARA Bio). The cDNAs encoding wild-type and ITAM mutant FcRγ (FcRγ-ΔITAM) were previously described[32]. *Spe*I-*Not*I-excised cDNA fragments expressing full-length or ΔITAM FcRγ were inserted into pMX-IRES-rCD2

vector. FcRγ-L21A and FcRγ-D11A mutants carrying point mutations in the transmembrane portion were created by PCR and introduced into the same vector. cDNAs expressing wild-type FcRγ and mutant FcRγs (ΔITAM, L21A, D11A) tagged with Flag at their C termini were generated by PCR; these were cloned also into pMX-IRES-rCD2 vector. For the construction of vectors for FcRγ-DAP12 fusion protein, see **Supplementary Methods**.

Retroviral infection. Retrovirus vectors created as above were used for transduction into BM-derived basophils and Y16 pro B cell line[31]. These retroviral constructs were transiently transfected into the packaging cell line Phoenix using FuGene-6 reagent (Roche Diagnostics). Retrovirus-containing supernatants were collected 48 h after transfection, concentrated 10-fold by centrifugation and added to 12-well plates, which were processed by sequential treatment with RetroNectin solution (50 μg/ml, Takara Bio) in PBS for 2 h at room temperature followed by 2% bovine serum albumin in PBS for 30 minutes. After incubating the plates with the virus supernatants for 4 h at 30°C, BM-derived basophils (0.5 or 1 x 10^6 cells/ml) were added and infected for 2 days. The BM-derived basophils used for infection were prepared by culturing only for 10 days, instead of 13 days, and enriched for c-kit$^-$ cells. Infected BM-derived basophils were starved for 12 to 18 hours, and rCD2$^+$ cells were enriched with IMag beads and AutoMACS, and used for stimulation. Y16 cell line was infected similarly with the retrovirus vectors carrying wild-type FcRγ, C-terminal Flag-tagged FcRγ or its mutants, and cells stably expressing rCD2 were selected using IMag beads and AutoMACS. For infection with Syk shRNA-expressing retrovirus and selection of infected cells, see **Supplementary Methods** online.

Immunoprecipitation and immunoblotting. BM-derived basophils were 'starved' in the absence of IL-3 for 12-18 hours followed by stimulation with IL-3 (20 ng/ml) for 10 minutes. Freshly isolated BM basophils were enriched as above and stimulated with IL-3 (5 ng/ml) for 2, 5 or 10 minutes. These cells and Y16 were lysed with lysis buffer containing 10 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% (volume/volume) Nonidet P-40, 0.5 mM EDTA, 10 mM NaF, 1mM sodium orthovanadate and protease inhibitor cocktail (Roche Diagnostics). Immunoprecipitation was carried out by incubating these cell lysates with antibodies (2 μg) and protein G–Sepharose (GE Healthcare) at 4°C for 2 hours. These immunoprecipitates or whole cell lysates were boiled in sample buffer, separated by SDS-PAGE and electrophoretically transferred to PVDF membranes, followed by immunoblotting with various antibodies. Blots were developed with Immobilon Western reagent (Millipore) and analyzed using CoolSaver (Atto, Japan).

T_H1/T_H2 differentiation *in vitro*. T_H1 and T_H2 differentiation were induced *in vitro* using OT-II TCR tg spleen cells as described[20]. Briefly, red blood cell-lysed spleen cells ($1x10^6$) were depleted of TER119$^+$, NKG2D$^+$ and B220$^+$ cells and cultured with 0.5 μM chicken ovalbumin peptide (residues 323-339). The starting spleen cell preparations contained 2-3% CD11c^{high} cells (DCs) and 0.8-1.0% DX5$^+$IL-3Rα$^+$ cells (basophils). Detailed methods used for culturing CD4$^+$ T cells and APCs and supplementation of basophils are described in **Supplementary Methods** online. T cells recovered on day 5 from cultures established as above were restimulated with plate-bound anti-CD3 (10

μg/ml) and anti-CD28 (1 μg/ml) for 5 hours, and stained with PE-anti-IL-4 and FITC-anti-IFN-γ (BD Biosciences) using the Cytoperm-Cytofix kit Plus (BD Biosciences) according to the instruction by the supplier. To detect IL-3 and 'early' IL-4 production, culture supernatants were collected from the primary cultures on day 2, and IL-3 and IL-4 concentrations were determined using OptEIA kits (BD Biosciences) and Mouse ELISA kits (e-Bioscience), respectively.

***T. spiralis* infection.** *T. spiralis* larvae were isolated from the skeletal muscles of orally infected mice by digestion with pepsin and used for inoculation (300 larvae/mouse) as described[49]. Ten or eleven days later, mice were sacrificed, and cells were obtained from spleen, BM and mesenteric lymph nodes for enumerating the numbers of basophils and for examining ST2 and CD62L expression on $CD4^+$ cells by flowcytometry. The amounts of IL-5 in sera were also measured using OptEIA kits (BD Biosciences).

Statistical analysis. Statistical significance was calculated with Mann-Whitney U test.

Accession Code. UCSD-Nature Signaling Gateway http://www.signaling-gateway.org: A001261, A000040, A002396

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AUTHOR CONTRIBUTION
S.H. designed and did experiments and wrote the manuscript; S.Y. helped vector construction and provided critical reagents, Y.S. did experiments; T.K., H.K., T.T. and T.S. provided critical reagents; M.T. and K.S. did the *T. spiralis* infection experiments; and S.T. designed and supervised research and wrote the manuscript.

The authors declare no financial conflict.

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FIGURE LEGENDS

Figure 1. Normal development and proliferative responses to IL-3 of basophils in *Fcer1g*$^{-/-}$ mice. (**a**) Flowcytometry wild-type (Control) and *Fcer1g*$^{-/-}$ (KO) spleen and BM cells for expression of IL-3 receptor α-chain (IL-3Rα) and DX5 (top). Numbers in dot plots indicate the percentages of IL-3Rα^{+}DX5^{+} basophils, which express FcεRIα but not c-kit (below). Open histograms represent isotype controls. (**b**) Absolute numbers of basophils (IL-3Rα^{+}DX5^{+}) within spleen and BM in control and *Fcer1g*$^{-/-}$ (KO) mice. Each symbol represents an individual animal (n=5 for each genotype). (**c**) Cytospin morphology of sorted basophils from BM. (**d**) Percent basophils in spleen and BM in uninfected (–) or *T. spiralis*-infected (Ts) mice. Data represent the means and s.d. of three animals per each group. *$P < 0.05$. (**e**) Numbers of IL-3Rα^{+}c-kit^{-} cells recovered from BM cultures supplemented with IL-3. Data are the means and s.d of duplicate cultures. (**f**) Surface expression of IL-3Rα and βc on basophils freshly prepared from BM (DX5^{+}c-kit^{-}). Open histograms represent isotype controls. (**g**) Flowcytometry for intracellular expression of phospho-STAT5 in basophils (DX5^{+}c-kit^{-}NK1.1^{-}) from BM cells stimulated with IL-3 (filled histograms) or left untreated (open histograms). Data are representative of more than four (**a**), two (**c**,**g**), or three (**d**-**f**) independent examinations.

Figure 2. Impaired IgE-independent, IL-3-induced IL-4 production by FcRγ-deficient basophils. (**a,b**) ELISA for IL-4 production by *Rag1*$^{-/-}$ (RAG-KO) or *Fcer1g*$^{-/-}$ (FcRγ-KO) basophil-enriched BM cells (19, 21 and 17% basophils, respectively) stimulated with the indicated amounts of IL-3 (**a**) or in the absence (–) or presence (+) of 0.5 μg/ml ionomycin (**b**) for 24 hours. (**c**) Flowcytometry for intracellular expression of IL-4 in basophil-enriched BM cells (c-kit^{-}) stimulated with IL-3 (5 ng/ml) for 18 hours. (**d**) ELISA for IL-4 production by RAG1-deficient spleen cells (0.7% basophils) upon stimulation with IL-3 in media supplemented with either fetal bovine serum or serum from *Rag1*$^{-/-}$ mice for 24 hours. (**e**) RT-PCR for IL-4 mRNA in basophil-enriched BM cells stimulated with IL-3 for 6 hours in the presence or absence of cycloheximide. Data represent the means and s.d. of two independent cultures. (**f**) ELISA for IL-4 production by wild-type (control) and *Fcer1g*$^{-/-}$ 'starved' and enriched BM-derived basophils stimulated with IL-3. (**g**) Flowcytometry for IL-3Rα and βc expression on the indicated 'starved' c-kit^{-} BM-derived basophils (shaded histograms); isotype control (open histograms). (**h**) ELISA for IL-4 production from control (0.7% basophils) and DAP12-KO (1.3% basophils) spleen cells stimulated with IL-3 for 24 hours. (**i**) ELISA for IL-4 production by unstarved wild-type and FcRγ-deficient (FcRγ-KO) BM-derived basophils stimulated with CD200R3 for 24 hours. Columns and error bars indicate the means and s.d., respectively, of duplicate cultures in **a**, **b**, **d, f, h** and **i**. Representative of two (**c,d,h**), three (**b,e,g, i**), or more than four (**a**,**f**) individual trials.

Figure 3. Constitutive and functional association of FcRγ with βc in basophils. (**a**) Immunoblot on lysates of BM-derived basophils from wild-type (control) or *Fcer1g*$^{-/-}$ (KO) mice 'starved' and then stimulated with IL-3 (+) or left untreated (–) for 10 minutes followed by immunoprecipitation (IP) with anti-βc and blot (IB) with anti-FcRγ or with anti-βc. Whole cell lysates (WCL) from the same cells were analyzed with antibodies indicated. (**b**) Immunoblot on lysates of basophils enriched from wild-type

BM (26% basophils) left untreated (–) or stimulated with IL-3 (+) for 5 minutes followed by immunoprecipitation (IP) with anti-FcRγ and immunoblot (IB) with either anti-Syk or anti-FcRγ. (**c**) Immunoblot with anti-phospho-Syk (p-Syk) or anti-Syk of cell lysates from control or FcRγ-deficient basophil-enriched BM cells (48% and 45% basophils, respectively) treated as in **b**. (**d**) IL-4 production by BM-derived basophils transduced with the vector for control (C) or a dominant negative Syk mutant (D), and enriched (80% and 76% $rCD2^+$c-$kit^-Fc\varepsilon RI\alpha^+$, respectively) upon stimulation with IL-3 (20 ng/ml) or ionomycin (0.1 μg/ml, Ion). (**e**) ELISA for IL-4 production by BM-derived basophils expressing control (C) or Syk shRNA (sh) (70% and 50% $rCD2^+$c-$kit^-Fc\varepsilon RI\alpha^+$, respectively) stimulated as in **d**. Data represent the means and s.d. of duplicate cultures. Repeated thrice (**a**,**d**) or twice (**b**,**c**,**e**) with similar results.

Figure 4. Essential role for FcRγ ITAM in IL-3-induced IL-4 production. (**a**) Wild-type (WT) and mutant FcRγ (ΔITAM) with two tyrosine (Y) residues in the ITAM replaced with phenylalanine (F) inserted into the retroviral vectors. (**b**) Flowcytometry for cell surface expression of FcεRIα and intracellular expression of FcRγ (cFcRγ) by FcRγ-deficient BM-derived basophils transduced with retrovirus vectors ($rCD2^+$c-kit^-). Control represents those transduced with control vector. (**c**) IL-4 production by 'starved' FcRγ-deficient BM-derived basophils expressing none (control), wild-type FcRγ (WT) or FcRγ-ΔITAM (75, 65 or 72% $rCD2^+$c-kit^-, respectively) in response to IL-3. The means and s.d. of duplicated cultures are indicated. Representative of more than four independent experiments (**a-c**).

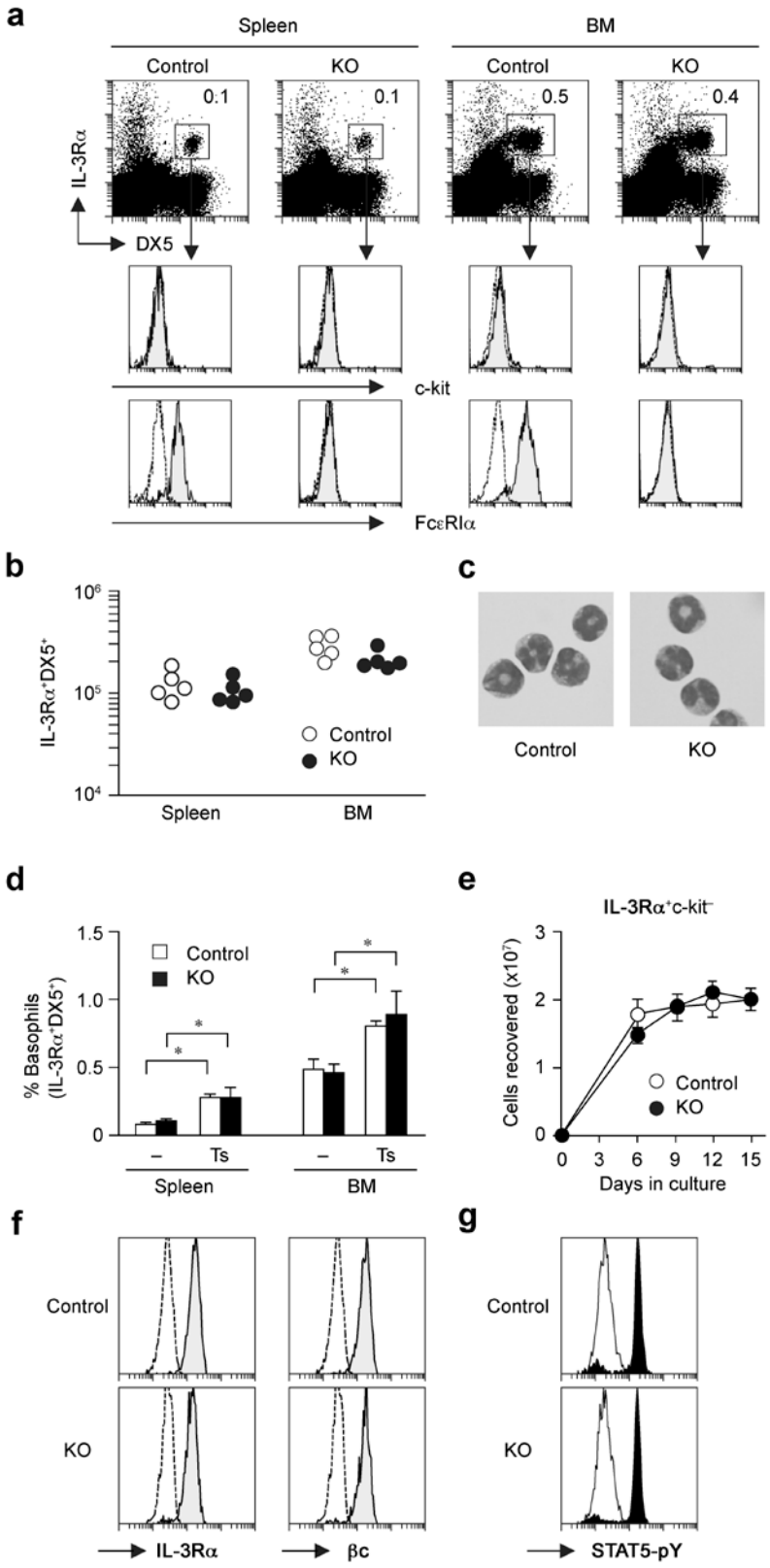
Figure 5. Differential requirement for transmembrane amino acids in FcRγ in IL-3 responses. (**a**) Wild-type (WT) and FcRγ transmembrane (TM) mutants (L21A) and (D11A) used for transducing BM-derived basophils. (**b**) Flowcytometry for surface expression of FcεRIα and intracellular expression of FcRγ (cFcRγ) in FcRγ-deficient BM-derived basophils transduced with the vectors shown in **a** or control vector. FcεRI and cFcRγ expression was examined in gated $rCD2^+$c-kit^- cells. (**c**) ELISA for IL-3-induced IL-4 production by 'starved' FcRγ-deficient BM-derived basophils expressing none (control), wild-type FcRγ (WT), L21A mutant or D11A mutant (70, 66, 57 or 50% $rCD2^+$c-kit^- cells, respectively). The means and s.d. of duplicated cultures are indicated. Representative of three independent experiments (**a-c**).

Figure 6. Differential physical association of FcRγ mutants with βc. Immunoblot of lysates of Y16 cells expressing Flag-tagged wild-type FcRγ (WT), FcRγ-ΔITAM, FcRγ-L21A or FcRγ-D11A. Immunoprecipitation (IP) with anti-βc, followed by immunoblotting (IB) with anti-Flag or anti-βc. Whole cell lysates were also immunoblotted with these antibodies. Representative of three independent experiments.

Figure 7. FcRγ-deficient basophils failed to support T_H2 differentiation i*n vitro*. (**a-d**) Flowcytometry for IL-4- or IFN-γ-producing T cells from spleen cells prepared from control or *Fcer1g*$^{-/-}$ (KO) mice expressing OT-II TCR transgene. (**a**) Cytokine production was examined on gated $CD4^+$ T cells. (**b**) T_H cell differentiation in cultures

of splenic $CD4^+$ T cells isolated from OT-II TCR-tg control or *Fcer1g*$^{-/-}$ (KO) mice in the presence of wild-type APCs. (**c**) T_H cell differentiation in cultures of wild-type OT-II TCR-tg $CD4^+$ T cells with APCs prepared from control or *Fcer1g*$^{-/-}$ (KO) mice. (**d**) T_H cell generation in cultures of spleen cells from OT-II TCR-tg *Fcer1g*$^{-/-}$ mice were supplemented with exogenous IL-4, 2 x 10^4 basophil-enriched BM cell preparations from control (+ Control, 42% basophils) or *Fcer1g*$^{-/-}$ mice (+ KO, 47% basophils). '+ Control ΔBs' = BM celld depleted of $DX5^+$ basophils (1.2% basophils) were added to the cultures. (**e**, **f**) ELISA for IL-3 (**e**) or IL-4 (**f**) produced on day 2 in cultures of whole (–) or basophil-depleted (ΔBs) spleen cells from wild-type (control) or FcRγ-deficient (KO) OT-II TCR-tg mice. Data are the means and s.d. of duplicated cultures. Representative results of more than three (**a**,**d**), three (**e**,**f**) or two (**b**,**c**) experiments.

Figure 1. Taki



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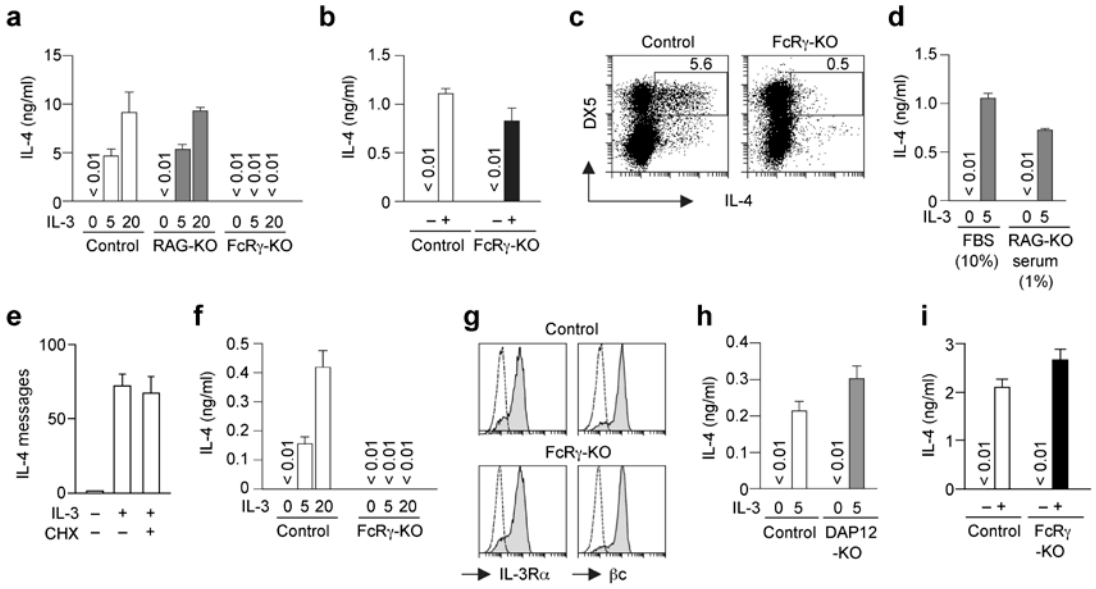

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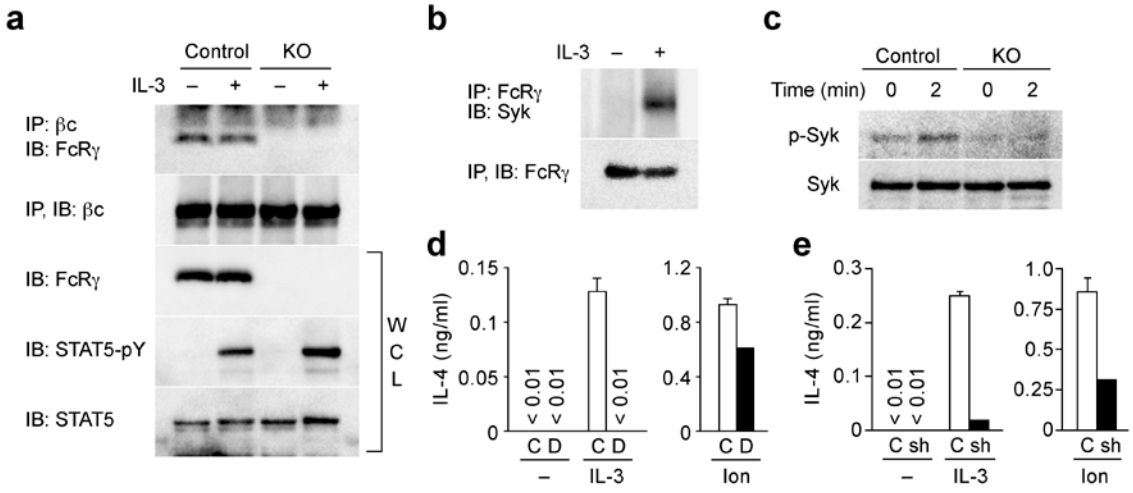

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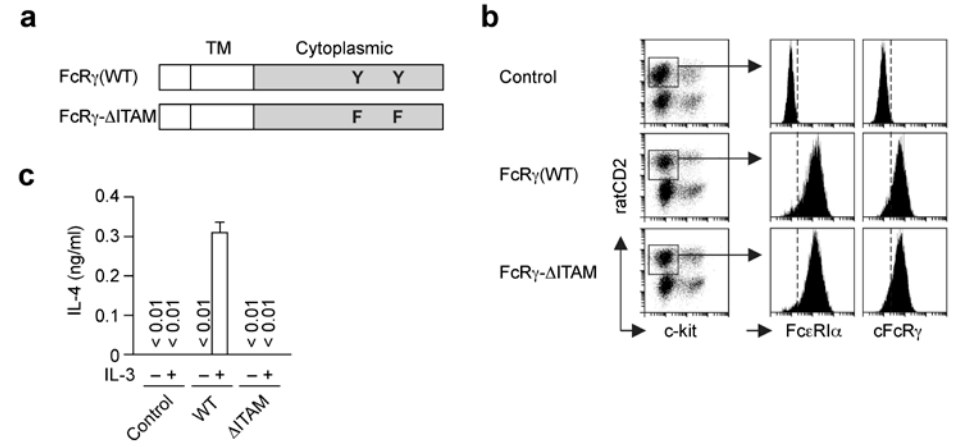

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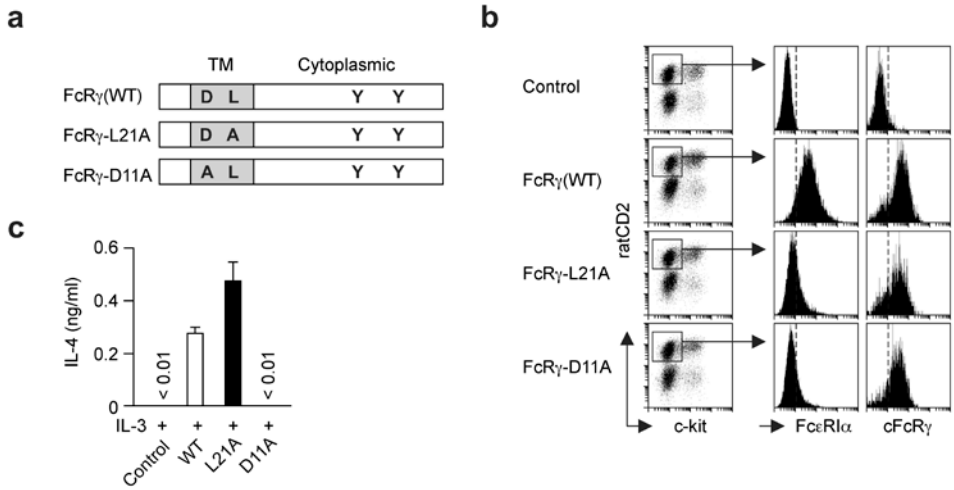

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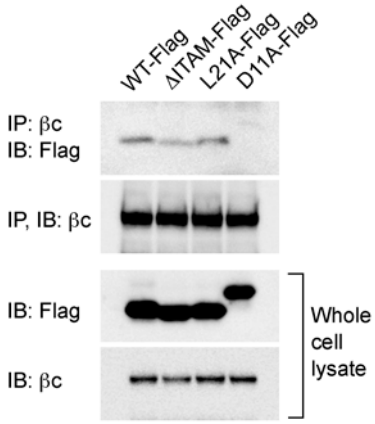

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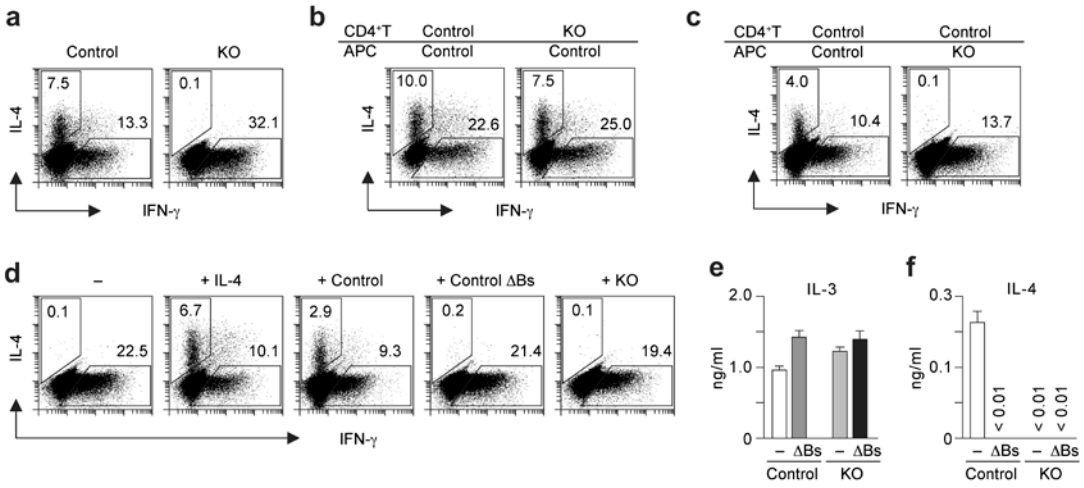
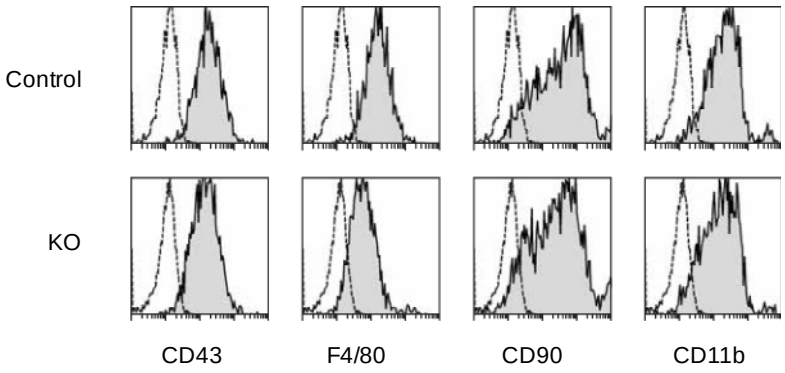
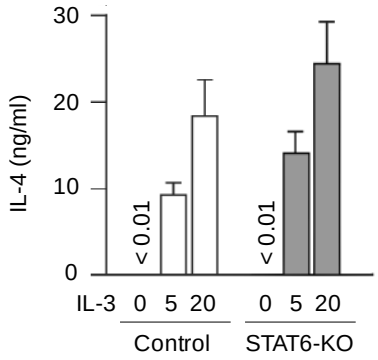
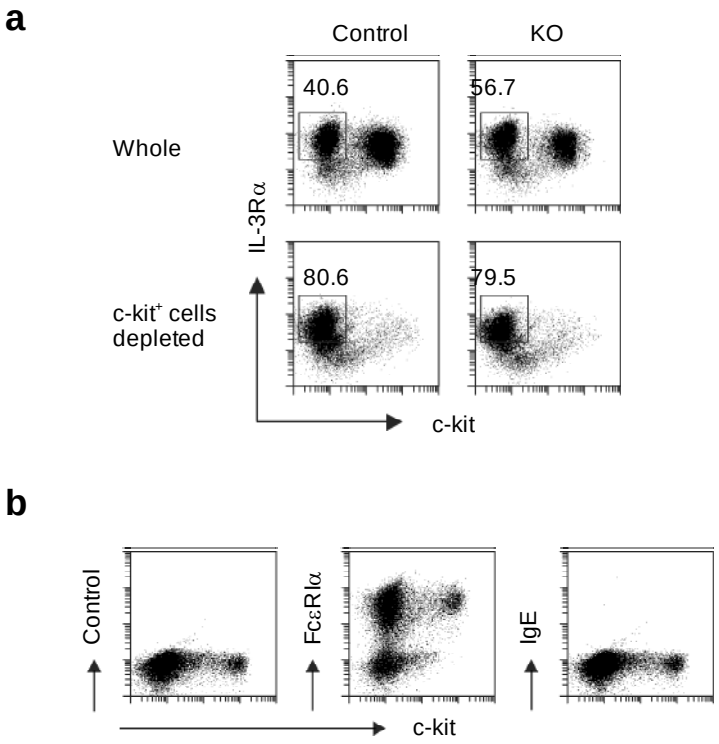




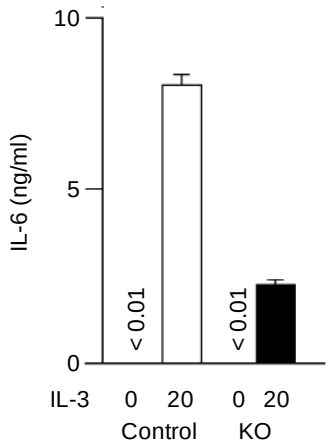
Supplementary figure 1. Surface phenotype of FcRg-deficient basophils. Freshly isolated BM cells from control and *Fcer1g*$^{-/-}$ (KO) mice were stained with antibodies indicated. Shown are the histograms (shaded) for the gated IL-3R α^{+}DX5^{+}c-kit^{-} cells. Both control and FcRγ-deficient basophils were negative for NK1.1 and Gr-1 (not shown). F4/80 staining was slightly lower on FcR g-deficient basophils than on control, the reason for this difference being unclear at present. Dotted histograms represent unstained control. Representative histograms of three pairs of animals.



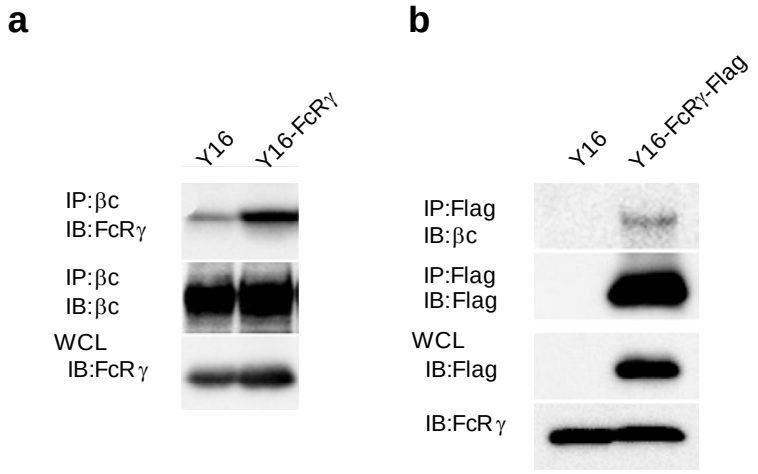
Supplementary figure 2. ELISA for IL-4 production from STAT6-deficient basophils. Basophil-enriched BM cells were prepared and stimulated with the indicated concentrations of IL-3, as in **Fig.2a**. Data represent the means and s.d. of duplicated cultures. Representative of two independent experiments.



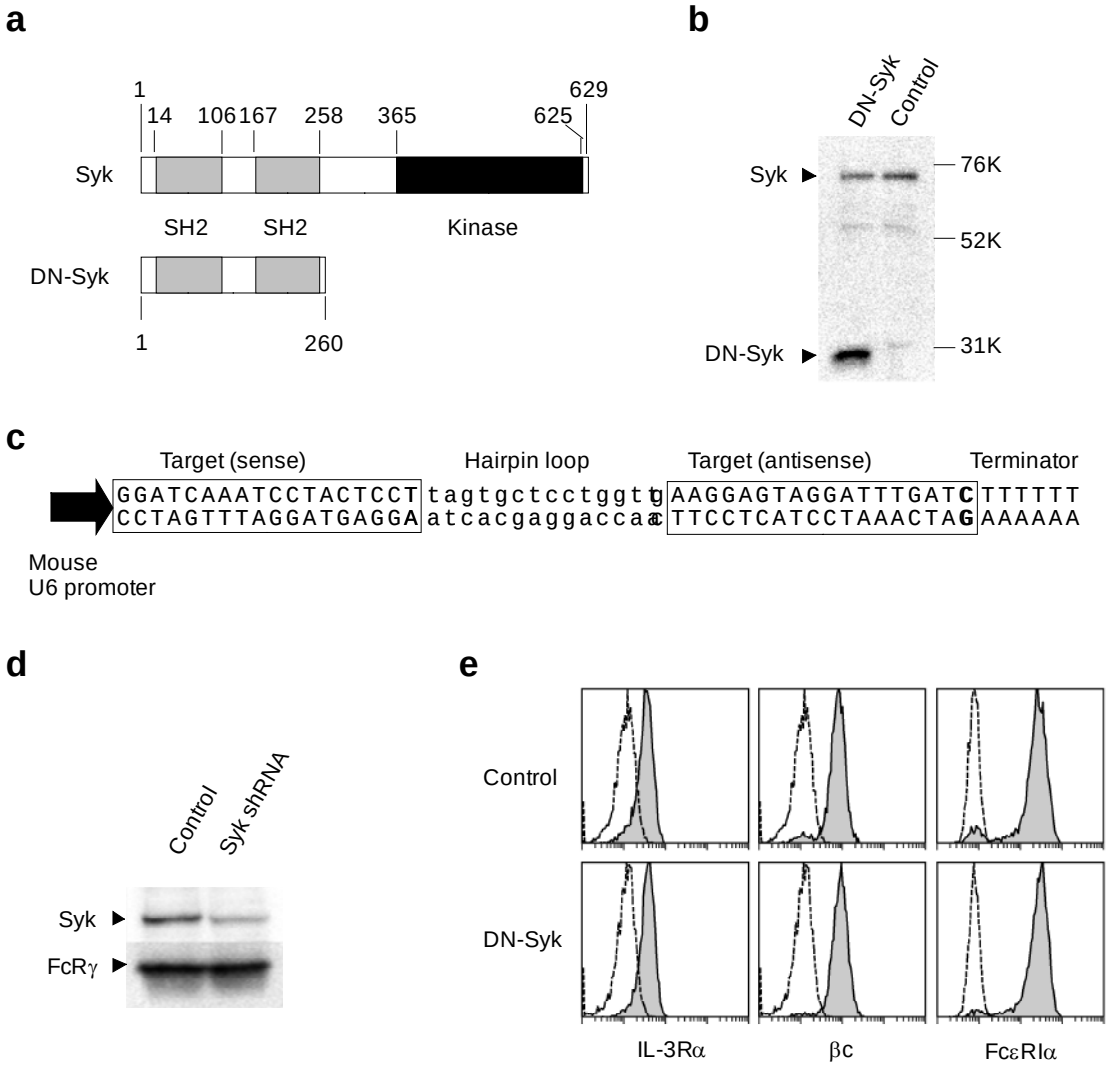
Supplementary figure 3. Enrichment of 'starved' BM -derived basophils. (**a**) BM-derived basophils derived from control and *Fcer1g*$^{-/-}$ (KO) mice were starved as in **Fig.2f** and stained with IL-3R a and c-kit antibodies untreated (Whole) or after depletion of c-kit^{+}cells. The numbers shown are the percentages of cells within the gates. (**b**) BM cells prepared from wild-type mice were cultured in IL-3 for 10 days, and stained with either PE-anti-FceRIa or PE-anti-IgE. Note that both c-kit^{-} cells (basophils) and c-kit^{+} cells (mast cells) express Fc eRI but do not bear IgE, indicating that their Fc eRI is empty. Representative of at least three (**a**) and two (**b**) independent experiments.



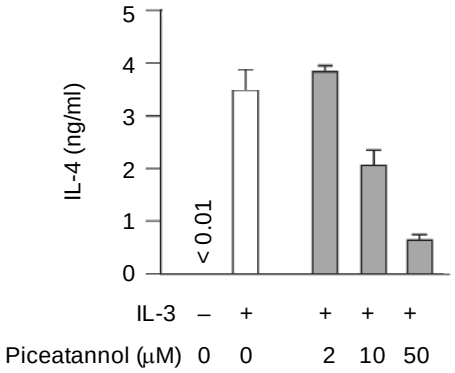
Supplementary figure 4. Impaired production of IL-6 by FcRγ-deficient basophils in response to IL-3. Basophil-enriched BM cells prepared from control or *Fcer1g*$^{-/-}$ mice (KO) were stimulated with IL-3 (20 ng/ml). The percentages of basophils in these cell preparations were 30-40%. The amounts of IL-6 were determined by ELISA. Data represent the means and s.d. of duplicate cultures. Representative of more than three independent trials.



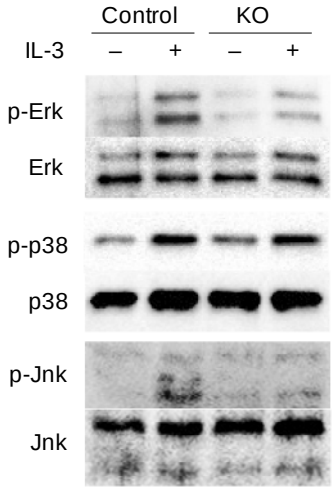
Supplementary figure 5. Association of βc with both endogenous and exogenous FcRγ in Y16. (**a**) Y16 cells were transduced with the retroviral vector carrying wild-type FcRγ, and successfully transduced cells (rCD2 $^{+}$) were sorted (Y16-FcRγ). Cell lysates from Y16 or Y16-FcRγ were immunoprecipitated (IP) with anti- βc, followed by immnoblotting (IB) with anti-FcR γ. Whole cell lysates (WCL) were also blotted with anti-FcRγ. Note that the amounts of FcR γ co-precipitated with βc from Y16-FcRγ were higher than those from Y16, as were those of total FcR γ. (**b**) Reciprocal immunoprecipitation. IP and IB were carried out with the indicated combinations of antibodies on parental Y16 and Y16 expressing Flag-tagged wild-type FcR γ (Y16-FcRγ-Flag). Representative of four (**a**) and two (**b**) independent experiments.



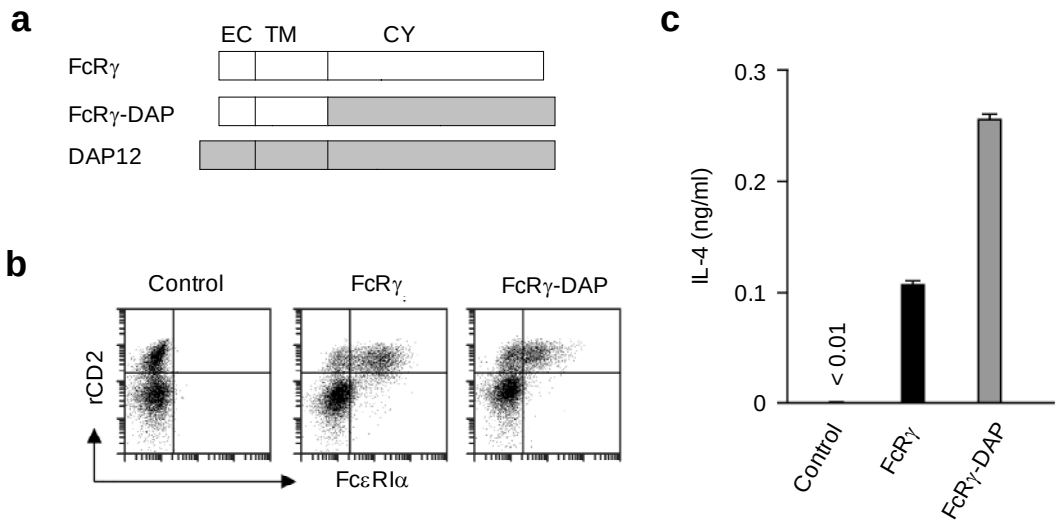
Supplementary figure 6. Expression of DN-Syk and shRNA-mediated down modulation of Syk expression. (**a**) Schematic representation of the domain structure of Syk and DN-Syk. The amino acid positions delineating the SH2 and kinase domains are from the UniProt database (http://www.pir.uniprot.org/). (**b**) Relative amounts of Syk and DN-Syk in BM-derived basophils transduced with control or DN-Syk retroviruses, as revealed by immunoblotting with anti-Syk-N-terminus antibody. (**c**) The Syk-shRNA sequence inserted into pSINsi-mU6 vector (TAKARA Bio). Boxed are the sense and antisense Syk target sequences flanking the hairpin loop sequence (lower case). An unrelated sequence (see Methods) and its antisense sequence were used for control vector. (**d**) The amounts of Syk relative to those of FcR γ in BM-derived basophils transduced with control or Syk-shRNA-expressing vectors. Representative of two independent experiments. (**e**) Cell surface staining for IL-3R α, βc and FcεRIα on BM-derived basophils transduced with DN-Syk or control retroviruses. Dotted histograms represent control staining. Expression of these cell surface receptors was also unaffected in BM-derived basophils expressing Syk-shRNA (data not shown). Representative of three (**b**, **e**) and two (**d**) independent experiments.



Supplementary figure 7. The Syk inhibitor Piceatannol inhibited IL-3-induced IL-4 production by basophils. Basophil-enriched wild-type BM cells prepared as in **Fig.2a** (25% basophils) were stimulated with IL-3 in the presence of the indicated concentrations of Piceatannol for 24 hours, and IL-4 production was measured by ELISA. Data represent the means and s.d. of duplicated cultures. Repeated twice with similar results.

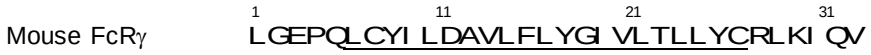


Supplementary figure 8. MAPK activation induced by IL-3 in the absence of FcR γ. Whole cell lysates were prepared from wild-type and FcR γ-deficient (KO) basophil-enriched BM cells (38.1% and 32.0% pure, respectively) stimulated for 10 min as in **Fig.3b,** and subjected to immunoblot analysis with antibodies against the native and phosphorylated forms of the MAPK p38, Erk and Jnk. Representative of two independent experiments.



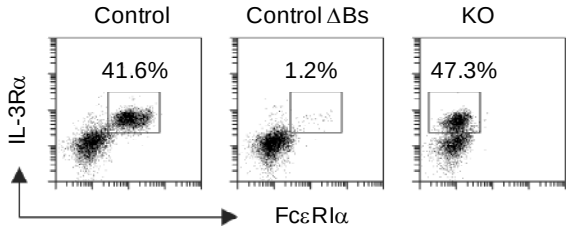
Supplementary figure 9. The cytoplasmic portion of DAP12 could replace that of FcRγ. (**a**) Schematic representation of the structure of a chimeric molecule comprising of the extracellular (EC) and transmembrane (TM) portions of FcR γ fused with the cytoplasmic (CY) portion of DAP12 (FcRγ-DAP). (**b**) Surface FcεRIα expression on FcRγ-deficient BM-derived basophils transduced with the vectors for wild-type FcR γ or FcRγ-DAP. Shown are the data for c-kit⁻ cells. (**c**) IL-3-induced IL-4 production by 'starved' FcRγ-deficient BM-derived basophils expressing none (Control), wild-type FcRγ or FcRγ-DAP. In the absence of IL-3 stimulation, IL-4 production was undetectable (< 0.01 ng/ml). Data represent the means and s.d. of duplicate cultures. Note that FcRγ-DAP was more potent in transducing IL-3 signals for IL-4 production (**c**), despite its apparent inefficiency in transporting Fc εRI to the surface (**b**), compared with wild-type FcRγ, potentially suggesting that FcR γ and DAP12 cytoplasmic portions might not be functionally identical. Repeated twice with similar results.

a

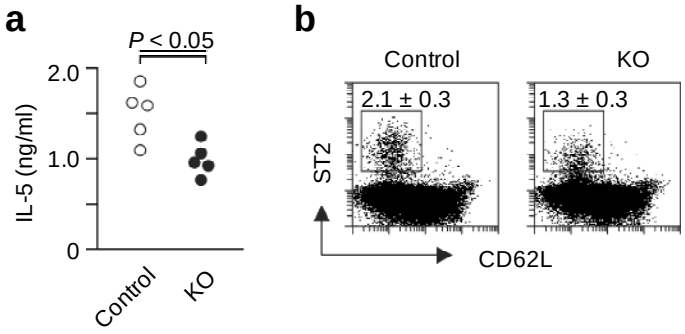


b

Supplementary figure 10. Primary structure of FcR γ and various FcRγ-associated receptors. (**a**) Amino acid sequence of the extracellular and potential TM portions of FcRγ is shown in the single letter format. TM sequence is underlined, and amino acid positions in the mature protein are given. (**b**) Amino acid sequences of the potential TM portions of various FcR γ-associated receptors. The canonical arginine residues are boxed, and the two amino acids in β_{IL-3} different from those in βc underlined. These sequences were taken from the UniPlot database (http://www.pir.uniprot.org/).



Supplementary figure 11. BM cell preparations used for supplementing the cultures of FcRg-deficient OT-II TCR transgenic spleen cells. Basophil-enriched BM cells were prepared from control or *Fcer1g*$^{-/-}$ (KO) mice as in **Fig.2a**. Basophils (DX5^{+}) were additionally depleted from the control cell preparation (Control DBs). In 'Control' and 'Control DBs' basophil preparations, CD11c^{high} cells (DCs) were less than 0.5% and 1%, respectively (not shown). Shown are the surface staining profiles for IL-3R a and FceRIa. The numbers are for the percentages of cells within the gates that represent basophils. Repeated at least three times with similar results.



Supplementary figure 12. Observations suggesting impaired T_H2 development *in vivo* in *Fcer1g*$^{-/-}$ mice. Serum IL-5 levels (**a**) and the expression of ST2 and CD62L on CD4^{+} T cells in mesenteric lymph nodes (**b**) in control or *Fcer1g*$^{-/-}$ (KO) mice at 10 day after infection with *T. spiralis*. In **a**, each symbol represents an individual animal (n = 5 for each genotype). Cumulative data of two independent infections. $P < 0.05$. Dot plots in **b** are representative of three animals of each genotype. Numbers shown are the means and s.d. of values in these animals that differ significantly between control and *Fcer1g*$^{-/-}$ mice ($P < 0.05$).

Supplementary Methods.

PCR primers for quantitative RT-PCR. PCR primers used for quantitative RT-PCR were as follows:
β-actin (Applied Biosystem), sense: 5'-GCTTCTTTGCAGCTCCTTCGT-3', antisense: 5'-AGCGCAGCGATATCGTCAT-3',
IL-4 (Takara Bio), sense: 5'-TCTCGAATGTACCAGGAGCCATATC-3', antisense: 5'-AGCACCTTGGAAGCCCTACAGA-3'

Plasmid construction for FcRγ-DAP12 fusion protein. The extracellular and transmembrane portions of FcRγ, corresponding to amino acid position 1-26, and the cytoplasmic portion of DAP12, corresponding to amino acid position 47-93, were separately cloned by RT-PCR. These cDNAs were sequentially cloned into pMX-IRES-rCD2.

Derivation and 'starvation' of BM-derived basophils. Whole BM cells (2.5×10^7) were cultured in 10 ml of 10% fetal calf serum-containing RPMI1640 medium (Nissui, Japan) supplemented with recombinant murine IL-3 (5 ng/ml) for 13 days with medium changed every 3 days. Recombinant murine IL-3 was produced by a cell line transfected with murine IL-3 expression vector and partially purified. BM-derived basophils were enriched by depleting mast (c-kit$^+$) cells using IMag beads, washed extensively, seeded at 2×10^6/ml and 'starved' for 12 to 18 hours. 'Starved' BM-derived basophils were used for stimulation with IL-3.

Introduction of shRNA expression vectors. Wild-type BM cells cultured for only 4 days in IL-3 were infected with the retrovirus for pSINsi-mu6 vector for targeting Syk (Syk-shRNA) or control vector (see **METHODS**) for 2 days. Infected cells were then selected for successfully transduced cells by culturing with G418 (Roche Applied Science, 0.5 mg/ml) in the presence of IL-3 for 6 days, starved and stimulated with IL-3 or ionomycin for 24 hours for IL-4 production.

Examination of the effects of FcRγ deficiency in CD4$^+$ T cells, APCs and basophils on T_H2 generation *in vitro*. In the experiments depicted in **Fig. 7b**, 1×10^6 CD4$^+$ T cells purified from spleen by IMag beads (regularly >90% pure) prepared from OT-II TCR tg wild-type or *Fcer1g*$^{-/-}$ mice were stimulated with 0.5 μM chicken ovalbumin peptide in the presence of 2.5×10^5 splenic APCs purified by removing TCRβ$^+$, B220$^+$, NKG2D$^+$ and TER119$^+$ cells from spleen cells from C57BL/6 mice. In other experiments (**Fig. 7c**), CD4$^+$ T cells purified similarly from wild-type OT-II TCR-tg mice were stimulated as above in the presence of 2.5×10^5 splenic APCs prepared from control or *Fcer1g*$^{-/-}$ mice. For supplementing exogenous basophils (**Fig. 7d**), BM basophils enriched from wild-type or *Fcer1g*$^{-/-}$ mice (see legends for **Fig. 7** and **Supplementary Fig. 11** for the purities) were added to the cultures of FcRγ-deficient OT-II TCR-tg spleen cells (1×10^6) prepared by removing B, NK and erythroid cells. Recombinant mouse IL-4 (25 ng/ml; R&D systems) was also included as a positive control.