in mice [16–18]. Recently, Fillatreau et al. demonstrate that B cell-derived IL-10 plays a key role in controlling the pro-inflammatory type 1 immune response and EAE [19]. In addition, IL-17 and IL-23 play a crucial role in the development of EAE [20,21]. In a specific condition, it is shown that myelin-specific Abs contribute to the EAE onset. Transgenic mice engineered to produce high titers of autoantibodies against MOG both accelerate and exacerbate EAE [22,23]. $Lyn^{-/-}$ which show IgM hyperglobulinemia and elevation in serum anti-IgM MOG antibodies levels have higher clinical and pathological severity of MOG-induced EAE when compared with wild type [24]. In system of EAE which used normal mice, it remains unclear whether an anti-myelin antibody contributes to the onset of EAE.

The B cells in the immune system use multiple genetic mechanisms such as gene rearrangement, class switching and somatic hypermutation to drive the generation of antibody diversity. Recently, activation-induced cytidine deaminase (AID), which is involved in regulation of the DNA modification step of both class switching and somatic hypermutation, has been identified [25,26]. Examining the profiles of expressed genes revealed expression of the AID gene to be largely restricted to germinal center B cells, memory B cells and activated B cells, but not other cells including T cells, suggesting that AID is not involved in T cell function [27,28]. $AID^{-/-}$ with circulating B cells produced IgM which may be low affinity antibodies due to the lack of somatic hypermutation allowing the generation of antibodies with improved affinity for antigen, but not IgG, IgA and IgE [25]. Accordingly, $AID^{-/-}$ is suitable for in vivo examination to evaluate the effects of MOG-specific antibodies on the development of EAE.

To clarify the effect of antibodies on the development of chronic non-remitting EAE and to conform whether AID is not involved in Th1-mediated immune response, we studied severity of EAE in $AID^{-/-}$ which have normal levels of circulation B cells, and defect of IgG, IgA and high affinity IgM.

2. Materials and methods

2.1. Animals and reagents

Established $AID^{-/-}$, $AID^{+/-}$ and $AID^{+/+}$ eighth backcrossed from (CBA × C57BL/6) × C57BL/6 to C57BL/6 were maintained in the animal facility of Shinshu University and used between 8- and 10-week old. Mice were housed and cared for in a nationally approved facility in accordance with the National Institutes of Health guidelines of the United States. The synthetic mouse MOG peptides 35–55 (Met–Glu–Val–Gly–Trp–Tyr–Arg–Ser–Pro–Phe–Ser–Arg–Val–Val–His–Leu–Try–Arg–Asn–Gly–Lys), obtained from Sawady Technology Co., Ltd. (Tokyo, Japan), were synthesized using standard F-moc chemistry; purity (>90% pure) was determined using reverse phase HPLC.

2.2. Immunization

$AID^{-/-}$, $AID^{+/-}$ and $AID^{+/+}$ were injected with MOG35–55 peptide (100 μg) dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (CFA), supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (Difco, H37Ra) or CFA alone in hip (50 μl) and in the nape of the neck (50 μl) as previously described [29,30]. Immediately before and 2 days after injection of the emulsion, the mice received an intravenous injection of 300 ng pertussis vaccine in 100 μl of phosphate-buffered saline (PBS). The mice were assessed daily for clinical signs of disease; signs of neurological dysfunction were graded using the following system: normal, 0; limp tail, 1; hind leg weakness, 2; hind leg paralysis, 3; fore leg paralysis, 4; moribund or death, 5. The batches of the peptide and adjuvant used were identical throughout the present studies.

2.3. Serum collection

Serum samples were prepared from the peripheral blood obtained by tail vein puncture every week up to 10 weeks after immunization, and were stored at −30 °C until use.

2.4. Histological examination of tissues

Mice were anesthetized with an i.p. injection of pentobarbital and exsanguinated by transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on day 21 after immunization. Whole CNS tissues, including an entire portion of the spinal cord and optic nerves, were carefully dissected free from the cranium and spinal canal, then immersed overnight in the same fixative solution at room temperature. The CNS was longitudinally and coronally sectioned along the rostrocaudal axis. The tissue slices were dehydrated in a graded series of ethanol, cleared in Hemo-D and embedded in paraffin. Serial sections of 5 μm thickness were mounted on silane-coated glass slides. For light microscopic examination, sections were stained using hematoxylin–eosin and Klüver–Barrera (luxol fast blue–cresyl violet) procedures.

2.5. ELISA

Ninety-six-well microtiter plates (Nunc-Immunoplate, Denmark), pretreated with 0.2% glutaraldehyde, were coated (50 μl/well) with MOG35–55 peptide diluted to a concentration of 1 μg/ml in carbonate buffer (pH 9.6) as previously described [30,31] for MOG-specific-antibody titration. For immunogloblin titration, microtiter plates were coated (50 μl/well) with rabbit anti-mouse IgG (rabbit anti-mouse IgG (H + L), Wako pure chemical Industry, Osaka, JP) or rat anti-mouse IgM (LO-MM9, Monosan, NL) or rat anti-mouse IgA (LO-MA7, Monosan, NL) diluted to a concentration of 0.2 μg/ml in carbonate buffer (pH 9.6). After incubation at 37 °C for 3 h, the plates were washed three times with PBS containing 0.1% Tween-20 and blocked with 2% BSA–PBS at 4 °C overnight. The plates were then incubated with 50 μl of 1/1000 dilutions of mouse serum for 1 h at room temperature.

For MOG-specific-antibody titration, mouse standard serum (Nordic Immunological Lab., Ltd., The Netherlands) was used as negative control. After washing three times, 1/2000 diluted alkaline phosphatase labeled goat anti-mouse IgG (KPL Inc., MD), rat anti-mouse IgA (Zymed, San Francisco, CA) or rat anti-mouse IgM (Zymed) was added to the plates and incubated for 1 h at room temperature. The reaction products were visualized using *p*-nitrophenyl phosphate as substrate. The plates were then read at 405 nm with a microplate reader (Tosoh Co., Yamaguchi, Japan), and OD 405 level of each sample was compared.

2.6. T cell proliferation assay

At 2 weeks after immunization, mice were sacrificed. Spleen cells were harvested from three arbitrarily selected mice in each group. Cells (1×10^5) were cultured in 96-well flat bottom microculture plates in RPMI-1640 medium with 10% fetal calf serum. Triplicate cultures were stimulated with 2.0 μg/ml of MOG35–55, 0.5 μg/ml phytohemagglutinin (GIBCO, Grand Island, NY) or none at 37 °C in a 5% CO_2 incubator. During the last 12 h of the 4 days of culture, 1 μCi of ^{3}H-thymidine was added and the cell proliferation

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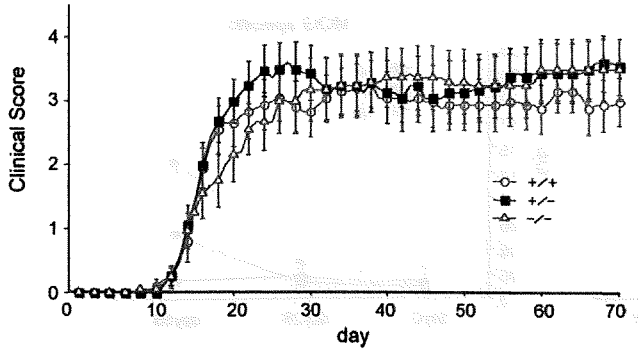

Fig. 1. Induction of EAE. EAE in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n = 18$ in each group) was induced following immunization with MOG35–55, as described in Section 2. The severity of EAE is presented as mean clinical score ± S.E.M. in each group.

was estimated by ^{3}H-thymidine incorporation using a scintillation counter and expressed as cpm. The stimulation index (S.I.) was calculated as cpm with stimulation/background cpm. S.I. was considered significant only if it exceeded background by at least threefold.

2.7. Statistical analysis

Clinical scores were analyzed using the repeated measure ANOVA (StatView, Abacus Concepts, Berkeley, CA). Other results were statistically evaluated using an unpaired Student's *t*-test. All values are expressed as the mean ± S.D. and $P < 0.05$ was taken as indicating statistical significance.

3. Results

3.1. Clinical manifestation

Following immunization with MOG35–55, all 18 mice of each AID$^{-/-}$, AID$^{+/-}$ or AID$^{+/+}$ exhibited same disease courses of the typical MOG peptide-induced EAE. In AID$^{-/-}$ group, mean onset day was 16.6 ± 6.3, and the maximum disease score was 3.8 ± 1.4 and cumulative disease score from days 0 to 70 was 157.7 ± 104.2. The first symptoms of the disease, weight loss and limp tail, were observed 8–33 days after immunization with the mean onset day of 16.6 ± 6.3 in the AID$^{-/-}$ (Fig. 1). In AID$^{+/-}$ and AID$^{+/+}$ groups, the disease severities were same compared with AID$^{-/-}$ indicated by the same mean onset day, the same mean maximum clinical score and cumulative disease score. The mean onset day, the maximum disease score and cumulative disease score were 18.6 ± 11.5, 4.0 ± 0.8 and 165.5 ± 78.6 in AID$^{+/+}$ group, and 17.3 ± 10.0, 4.2 ± 1.0 and 171.7 ± 106.6 in AID$^{+/-}$ group, respectively (Fig. 1). No statistical difference in clinical manifestations was found according to AID genetics.

3.2. Histology

Each mouse immunized with MOG35–55 from the three genetic groups showed inflammatory lesions and demyelination localized mostly in the lumbar spinal cord. The numbers of demyelinating lesions and leukocytes in the lesions were similar among the three groups (Fig. 2).

3.3. Antibody synthesis

The antibody isotype reactivity of serum from each group was tested using ELISA at 0, 4 and 8 weeks after immunization. Serum IgG and IgA were not detected in AID$^{-/-}$ group throughout the study (Fig. 3 and data not shown). IgM antibody titers in AID$^{-/-}$ group were higher than those of the other two groups (Fig. 3). Serum anti-MOG35–55 IgG titers of AID$^{-/-}$ group were on baseline value, and were significantly lower than those of AID$^{+/+}$ and AID$^{+/-}$ groups at any time point (Fig. 3). Furthermore, anti-MOG35–55 IgM antibody titers of AID$^{-/-}$ were significantly higher than those of other two groups at 4 weeks (Fig. 3). The production of IgA against MOG35–55 was not observed in any mice (data not shown).

3.4. MOG35–55-specific T cell proliferation

To determine the effect of disruption of the AID gene on T cell function after MOG immunization, we examined T cell proliferative response of spleen cells following stimulation by MOG35–55. Spleen cells of AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ showed positive response to MOG35–55 stimulation. AID$^{+/+}$ group showed tendencies of higher proliferation with MOG35–55 stimulation, but was not statistically significant (Fig. 4).

4. Discussion

Demyelination of MS is thought to be a multifactorial including T cell, antibodies, complement activation and direct effect of inflammatory cytokines. AID$^{-/-}$ with normal number of circulating B cells displayed no serum IgG, IgA and anti-MOG IgG, but modestly increased levels of serum IgM and anti-MOG IgM. The proliferative response was not significant statistically among the groups. AID$^{-/-}$ displayed no difference of clinical and pathological severity of chronic non-remitting EAE compared with AID$^{+/+}$

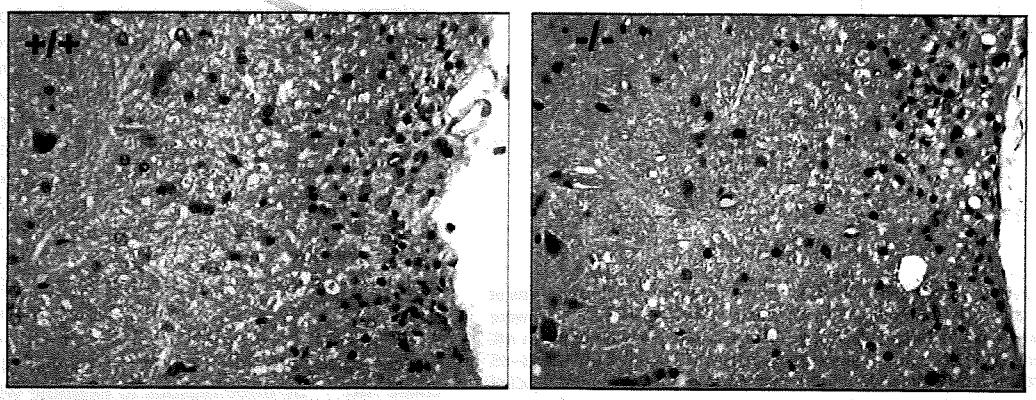

Fig. 2. Histology. Spinal cords were isolated from EAE mice randomly selected from AID$^{-/-}$ (clinical score: 2, 2 and 3), AID$^{+/-}$ (clinical score: 2, 2 and 3) and AID$^{+/+}$ (clinical score: 2, 2 and 2) mice on day 21. The numbers of demyelinating lesions and leukocytes in the lesions were similar among the three groups.

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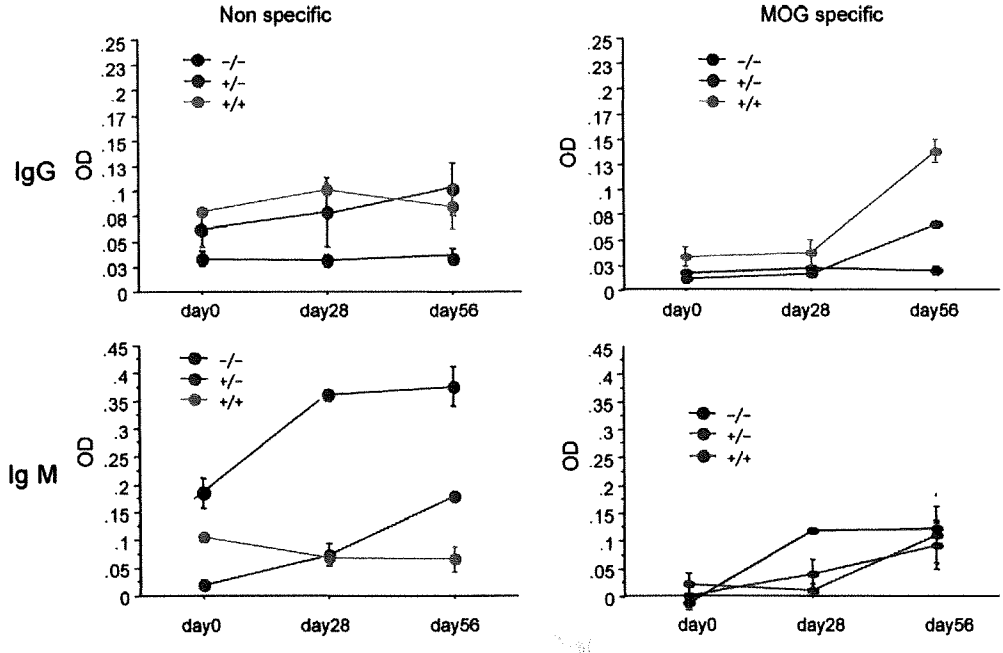

Fig. 3. Serum levels of antibodies. The mice in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n = 5$ in each) were immunized with MOGp35–55. Sera were collected on days 0, 28 and 56, and total IgG and IgM and anti-MOG IgG and anti-MOG IgM were measured by ELISA. The OD 450 is presented by the mean value and S.D. of three separate measurements of each sample. *$P = 0.10$ and **$P = 0.08$. Q1

and AID$^{-/-}$. MS is a chronic neurodegenerative disease characterized by central nervous system inflammation and demyelination. The clinical heterogeneity of MS is well described and numerous animal models: EAE has been developed in different species of animals induced by different autoantigens [2]. Each represents certain aspects of MS. Immunization of MOG35–55 in C57BL/6 mice develops a chronic non-remitting type of demyelinating disease which resembles chronic MS. Thus, our studies by mice chronic MS model demonstrated that antibodies did not contribute to the regulation of the development of chronic non-remitting EAE in C57BL/6 mice.

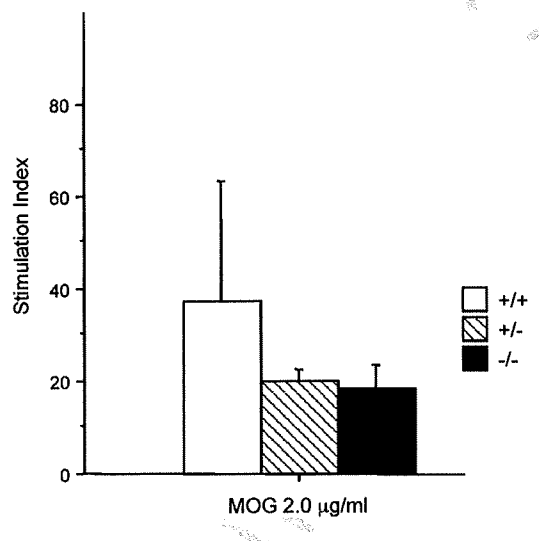

Fig. 4. MOG35–55-specific T cell proliferation. Cells were isolated from spleens of AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ mice on day 14 after immunization. Cells (1×10^5) were cultured in 96-well flat bottom microculture plates in RPMI-1640 medium with 10% fetal calf serum. Triplicate cultures were stimulated with 2.0 μg/ml of MOG35–55 at 37 °C in a 5% CO_2 incubator for 4 days. During the last 12 h of the culture, 1 μCi of ^{3}H-thymidine was added and the cell proliferation was estimated by ^{3}H-thymidine incorporation using a scintillation counter. Mean stimulation index (S.I.) ± S.D. in each group was shown.

Although EAE is considered a prototypic Th1-mediated autoimmune disease, a number of studies have shown a role for B cells in the development of EAE [32]. There is much evidence to implicate B cells, plasma cells and their products in the pathogenesis of MS [19,32]. Despite unequivocal evidence that EAE, the animal model for MS, is initiated by myelin-specific T cells, there is accumulating evidence of a role for B cells, plasma cells and their products in EAE pathogenesis [24]. In the earlier studies, depletion of B cells lead to the resistance of MBP-induced EAE [33,34]. In MOG Ag-induced EAE, Lyons et al. [14] suggested that B cells were required for development of EAE induced following immunization with whole human MOG protein, but not for the EAE induced following immunization with MOGp35–55 [16,17]. MOG is expressed on the cell surface, and therefore is a potential target for autoantibodies [35]. EAE is accelerated with increased severity when high levels of MOG-specific Ab are produced genetically in mice. In transgenic mice that were engineered to produce MOG-specific autoantibodies, severity of EAE was increased, suggesting an important role of Ag-specific antibodies in EAE [22]. In the marmoset, the administration of MOG antigens can increase concentrations of pathogenic autoantibodies and in some circumstances cause a severe worsening of EAE after recovery from the acute attack of EAE [36]. Lyn$^{-/-}$ with IgM hyperglobulinemia and elevation in serum anti-IgM MOG Ab levels have higher clinical and pathological severity of MOG-induced EAE [24]. However, as for lyn$^{-/-}$ system, possibilities that lyn contributes to T cell function remain. Disruption of lyn gene may interfere with CD40 signaling in antigen presenting cells [37], and consequently decrease IL-12 production and indirectly affect the development of a Th1 response.

Studies on mechanism of autoantibody IgG-mediated demyelination in EAE indicate that these autoantibodies enhance inflammation in the CNS via activation of complement [38] or directly through Ab-dependent cell-mediated cytotoxicity [39]. We chose AID$^{-/-}$ by three reasons to examine whether an MOG antibody, in particular about MOG IgG antibody, contributed to the onset of EAE equally. First, AID is involved in regulation or catalysis

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of the DNA/RNA modification step of both class switching recombination and somatic hypermutation, subsequently AID$^{-/-}$ do not produce IgG and IgA, and IgM synthesized by AID$^{-/-}$ is low affinity antibody, indicating that the Ag-specific IgM may not act sufficiently [25,40]. Second, there is not manifestation of AID expression in T cells and AID may not contribute T cell function. The patients with the autosomal recessive form of hyper-IgM syndrome (Hyper-IgM syndrome type II), which is caused by mutations of AID genes and have less sever clinical courses, have normal numbers and normal functions of circulating T cells, indicating that AID is restricted to immunoglobulin editing [41]. Third, normal numbers of B cells exist in AID$^{-/-}$. Therefore, we can exclude the influence of B cell itself.

In conclusion, our studies show that AID does not play an important role in the development of chronic non-remitting EAE in C57BL/6 mice. Although there is a tendency to compartmentalize autoimmune diseases into those that are T cell mediated and those that are B cell dependent (antibody mediated), these boundaries have not been clear-cut. The role of autoantibodies, anti-MOG IgG and unmutated anti-MOG IgM, is not crucial to mediate chronic non-remitting EAE in C57BL/6 mice, at least in the AID$^{-/-}$ system lacking IgG and mutated IgM. Also, since EAE is Th1-mediated disease, our data confirm that AID is not involved in the function of Th1 cells. Our studies can contribute not only to the understanding of EAE but also the development of therapeutic strategies of MS.

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nature immunology

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

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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 show here that basophils lacking FcRγ developed normally and proliferated efficiently in response to interleukin 3 (IL-3) but were very impaired in IL-3-induced production of IL-4 and in supporting T helper type 2 differentiation. Through its transmembrane portion, FcRγ associated constitutively with the common β-chain of the IL-3 receptor and signaled by recruiting the kinase Syk. Retrovirus-mediated complementation demonstrated the essential function of the ITAM of FcRγ in IL-3 signal transduction. Our results identify a previously unknown mechanism whereby FcRγ functions to 'route' selective cytokine-triggered signals into the ITAM-mediated IL-4 production pathway.

Cytokines are critical for many biological processes, including immune responses. Notably, not only do 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 this unique nature of cytokine signaling is the interleukin 3 (IL-3)–IL-5–granulocyte-macrophage colony-stimulating factor (GM-CSF) system, in which the signal-transducing β-subunit, called the 'common β-chain' (βc) or 'Csf2rb' (A001261), is shared by the receptors for those cytokines and provides the molecular basis for their functional redundancy[5,6]. However, IL-5 can expand eosinophil populations, support the generation of B-1 cells and augment immunoglobulin A (IgA) class switching, whereas GM-CSF is involved in the functional maturation of natural killer (NK) T cells as well as the proliferation and differentiation of macrophages, granulocytes and dendritic cells (DCs)[7,8]. Likewise, IL-3 expands the populations of a variety of hematopoietic progenitor cells, activates phagocytosis and cytotoxicity in eosinophils and induces the production of IL-4 and IL-6 by basophils[9,10]. Such pleiotropy in the actions of these '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 identified mainly for functions such as hematopoietic progenitor cell proliferation and survival in studies of cytokine-dependent cell lines; for other effector functions in mature cells, the pathways remain mostly uncharacterized[11]. Given that several signal-transducing molecules associate directly and indirectly with distinct regions of βc, βc seems to serve as a platform from which 'downstream' signaling pathways diverge[6]. The best established intracellular signaling pathway for βc signals is the one initiated by the activation of Jak protein kinases followed by the tyrosine phosphorylation and activation of transcription factor STAT5 molecules, which induce the 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), in contrast, are well known as being critical for signal transduction by various immunoreceptors, such as lymphocyte antigen receptors and Fc receptors for IgE (FcεRI) and IgG (FcγR)[15]. Those immunoreceptors indirectly recruit Syk and Zap70 through transmembrane adaptor molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs), such as CD3ζ, immunoglobulin-α, immunoglobulin-β, Fc receptor common γ-chain (FcRγ) and DAP12 (ref. 16). After 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 the many events inducible by 'βc cytokines' are mediated by Syk[14].

The importance of basophils as a regulators of T helper type 2 (T_H2) responses has become increasingly apparent on the basis of the

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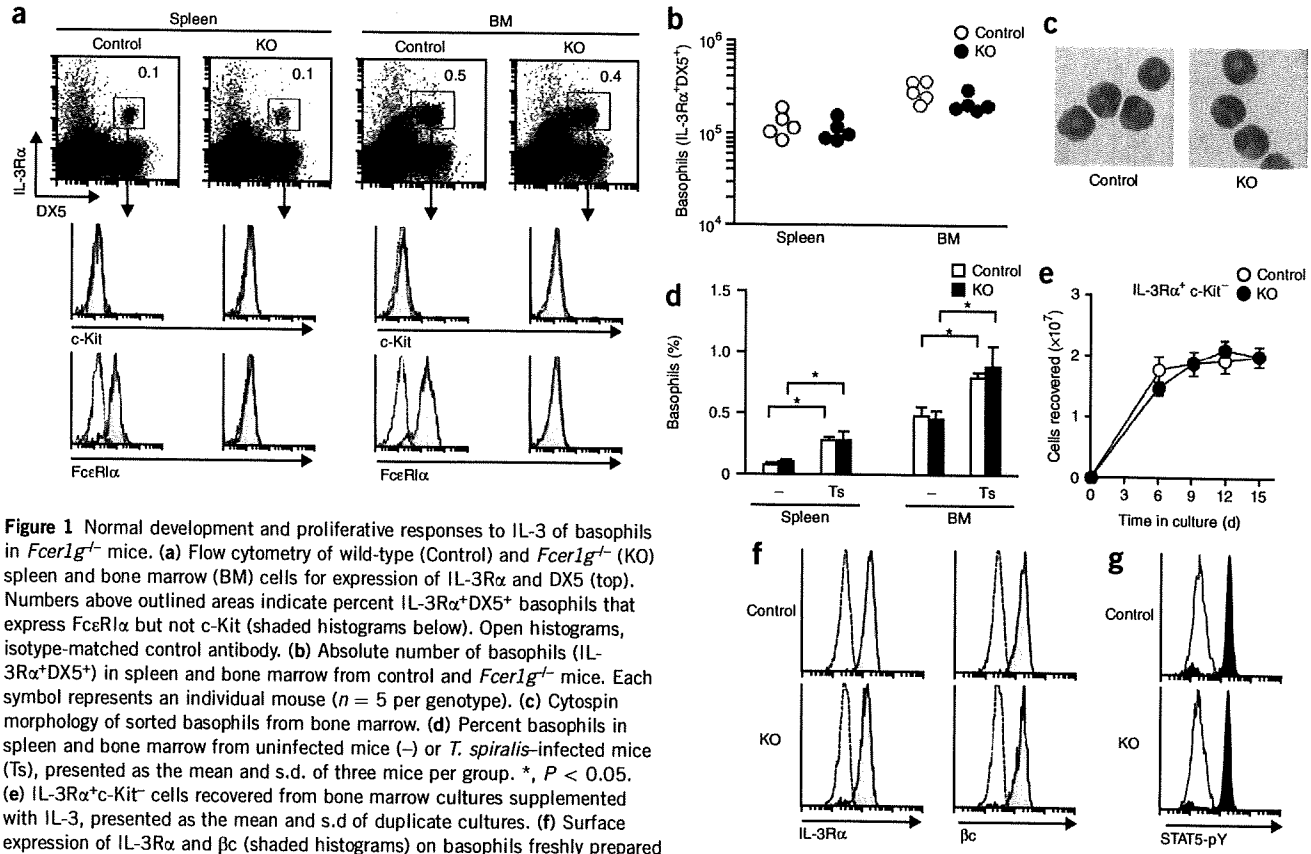

Figure 1 Normal development and proliferative responses to IL-3 of basophils in *Fcer1g*$^{-/-}$ mice. **(a)** Flow cytometry of wild-type (Control) and *Fcer1g*$^{-/-}$ (KO) spleen and bone marrow (BM) cells for expression of IL-3Rα and DX5 (top). Numbers above outlined areas indicate percent IL-3Rα$^+$DX5$^+$ basophils that express FcεRIα but not c-Kit (shaded histograms below). Open histograms, isotype-matched control antibody. **(b)** Absolute number of basophils (IL-3Rα$^+$DX5$^+$) in spleen and bone marrow from control and *Fcer1g*$^{-/-}$ mice. Each symbol represents an individual mouse ($n = 5$ per genotype). **(c)** Cytospin morphology of sorted basophils from bone marrow. **(d)** Percent basophils in spleen and bone marrow from uninfected mice (–) or *T. spiralis*–infected mice (Ts), presented as the mean and s.d. of three mice per group. *, $P < 0.05$. **(e)** IL-3Rα$^+$c-Kit$^-$ cells recovered from bone marrow cultures supplemented with IL-3, presented as the mean and s.d of duplicate cultures. **(f)** Surface expression of IL-3Rα and βc (shaded histograms) on basophils freshly prepared from bone marrow (DX5$^+$c-Kit$^-$). Open histograms, isotype-matched control antibody. **(g)** Flow cytometry of the intracellular expression of phosphorylated STAT5 (STAT5-pY) in basophils (DX5$^+$c-Kit$^-$NK1.1$^-$) from bone marrow 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 experiments.

observation that these cells produce IL-4 in response to various stimuli, including IL-3, parasite constituents and dietary allergens, as well as crosslinkage of FcεRI (ref. 18). Basophils may be indispensable in the generation of T_H2 cells in an IL-3-dependent way *in vitro*, and acceleration of T_H2 differentiation results after the population expansion of basophils *in vivo* by continuous IL-3 exposure[19] or in the absence of the transcription factor IRF2 (ref. 20). For the initiation of T_H2 responses, IL-3 is of particular interest among those molecularly heterogeneous basophil stimulants because it can directly induce IL-4 production in resting basophils[20], whereas other stimuli, such as FcεRI crosslinkage and the allergen protease papain, seem to require prior activation of basophils for IL-4 induction[21–23]. As mentioned above, however, there is very limited information on the IL-3-triggered signaling pathway for IL-4 production, an important effector function of basophils.

We show here that FcRγ is a constitutive component of IL-3R and that it was essential, through its ITAM, in the IL-3-induced production of IL-4 and in supporting T_H2 differentiation by basophils. The involvement of FcRγ in IL-4 production seemed to be selective, as another function of IL-3, the promotion of basophil proliferation, was not affected by FcRγ deficiency. We further show that the mechanism for the association of FcRγ with βc through their transmembrane portions is distinct from that for the association of FcRγ with other immunoreceptors, including FcεRI and FcαRI. Our study identifies a hitherto undescribed function for FcRγ whereby it 'channels' 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 mouse basophils are defined usually as FcεRI$^+$c-Kit$^-$ cells in bone marrow and spleen, basophils from mice lacking FcRγ (*Fcer1g*$^{-/-}$)[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] allowed us to unequivocally identify basophils even in *Fcer1g*$^{-/-}$ mice (**Fig. 1a**). Thus, in littermate control mice, basophils (IL-3Rα$^+$DX5$^+$) expressed FcεRI but not c-Kit, whereas FcεRI was absent on FcRγ-deficient basophils, as expected (**Fig. 1a**). Basophils were present at equivalent frequencies in the bone marrow and spleen of *Fcer1g*$^{-/-}$ and control mice (**Fig. 1b**) and were morphologically indistinguishable (**Fig. 1c**) and expressed a more or less similar array of surface molecules in *Fcer1g*$^{-/-}$ and control mice (**Supplementary Fig. 1** online). Furthermore, infection by the nematode *Trichinella spiralis* induced the population expansion of basophils in spleen and bone marrow equally in wild-type and *Fcer1g*$^{-/-}$ mice (**Fig. 1d**). These observations collectively indicate that FcRγ is not required for the development, maturation, population expansion or migration of basophils *in vivo*. In addition, FcRγ-deficient basophils proliferated as efficiently as control basophils in response to IL-3

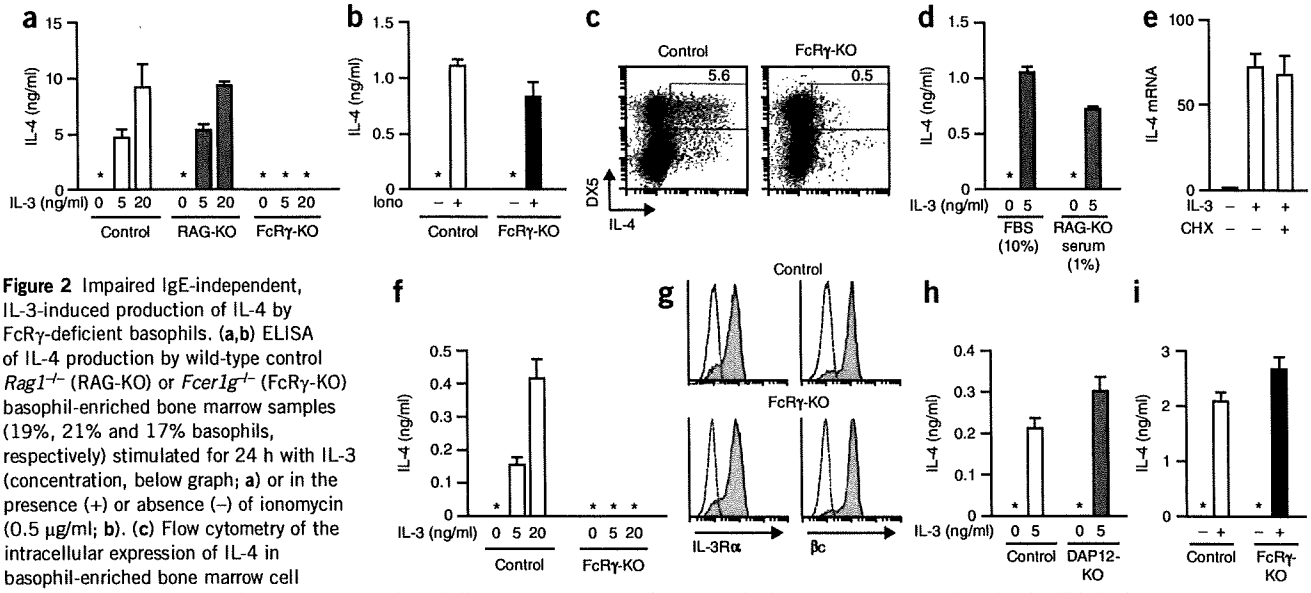

Figure 2 Impaired IgE-independent, IL-3-induced production of IL-4 by FcRγ-deficient basophils. (**a**,**b**) ELISA of IL-4 production by wild-type control *Rag1*$^{-/-}$ (RAG-KO) or *Fcer1g*$^{-/-}$ (FcRγ-KO) basophil-enriched bone marrow samples (19%, 21% and 17% basophils, respectively) stimulated for 24 h with IL-3 (concentration, below graph; **a**) or in the presence (+) or absence (–) of ionomycin (0.5 μg/ml; **b**). (**c**) Flow cytometry of the intracellular expression of IL-4 in basophil-enriched bone marrow cell samples (c-Kit$^-$) stimulated for 18 h with IL-3 (5 ng/ml). Numbers above outlined areas indicate percent DX5$^+$IL-4$^+$ cells. (**d**) ELISA of IL-4 production by *Rag1*$^{-/-}$ spleen cells (0.7% basophils) after stimulation with IL-3 in medium supplemented for 24 h with either FBS or serum from *Rag1*$^{-/-}$ mice. (**e**) RT-PCR analysis of IL-4 mRNA in basophil-enriched bone marrow cell samples stimulated for 6 h with IL-3 in the presence or absence of cycloheximide (CHX). Expression is expressed as means and s.d., in arbitrary units. (**f**) ELISA of IL-4 production by 'starved' and enriched wild-type and *Fcer1g*$^{-/-}$ bone marrow–derived basophils stimulated with IL-3. (**g**) Flow cytometry of the expression of IL-3Rα and βc (shaded histograms) on 'starved' c-Kit$^-$ bone marrow–derived basophils; open histograms, isotype-matched control antibody. (**h**) ELISA of IL-4 production by wild-type spleen cells (0.7% basophils) and DAP12-knockout spleen cells (DAP12-KO; 1.3% basophils) stimulated for 24 h with IL-3. (**i**) ELISA of IL-4 production by 'nonstarved' wild-type and *Fcer1g*$^{-/-}$ (FcRγ-KO) bone marrow–derived basophils stimulated for 24 h with anti-CD200R3. *, concentration less than 0.01 ng/ml. Data are representative of two (**c**,**d**,**h**), three (**b**,**e**,**g**,**i**) or more than four (**a**,**f**) individual experiments (error bars, s.d. of duplicate cultures (**a**,**b**,**d**,**f**,**h**,**i**)).

in vitro (**Fig. 1e**). The amount of cell surface–expressed IL-3Rα and βc was equivalent in wild-type and FcRγ-deficient basophils (**Fig. 1f**), and STAT5 phosphorylation, an immediate 'downstream' event induced by IL-3, was also unaffected by FcRγ deficiency (**Fig. 1g**). These results indicate that FcRγ is dispensable at least for IL-3R expression and 'downstream' signals for the proliferation of basophils.

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

Despite the normal proliferation of FcRγ-deficient basophils, we found that basophil-enriched bone marrow samples prepared from *Fcer1g*$^{-/-}$ mice failed to produce IL-4 when stimulated *in vitro* with IL-3 in conditions in which cells from control mice produced considerable IL-4 (**Fig. 2a**); in contrast, their production of IL-4 in response to ionomycin was similar to that of control cells (**Fig. 2b**). Bone marrow basophils enriched from recombination-activating gene 1–deficient (*Rag1*$^{-/-}$) mice, which lack both T cells and B cells, produced as much IL-4 as did wild-type bone marrow cells (**Fig. 2a**), and IL-4 was present mainly in DX5$^+$ cells among IL-3-stimulated bone marrow cells from control mice but not in DX5$^+$ from *Fcer1g*$^{-/-}$ mice (**Fig. 2c**), which excluded the possibility of involvement of other contaminating cells in IL-4 production by basophil-enriched bone marrow samples.

The failure of FcRγ-deficient basophils to produce IL-4 might have been due to their lack of surface FcεRI and FcγR, which could possibly induce IL-4 production together 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 considerable IL-4 even when cultured in medium containing immunoglobulin-deficient serum from *Rag1*$^{-/-}$ mice (**Fig. 2d**). In addition, basophils derived from *Stat6*$^{-/-}$ mice, which lack 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 production of IL-4 independently of the lack of signals through FcεRI or FcγR. 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 new protein synthesis (**Fig. 2e**).

When cultured *in vitro* in the presence of IL-3, basophil populations were expanded from bone marrow cells[28] and could be purified by the removal of c-Kit$^+$ mast cells (**Supplementary Fig. 3a** online). We found that wild-type bone marrow–derived basophils prepared as described above produced considerable IL-4 in response to IL-3 after they were incubated in the absence of IL-3 ('starved') for several hours (**Fig. 2f**); the cells stained with antibody to FcεRIα (anti-FcεRIα) but not with anti-IgE (**Supplementary Fig. 3b**), which confirmed the IgE independence of IL-4 production in response to IL-3 stimulation. In contrast, basophils from *Fcer1g*$^{-/-}$ mice prepared in a similar way failed to produce IL-4 (**Fig. 2f**) even though they had normal expression of IL-3Rα and βc (**Fig. 2g**). These results, together with the data reported above (**Fig. 1**), indicate that IL-3 signals diverge into at least two distinct pathways in basophils, with one for proliferation and one for IgE- and IgG-independent IL-4 production; only the latter pathway requires FcRγ. We also found that IL-3-induced production of IL-6, another basophil cytokine linked to T_H2 responses[29], was impaired if not completely abolished in basophil-enriched bone marrow samples from *Fcer1g*$^{-/-}$ mice (**Supplementary Fig. 4** online), which indicates that FcRγ is involved in the IL-3 signaling pathway leading to the production of IL-6 as well as IL-4.

Notably, basophil-enriched spleen cell samples from mice lacking another ITAM-containing adaptor, DAP12, showed no impairment in IL-3-induced IL-4 (**Fig. 2h**). In addition, DAP12 seemed to be

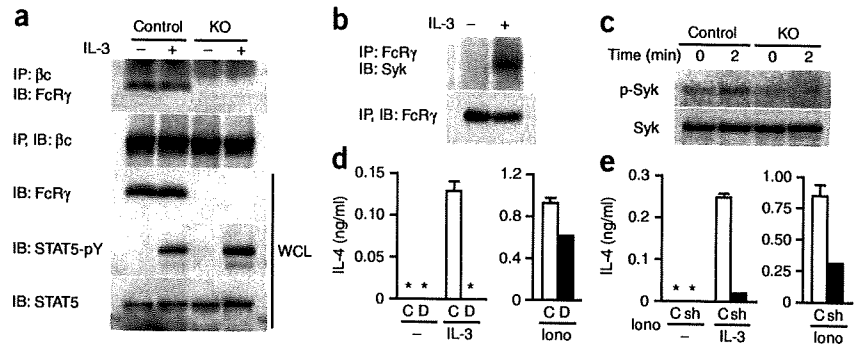

Figure 3 Constitutive and functional association of FcRγ with βc in basophils. (**a**) Immunoblot analysis of lysates of wild-type or *Fcer1g*$^{-/-}$ bone marrow–derived basophils 'starved' and then left untreated (–) or stimulated for 10 min with IL-3 (+), followed by immunoprecipitation (IP) with anti-βc and immunoblot (IB) with anti-FcRγ or anti-βc. WCL, immunoblot analysis of total and phosphorylated STAT5 and FcRγ in whole-cell lysates. (**b**) Immunoblot analysis of lysates of basophils enriched from wild-type bone marrow (26% basophils), then left untreated (–) or stimulated for 5 min with IL-3 (+), followed by immunoprecipitation with anti-FcRγ and immunoblot with anti-Syk or anti-FcRγ. (**c**) Immunoblot analysis of phosphorylated Syk (p-Syk) and total Syk in lysates of control or *Fcer1g*$^{-/-}$ basophil-enriched bone marrow cell samples (48% and 45% basophils, respectively) treated as described in **b**. (**d**) IL-4 production by bone marrow–derived basophils transduced with vector for the full-length Syk (C) or a dominant negative Syk mutant (D) and enriched (80% and 76% rCD2$^+$c-Kit$^-$FcεRIα$^+$, respectively) after stimulation with IL-3 (20 ng/ml) or ionomycin (Iono; 0.1 μg/ml). (**e**) ELISA of IL-4 production by bone marrow–derived basophils expressing a control (see Methods) (C) or Syk-specific (sh) shRNA (70% and 50% rCD2$^+$c-Kit$^-$FcεRIα$^+$, respectively) stimulated as described in **d**. *, concentration less than 0.01 ng/ml. Data represent experiments repeated three times (**a**,**d**) or two times (**b**,**c**,**e**) with similar results (mean and s.d. of duplicate cultures (**d**,**e**)).

expressed functionally in FcRγ-deficient bone marrow–derived basophils, as IL-4 production was induced normally after crosslinkage of the receptor CD200R3 (**Fig. 2i**), which has been shown to require DAP12 (ref. 30). Thus, IL-3 signals leading to IL-4 production requires FcRγ specifically, and DAP12 cannot functionally replace FcRγ.

FcRγ is a constitutive component of IL-3R

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

We also found that stimulation of basophil-enriched bone marrow samples with IL-3 induced recruitment of Syk to FcRγ (**Fig. 3b**) and resulted in more phosphorylation of Syk by a mechanism dependent on FcRγ (**Fig. 3c**). Furthermore, expression of a dominant negative Syk mutant (**Supplementary Fig. 6a,b** online) or short hairpin RNA (shRNA) specific for Syk (**Supplementary Fig. 6c,d**) abolished IL-3-induced production of IL-4 by bone marrow–derived basophils (**Fig. 3d,e**) without affecting the surface expression of either IL-3R or FcεRI (**Supplementary Fig. 6e**), as did the Syk inhibitor piceatannol (**Supplementary Fig. 7** online). In addition, IL-3-induced activation of mitogen-activated protein kinases, in particular Erk and Jnk, was substantially lower in the absence of FcRγ (**Supplementary Fig. 8** online). These results indicate that the FcRγ-Syk pathway is functionally involved in the IL-3-induced production of IL-4 by basophils. It was notable, in addition, that Syk and mitogen-activated protein kinases seemed to be phosphorylated to some extent even in unstimulated basophils, independently of FcRγ (**Fig. 3c** and **Supplementary Fig. 8**). Such 'background signals' might potentiate overall IL-4 production by basophils through as-yet-unknown signaling pathways other than the IL-3-FcRγ pathway, as ionomycin-induced production of IL-4 was diminished by Syk-specific shRNA and the dominant negative Syk mutant, albeit much less prominently than was IL-3-induced production of IL-4 (**Fig. 3d,e**).

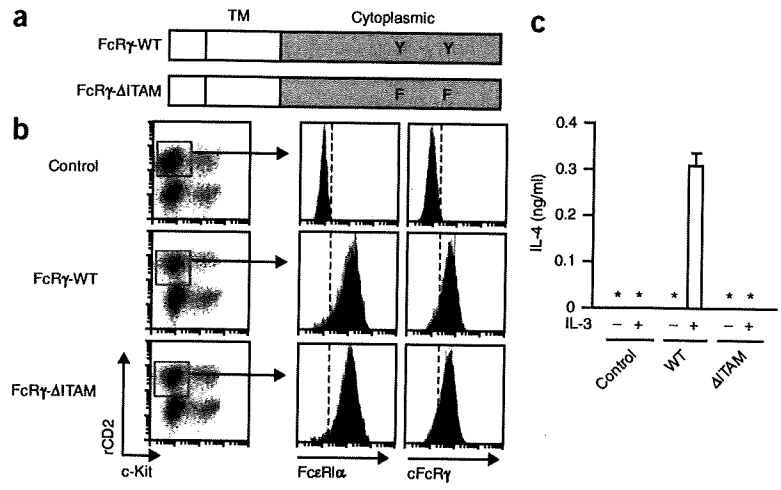

Figure 4 Essential function for FcRγ ITAM in IL-3-induced IL-4 production. (**a**) Wild-type FcRγ (FcRγ-WT) and mutant FcRγ (FcRγ-ΔITAM), with two tyrosine residues in the ITAM replaced with phenylalanine, inserted into retroviral vectors. TM, transmembrane portion. (**b**) Flow cytometry of the cell surface expression of FcεRIα and intracellular expression of FcRγ (cFcRγ) by *Fcer1g*$^{-/-}$ bone marrow–derived basophils transduced with retroviral vectors (rCD2$^+$c-Kit$^-$ cells; boxed at left). Control, cells transduced with empty vector. Dashed lines, boundary between positive and negative staining. (**c**) ELISA of IL-3-induced IL-4 production by 'starved' *Fcer1g*$^{-/-}$ bone marrow–derived basophils expressing no FcRγ (Control), wild-type FcRγ (WT) or FcRγ-ΔITAM (ΔITAM; 75, 65 or 72% rCD2$^+$c-Kit$^-$ cells, respectively). *, concentration less than 0.01 ng/ml; error bars, s.d. of duplicate cultures. Data are representative of more than four independent experiments.

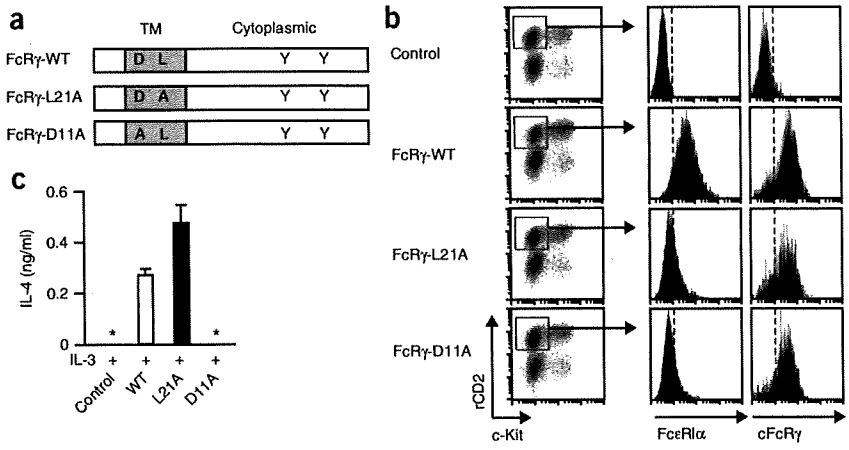

Figure 5 Different requirements for transmembrane amino acids in FcRγ for IL-3 responses. (**a**) Wild-type and FcRγ transmembrane mutants (L21A) and (D11A) used for transducing bone marrow–derived basophils. (**b**) Flow cytometry of the surface expression of FcεRIα and intracellular expression of FcRγ in *Fcer1g*$^{-/-}$ bone marrow–derived basophils transduced with the vectors in **a** or control vector, assessed in gated rCD2^{+}c-Kit^{-} cells (boxed at left). Dashed lines, boundary between positive and negative staining. (**c**) ELISA of IL-3-induced IL-4 production by 'starved' *Fcer1g*$^{-/-}$ bone marrow–derived basophils expressing no FcRγ (Control), wild-type FcRγ, or the L21A mutant or D11A mutant of FcRγ (70, 66, 57 or 50% rCD2^{+}c-Kit^{-} cells, respectively). *, concentration less than 0.01 ng/ml; error bars, s.d. of duplicate cultures. Data are representative of three independent experiments.

IL-4 production requires the FcRγ ITAM

In immunoreceptor signaling, Syk is recruited to the ITAM of FcRγ. To examine the function of the FcRγ ITAM in the production of IL-4 by basophils, we constructed retroviral vectors to complement FcRγ-deficient bone marrow–derived basophils with wild-type FcRγ and a mutant with a disrupted ITAM (FcRγ-ΔITAM; **Fig. 4a**). The ITAM of FcRγ is known to be dispensable for the transport of FcεRI to the surface of mast cells[32]. Consistent with that, when introduced retrovirally into FcRγ-deficient bone marrow–derived basophils, both wild-type FcRγ and FcRγ-ΔITAM restored the expression of FcεRI on the cell surface to a similar amount, mirroring the similar amounts of wild-type and mutant FcRγ (**Fig. 4b**). In contrast, wild-type FcRγ restored the IL-3-induced production of IL-4 by FcRγ-deficient bone marrow–derived basophils but FcRγ-ΔITAM did not (**Fig. 4c**), which demonstrates the essential function of the FcRγ ITAM in IL-3 signaling that leads to IL-4 production in basophils. It was also apparent that FcRγ-deficient basophils were otherwise sufficient in the 'machinery' required for IL-3 signal transduction for IL-4 production.

Structural requirements for the FcRγ-βc association

Even though DAP12 was unable to replace FcRγ in IL-3 signaling (**Fig. 2h,i**), the cytoplasmic portion of DAP12 was able to transduce IL-3 signals for IL-4 production when fused with the extracellular and transmembrane portions of FcRγ (**Supplementary Fig. 9** online), which suggests that the inability of DAP12 to replace FcRγ was due solely to the failure of its extracellular and transmembrane portions to associate with βc. Given that the transmembrane portion of FcRγ is important in its association with immunoreceptors, it is plausible that the same portion is also involved in its association with βc. We transduced FcRγ-deficient bone marrow–derived basophils with an FcRγ mutant containing an alanine residue in place of the leucine at position 21 in the transmembrane portion (FcRγ-L21A; **Fig. 5a** and **Supplementary Fig. 10a** online), which is a mutant known to be unable to bind to FcεRIα or FcαRI (ref. 33). Bone marrow–derived basophils expressing FcRγ-L21A indeed failed to express FcεRI on the surface even though they had similar expression of FcRγ-L21A and wild-type FcRγ protein (**Fig. 5b**). Notably, despite their inability to express FcεRI on the cell surface, 'starved' bone marrow–derived basophils expressing FcRγ-L21A produced IL-4 in response to IL-3 even more efficiently than did those expressing wild-type FcRγ (**Fig. 5c**), which indicates that the mode of the FcRγ-βc association is distinct from that of FcεRI and FcRγ. The relatively augmented IL-4 production by bone marrow–derived basophils expressing FcRγ-L21A might have been due to the greater availability of FcRγ-L21A molecules for association with βc, as FcεRI would not be able to compete with βc for this mutant. That observation further supports the FcεRI independence of IL-3-induced production of IL-4 by basophils. However, another mutant FcRγ with replacement of an aspartic acid with an alanine residue at position 11 also in the transmembrane portion (FcRγ-D11A; **Fig. 5a**) restored neither surface FcεRI expression (**Fig. 5b**) nor IL-3-induced production of IL-4 (**Fig. 5c**). Thus, Asp11 of the transmembrane portion is essential for not only the association of FcRγ with FcεRI but also for its transduction of IL-3 signals in basophils.

To directly examine the physical association of wild-type and mutant FcRγ with βc, we retrovirally introduced Flag-tagged wild-type and mutant FcRγ into Y16 cells. Both wild-type FcRγ and FcRγ-ΔITAM associated with βc in Y16 cells, as did FcRγ-L21A; however, FcRγ-D11A did not associate with βc (**Fig. 6**). Although FcRγ-D11A migrated slowly by SDS-PAGE, the protein seemed to be translated correctly, as both anti-FcRγ and anti-Flag (specific for the Flag tag at the carboxyl terminus) bound to FcRγ-D11A (**Fig. 6**). The results reported above indicate that for the association of FcRγ with βc, the ITAM and the transmembrane Leu21 are dispensable, whereas the transmembrane Asp11 is essential. The different requirement for Leu21 indicates that the physical and functional association of FcRγ and βc is distinct from that of FcRγ and FcεRI (**Supplementary Figure 10b**).

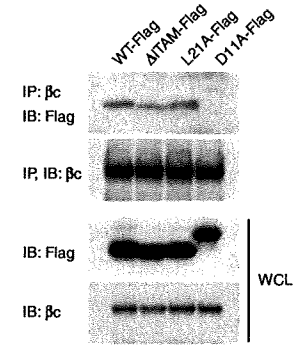

Figure 6 Different physical associations of FcRγ mutants with βc. Immunoblot of lysates of Y16 cells expressing Flag-tagged wild-type FcRγ, FcRγ-ΔITAM, FcRγ-L21A or FcRγ-D11A, analyzed with anti-Flag and anti-βc before (WCL) or after (IP) immunoprecipitation with anti-βc. Data are representative of three independent experiments.

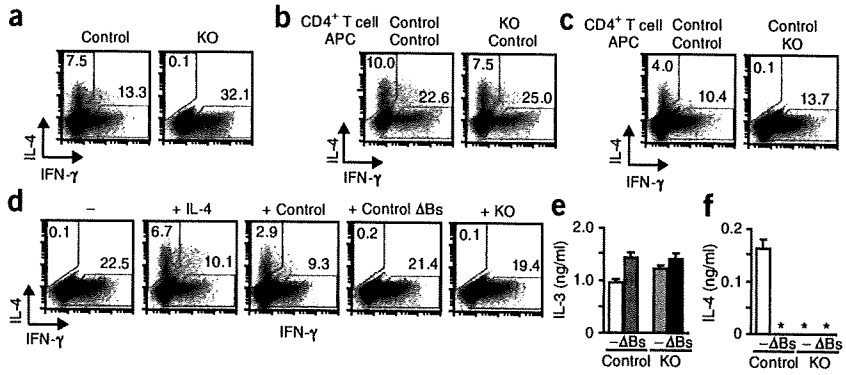

Figure 7 FcRγ-deficient basophils fail to support T_H2 differentiation *in vitro*. (**a–d**) Flow cytometry of T cells obtained from spleen cells prepared from control or *Fcer1g*$^{-/-}$ mice expressing the OT-II TCR transgene. Numbers in or above outlined areas indicate percent IL-4$^+$IFN-γ$^-$ cells (top left) or IL-4$^-$ IFN-γ$^+$ cells (bottom right). (**a**) Production of IL-4 and IFN-γ by gated CD4$^+$ T cells. (**b**) Helper T cell differentiation in cultures of splenic CD4$^+$ T cells from OT-II TCR-Tg control or *Fcer1g*$^{-/-}$ mice in the presence of wild-type APCs. (**c**) Helper T cell differentiation in cultures of wild-type OT-II TCR-Tg CD4$^+$ T cells with APCs prepared from control or *Fcer1g*$^{-/-}$ mice. (**d**) Generation of helper T cells in cultures of spleen cells from OT-II TCR-Tg *Fcer1g*$^{-/-}$ mice with no supplementation (–) or supplemented with exogenous IL-4 (+ IL-4), 2×10^4 basophil-enriched bone marrow cell preparations from control mice (+ Control; 42% basophils) or *Fcer1g*$^{-/-}$ mice (+ KO; 47% basophils), or bone marrow depleted of DX5$^+$ basophils (+ Control ΔBs; 1.2% basophils). (**e,f**) Production of IL-3 (**e**) or IL-4 (**f**) on day 2 in cultures of whole spleen cells (–) or basophil-depleted spleen cell samples (ΔBs) from wild-type or *Fcer1g*$^{-/-}$ OT-II TCR-Tg mice (mean and s.d. of duplicate cultures). *, concentration less than 0.01 ng/ml. Data are representative of more than three (**a,d**), three (**e,f**) or two (**b,c**) experiments.

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

We found that in contrast to spleen cells prepared from wild-type mice transgenic for the OT-II T cell receptor (OT-II TCR-Tg mice), those from OT-II TCR-Tg *Fcer1g*$^{-/-}$ mice did not differentiate into T_H2 cells in neutral conditions, as indicated by the impaired IL-4 production by differentiated T_H cells (**Fig. 7a**). We further found that OT-II TCR-Tg CD4$^+$ T cells purified from *Fcer1g*$^{-/-}$ mice differentiated readily into T_H2 cells when cultured in the presence of wild-type spleen samples depleted of T cells, B cells, NK cells and erythroid cells (called 'antigen-presenting cells' (APCs) here); CD11c^{hi} cells (DCs) and IL-3Rα$^+$DX5$^+$ cells (basophils) represented 10–12% and 3–4% of these cells, respectively (**Fig. 7b**). In contrast, 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 obtained from *Fcer1g*$^{-/-}$ mice (**Fig. 7c**). Notably, basophil-enriched cell preparations from wild-type mice compensated for the failure of OT-II TCR-Tg FcRγ-deficient spleen cells to generate T_H2 cells, as did exogenously supplemented IL-4, but basophil-enriched cell preparations from *Fcer1g*$^{-/-}$ mice did not (**Fig. 7d**). Although these basophil-enriched populations still contained unidentified cells other than DCs, basophils seemed to be responsible for the compensation, as depletion of DX5$^+$ cells made the preparations unable to restore the generation of T_H2 cells (**Fig. 7d** and **Supplementary Fig. 11** online). These observations collectively indicate that FcRγ expression is not required in CD4$^+$ T cells and DCs but is required in basophils for the efficient generation of T_H2 cells. T_H2 differentiation in this setting is has been shown to be completely dependent on IL-3 and IL-4 (ref. 20). Indeed, the *in vitro* cultures had considerable production of IL-3 independently of basophils and regardless of the *Fcer1g* genotype (**Fig. 7e**). IL-3 induced control basophils but not FcRγ-deficient basophils to produce 'early' IL-4 in those cultures, as IL-4 produced in the initial 2 d disappeared when wild-type cultures were depleted of basophils (**Fig. 7f**). Such 'early' IL-4 production was not present in FcRγ-deficient cultures at all (**Fig. 7f**). Thus, the inability of FcRγ-deficient spleen cells to support T_H2 differentiation seemed to be due to the failure of basophils to produce 'early' IL-4 in response to endogenously produced IL-3.

The *in vitro* observations reported above raised the possibility that *Fcer1g*$^{-/-}$ mice were defective in T_H2 responses. In agreement with that prediction, two reports have already shown that *Fcer1g*$^{-/-}$ mice fail to mount efficient T_H2 responses *in vivo*[34,35]. We also noted less-efficient production of serum IL-5, a T_H2 cytokine, in *Fcer1g*$^{-/-}$ mice than in control mice after infection with the T_H2-indicing nematode *T. spiralis*[36] (**Supplementary Fig. 12a** online), yet basophil population expansion in spleen and bone marrow occurred normally (**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 (ref. 37), were less frequent in mesenteric lymph nodes in *Fcer1g*$^{-/-}$ mice than in those of wild-type mice on day 10 of infection, and there was less ST2 on CD62L^{lo}CD4$^+$ T cells of *Fcer1g*$^{-/-}$ mice than on control cells (mean fluorescent intensity, 20.8 ± 5.3 and 11.5 ± 2.6, respectively; **Supplementary Fig. 12b**). Although we did not detect IL-4 in serum from uninfected or infected mice (less than 15 pg/ml), these results were in accordance with the *in vitro* observations (**Fig. 7a,f**) and suggested that *Fcer1g*$^{-/-}$ mice did not mount efficient T_H2 responses *in vivo* after infection with *T. spiralis*.

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 many 'downstream' signals, ensuring the functional pleiotropy of these cytokines. In this study, we found an unexpected function for the ITAM-bearing adaptor FcRγ in IgE-independent IL-3 signal transduction for IL-4 production but not for the proliferation or survival of basophils. As for the function of ITAM-bearing adaptors in nonimmunoreceptor signaling, both DAP12 and FcRγ are reported to be critical in the priming of macrophages by interferon-γ (IFN-γ) for enhanced IFN-α responses[38] and for 'outside-in' signaling through macrophage integrins[39]. In such cases, although Syk activation is essential, it remains to be determined if IFN-α receptors or integrins associate with FcRγ or DAP12, and the alternative possibility that other, unidentified FcRγ- and DAP12-associated receptors are involved in coupling IFN-α receptors or integrins functionally to the ITAM-mediated pathways cannot be excluded. Thus, this study is the first to our knowledge to demonstrate the incorporation of FcRγ into nonimmunoreceptors. Although DAP12 and FcRγ are redundant in the enhancement of IFN-α receptor and integrin signal transduction[38,39], FcRγ but not DAP12 was specifically required for IL-3 signals leading to IL-4 production in basophils, which emphasizes the uniqueness of this specific FcRγ function in IL-3 signal transduction.

It is well established that FcRγ associates with various cell surface receptors through an intramembrane interaction between the transmembrane portions of both partners. In a group of FcRγ-associated receptors, including FcαRI (CD89), NKp46, the platelet collagen receptor glycoprotein VI and the paired immunoglobulin-like receptor

A, an arginine residue in their transmembrane regions is required for interaction with the negatively charged aspartic acid residue of FcRγ[40]. In contrast, receptors such as FcεRI, FcγRI and FcγRIII lack the canonical transmembrane arginine but nevertheless are able to associate with FcRγ through mechanisms still not fully understood[33]. The same arginine residue is also absent from the transmembrane region of βc. In addition, we have shown here that Leu21 in the transmembrane region of FcRγ, a residue required for both arginine-dependent and arginine-independent associations[33], was dispensable for the physical and functional association of βc with FcRγ, which suggests that this association occurs by a mode distinct from those already known for FcRγ.

In contrast to various immunoreceptors, IL-3R was expressed normally even in the absence of FcRγ and was functionally competent in signaling for other events such as the proliferation of basophils. When participating in selective 'downstream' events elicited 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. An auxiliary function similar to that for FcRγ has been reported for FcαRI that can be expressed on the surface and mediate phagocytosis in neutrophils and macrophages but does not support other functions such as superoxide production and bacterial killing in the absence of FcRγ[41,42]. Integrins and IFN-α receptors can 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 unique to this cytokine system.

IL-3 and its related cytokines IL-5 and GM-CSF have been shown to induce recruitment of Syk to βc in a myeloid cell line and eosinophils[12,17]. In eosinophils, Syk is considered to be recruited directly to βc, possibly through ITAM-like 'Tyr-X-X-Leu' motifs (where 'X' is any amino acid) in the cytoplasmic region[12]. Although Syk could be recruited directly to βc in IL-3-stimulated basophils as well, independently of FcRγ, such recruitment and subsequent activation, if any, of Syk seems to be functionally irrelevant to IL-3 signal transduction for IL-4 production. In this context, it is known that mice but not humans have an additional βc-like molecule called 'β_{IL-3}' that can be a component of the receptor for IL-3 but not the receptors for IL-5 and GM-CSF[43]. Therefore, it is possible to speculate that only β_{IL-3} contributes to the IL-3 signaling pathway that leads to IL-4 production by recruiting Syk indirectly through FcRγ. However, as the structures of the transmembrane portions of βc and β_{IL-3} are almost indistinguishable, with nearly identical sequences but substitutions at only two positions, we do not consider it likely that only one of these two β-subunits associates selectively with FcRγ. Further studies are needed to explore the precise molecular mechanism of the FcRγ-βc association and to directly test those possibilities. Nevertheless, if both βc and β_{IL-3} associate 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.

The failure of FcRγ-deficient basophils to produce IL-4 in response to IL-3 resulted in the inability of mice infected with the T_H2-inducing nematode *T. spiralis* to support T_H2 differentiation *in vitro* and, conceivably, *in vivo*, which further substantiates the idea that basophils are the cells responsible for the early IL-4 production that leads to the initiation of T_H2 responses in allergy and helminth infection[18,21,44]. We also found 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 the production of IL-6, as well as IL-4, by basophils. The involvement of FcRγ in T_H2 responses *in vivo* has also been reported in studies in which *Fcer1g*$^{-/-}$ mice have shown inefficient T_H2 differentiation in lymph nodes after infection with *Leishmania major*[34] and milder airway inflammation than that of control mice[35]. The involvement of basophils was not examined in those studies, and instead such impaired type 2 immune responses were originally attributed to a 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 differed according to their means of inducing T_H2 responses or, alternatively, that basophils and DCs functioned with different timing, such as at the initiation and propagation phases, respectively, even though both 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], many cell species, not one single cell species, involved in T_H2 responses might be affected by FcRγ deficiency *in vivo*. Furthermore, as basophils also produce IL-4 in response to apparently FcRγ-independent stimulation through CD200R3, Toll-like receptor 2, the IL-18 receptor and other as-yet-unidentified 'receptors' for the proteases from house dust mites or parasites[28,30,45,46], the defect in FcRγ signals alone in basophils might not necessarily result in substantially impaired T_H2 responses. Nevertheless, as it is indispensable in both IL-3R- and FcεRI-mediated production of IL-4 in basophils, FcRγ may be more important in type 2 immune responses than considered before. In conclusion, the crosstalk identified here between the signaling pathways mediated by ITAM and those 'downstream' of the receptor for a 'βc cytokine' not only provides a new mechanism to account for the functional pleiotropy of some cytokines but also extends further the functional spectrum of ITAM-bearing adaptors beyond immunoreceptor signaling.

METHODS

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

Antibodies. Anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CD11b (M1/70), anti-CD117 (anti-c-Kit; 2B8), anti-CD123 (anti-IL-3Rα; 5B11), anti-CD49b (DX5), anti-CD90.2 (53-2.1), anti-F4/80 (BM8), anti-FcεRIα (MAR-1), anti-NKG2D (CX5), anti-NK1.1 (PK136), anti-TCRβ (H57-597), anti-B220 (RA3-6B2), anti-Sca-1 (D7), anti-Gr-1 (RB6-8C5), anti-Ter119 (TER119) and anti-Ly-6C (AL-21), as well as isotype-matched control antibodies, all conjugated to biotin, fluorescein isothiocyanate, phycoerythrin or allophycocyanin, were from e-Bioscience. Anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-βc (anti-CD131; JORO50), anti-IL-4 (BVD4-1D11) and anti-IFN-γ (XMG1.2), conjugated as described above, were from BD Biosciences. Fluorescein isothiocyanate–anti-ST2 (DJ8), biotin–anti–rat CD2 (anti-rCD2; OX34), and Alexa Fluor 647–anti–rabbit IgG (A21245) were from MD Biosciences, Cedarlane and Molecular Probes), respectively. These antibodies

were used for flow cytometry and/or cell separation. The antibody Ba91 (anti-CD200R3) has been described[30] and was used at a concentration of 1 μg/ml for stimulation of bone marrow–derived basophils. Polyclonal rabbit anti-Syk, anti-βc–β_{IL-3} (both from Santa Cruz Biotechnology, SC-1077 and SC-678 respectively) and anti-FcRγ (recognizing a carboxy-terminal epitope; Upstate Biotechnology, 06-727) were used for the detection of intracellular FcRγ, immunoprecipitation and immunoblot analysis. Monoclonal anti-STAT5 (89; BD Biosciences), monoclonal antibody to phosphorylated STAT5 (47; BD Biosciences) and monoclonal anti-Flag (M2; Sigma-Aldrich) were also used for immunoblot analysis. Monoclonal antibodies to the native and phosphorylated forms of the kinases p38 (27 and 30) and Erk (MK12 and 20A) were from BD Bioscience, and polyclonal anti-Jnk (9251 and 9252 to phosphorylated and total protein, respectively) was from Cell Signaling Technology.

Flow cytometry. After lysis of red blood cells with lysis buffer, cells were incubated with antibodies conjugated to biotin, fluorescein isothiocyanate, phycoerythrin or allophycocyanin. Fc-mediated nonspecific staining was blocked with anti-CD16/32 (2.4G2 hybridoma culture supernatant). Biotin-conjugated antibodies were visualized with phycoerythrin-indotricarbocyanine-streptavidin (BD Biosciences). A Cytomics FC500 flow cytometer and RXP software (Beckman-Coulter) were used for analysis of stained cells. In cells fixed and made permeable with Cytofix/Cytoperm Plus (BD Biosciences), FcRγ was stained with rabbit anti-FcRγ followed by development with Alexa Fluor 647–anti–rabbit IgG. Basophil-enriched bone marrow cell samples (described below) stimulated with IL-3 (5 ng/ml) were staining intracellularly for phosphorylated STAT5 for 15 min with Alexa Fluor 647–conjugated antibody (612599) according to the protocol provided by the supplier (BD Biosciences) with the slight modification that fixed cells were treated for only 20 min with 90% (vol/vol) methanol.

Basophil preparation and culture. Enrichment of bone marrow and splenic basophils was done as described[20] through the depletion of T cells, B cells, NK cells, natural killer T cells, mast cells and erythroid cells, DCs and granulocytes with anti-TCRβ, anti-B220, anti-NKG2D, anti-Sca-1, anti-Gr-1, anti-Ter119, anti-CD11c, anti-c-Kit and anti-Ly-6C and IMag beads (BD Biosciences). Bone marrow–derived basophils were obtained as described[28] with slight modification, followed by enrichment for c-Kit$^-$ cells and 'starvation' (**Supplementary Methods** online). The purity of fresh spleen, bone marrow and 'starved' bone marrow–derived basophils subjected to stimulation is described in figure legends where relevant. For analysis of morphology, basophils were enriched from bone marrow as described 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' bone marrow–derived basophils (1×10^5 cells) were stimulated for 24 h with IL-3 (5 or 20 ng/ml) and supernatants were collected for measurement of IL-4 with a mouse IL-4 enzyme-linked immunosorbent assay (ELISA) kit (eBioscience). A chemical inhibitor of Syk (piceatannol; Calbiochem) was added at various concentrations (**Supplementary Fig. 7**). The calcium ionophore ionomycin (Wako) was used at a concentration of 0.1 or 0.5 μg/ml. Intracellular IL-4 was analyzed by flow cytometry in basophils stimulated for 18 h with IL-3 and treated with GolgiStop (BD Biosciences) for the final 6 h. Cells were surface stained, fixed and made permeable and then were stained with phycoerythrin-conjugated anti-IL-4 (BD Biosciences). Total RNA was prepared from basophils stimulated for 6 h with IL-3 (5 ng/ml) and was converted to cDNA with the ImProm-II Reverse Transcription system (Promega). The Thermal Cycler Dice Real-Time System and a SYBR Premix Ex Taq kit were used for quantitative PCR analysis according to the manufacturer's instructions (Takara Bio; PCR primers, **Supplementary Methods**). IL-4 mRNA in each sample was calculated relative to that of β-actin determined by parallel amplification.

DNA construction. The cDNA encoding the dominant negative Syk mutant lacking the kinase domain was constructed by amplification of the portion of mouse Syk cDNA (**Supplementary Fig. 6a**) corresponding to amino acid positions 1–260 (1–261 of human Syk[48]) and was inserted into the pMX-IRES-rCD2 vector (modified from the original pMX-IRES-GFP vector provided by T. Kitamura). A sequence in Syk (target sequence; **Supplementary Fig. 6c**) and a control sequence (5′-TCTTAATCGCGTATAAGGC-3′) were used as target sequences together with their respective antisense sequences for construction of the retrovirus vectors expressing shRNA based on pSINsi-mU6, which contains a neomycin-resistance cassette (Takara). The cDNA constructs encoding wild-type FcRγ and FcRγ-ΔITAM have been described[32]. Fragments of cDNA excised with *SpeI* and *NotI* expressing full-length FcRγ and FcRγ-ΔITAM were inserted into the pMX-IRES-rCD2 vector. Constructs encoding FcRγ-L21A and FcRγ-D11A mutants, with point substitutions in the transmembrane portion, were created by PCR and were introduced into the pMX-IRES-rCD2 vector. The cDNA constructs expressing wild-type FcRγ and mutant FcRγ (FcRγ-ΔITAM, FcRγ-L21A and FcRγ-D11A) tagged with Flag at the carboxyl terminus were generated by PCR; these were cloned also into the pMX-IRES-rCD2 vector. Construction of vectors for the FcRγ-DAP12 fusion protein is described in the **Supplementary Methods**.

Retroviral infection. Retroviral vectors created as described above were used for transduction of bone marrow–derived basophils and the Y16 pro–B cell line[31]. These retroviral constructs were transiently transfected into the packaging cell line Phoenix with FuGene-6 (Roche Diagnostics). Retrovirus-containing supernatants were collected 48 h after transfection, were concentrated tenfold by centrifugation and were added to 12-well plates, which were treated for 2 h at room temperature (25 °C) with RetroNectin solution (50 μg/ml in PBS; Takara Bio), followed by 30 min in 2% (wt/vol) BSA in PBS. After incubation of the plates for 4 h at 30 °C with viral supernatants, bone marrow–derived basophils (0.5×10^6 or 1×10^6 cells per ml) were added, followed by infection for 2 d. The bone marrow–derived basophils used for infection were prepared by culture for only 10 d rather than 13 d and these samples were enriched for c-Kit$^-$ cells. Infected bone marrow–derived basophils were 'starved' for 12–18 h, were enriched for rCD2$^+$ cells with IMag beads and AutoMACS, and were used for stimulation. The Y16 cell line was infected similarly with retrovirus vector carrying wild-type FcRγ or FcRγ or its mutants tagged with Flag at the carboxyl terminus, and cells stably expressing rCD2 were selected with IMag beads and AutoMACS. Infection with retrovirus expressing Syk-specific shRNA and selection of infected cells is described in the **Supplementary Methods**.

Immunoprecipitation and immunoblot analysis. Bone marrow–derived basophils were 'starved' for 12–18 h in the absence of IL-3, followed by stimulation for 10 min with IL-3 (20 ng/ml). Freshly isolated bone marrow basophils were enriched as described above and were stimulated for 2, 5 or 10 min with IL-3 (5 ng/ml). Those cells and Y16 cells were lysed with lysis buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitor 'cocktail' (Roche Diagnostics). Samples were immunoprecipitated by incubation of cell lysates for 2 h at 4 °C with antibodies (2 μg) and protein G–Sepharose (GE Healthcare). Those immunoprecipitates and whole-cell lysates were boiled in sample buffer, separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes, followed by immunoblot analysis with various antibodies. Blots were developed with Immobilon Western reagent (Millipore) and were analyzed with Cool Saver (Atto).

T_H1-T_H2 differentiation *in vitro*. T_H1 and T_H2 differentiation was induced *in vitro* with OT-II TCR-Tg spleen cells as described[20]. Red blood cells in spleen samples were lysed; spleen cell samples (1×10^6) were depleted of Ter119$^+$, NKG2D$^+$ and B220$^+$ cells and then were cultured with 0.5 μM chicken ovalbumin peptide (residues 323–339). The starting spleen cell preparations were 2–3% CD11c^{hi} cells (DCs) and 0.8–1.0% DX5$^+$IL-3Rα$^+$ cells (basophils). Details of the methods used for culture of CD4$^+$ T cells and APCs and supplementation of basophils are provided in the **Supplementary Methods**. T cells recovered on day 5 from cultures established as described above were restimulated for 5 h with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) and were stained with phycoerythrin–anti-IL-4 and fluorescein isothiocyanate–anti-IFN-γ (BD Biosciences) with the Cytoperm/Cytofix Plus kit according to the instructions of the supplier (BD Biosciences). For detection of 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 measured with 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 were used for inoculation (300 larvae per mouse) as described[49]. At 10 or 11 d after inoculation, mice were killed and cells were obtained from spleen, bone marrow and mesenteric lymph nodes for counting of basophils and for analysis of the expression of ST2 and CD62L on $CD4^+$ cells by flow cytometry. IL-5 in serum was also measured with OptEIA kits (BD Biosciences).

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

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001261, A000040 and A002396.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.H. designed and did experiments and wrote the manuscript; S.Y. helped with vector construction and provided critical reagents; Y.S. did experiments; K.O., 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.

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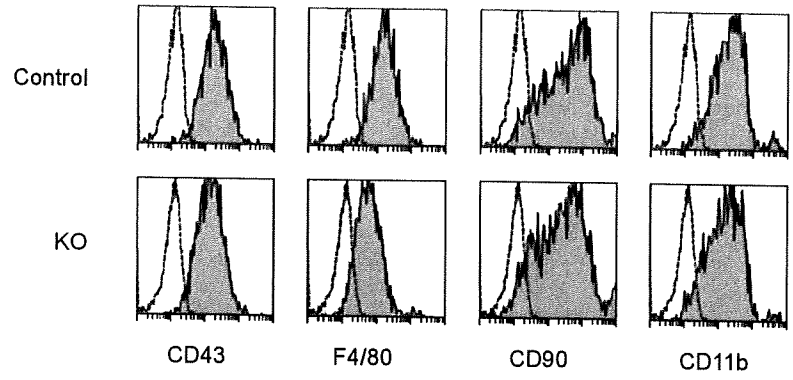
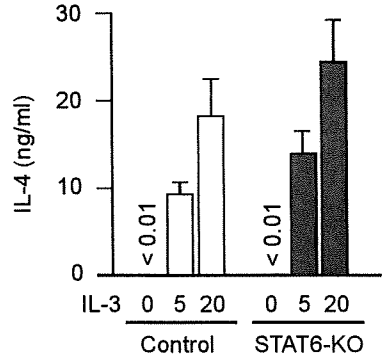
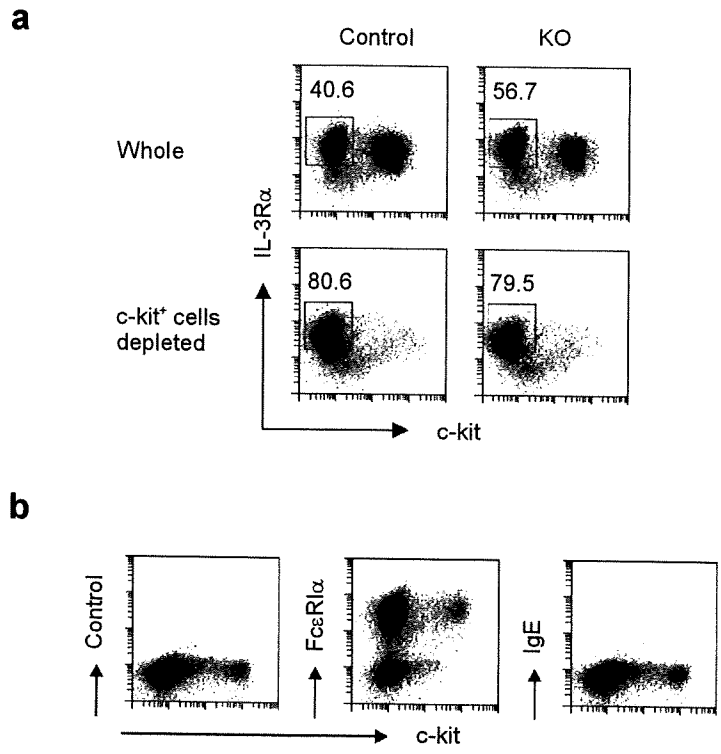


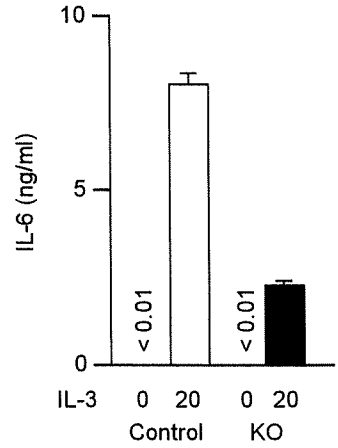
Supplementary figure 1. Surface phenotype of FcRγ-deficient basophils. Freshly isolated BM cells from control and *Fcer1g*$^{-/-}$ (KO) mice were stained with antibodies indicated. Shown are the histograms (shaded) for the gated IL-3Rα^{+}DX5^{+}c-kit^{-} cells. Both control and FcRγ-deficient basophils were negative for NK1.1 and Gr-1 (not shown). F4/80 staining was slightly lower on FcRγ-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.



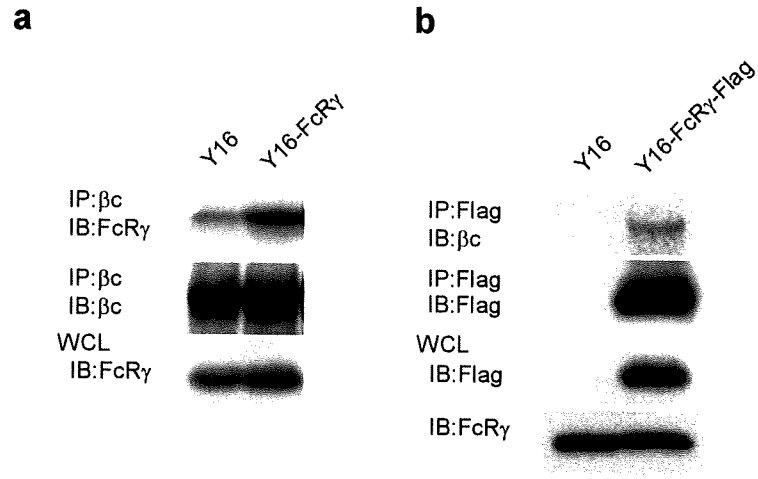
Supplementary figure 2. ELISA for IL-4 production from STAT6-deficient basophils. Basophil-enriched BM cells were prepared and stimulated with the indicated concentrations of IL-3, as in **Fig.2a**. Data represent the means and s.d. of duplicated cultures. Representative of two independent experiments.



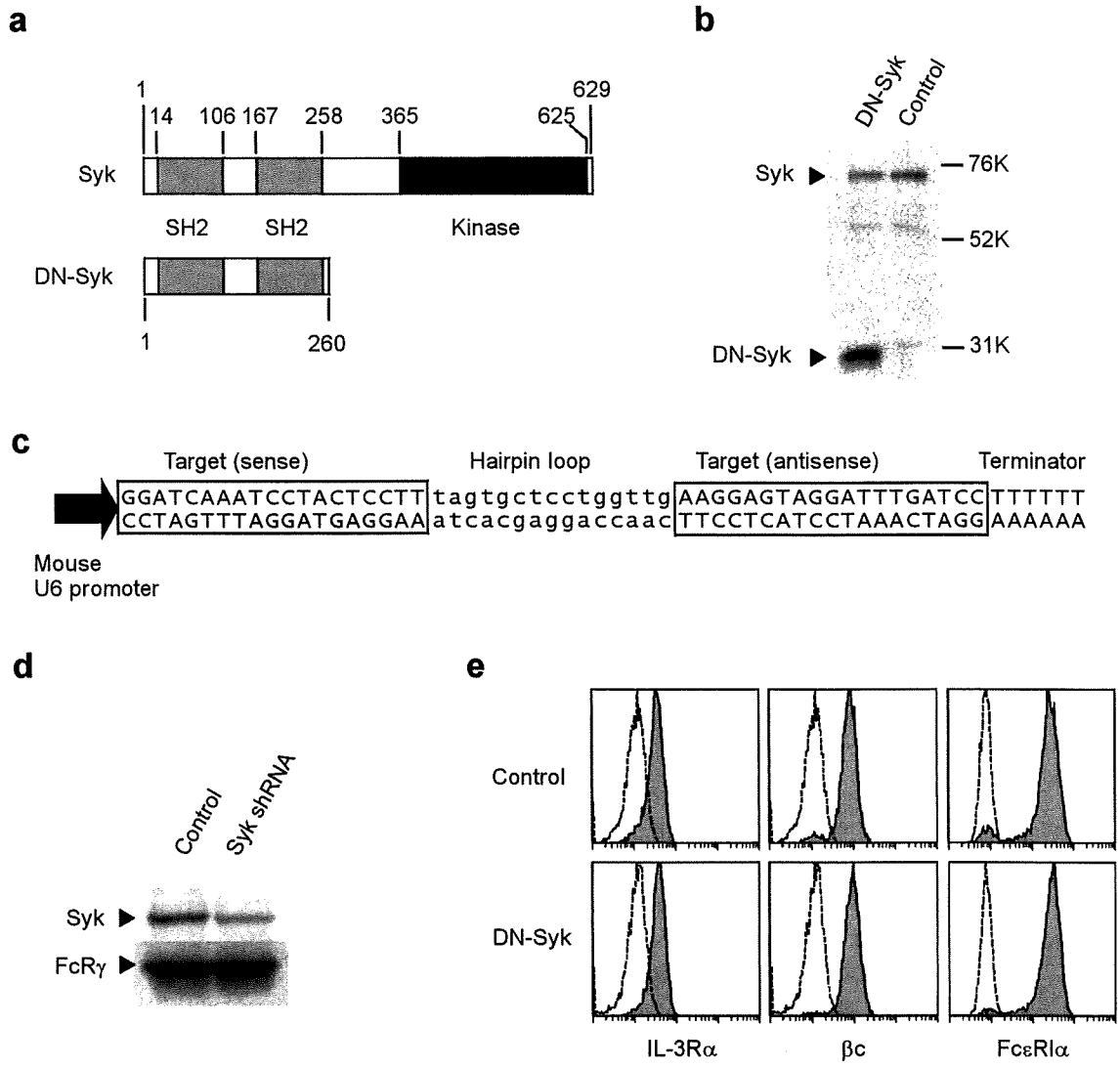
Supplementary figure 3. Enrichment of 'starved' BM-derived basophils. (**a**) BM-derived basophils derived from control and *Fcer1g*$^{-/-}$ (KO) mice were starved as in **Fig.2f** and stained with IL-3Rα and c-kit antibodies untreated (Whole) or after depletion of c-kit^{+}cells. The numbers shown are 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-FcεRIα or PE-anti-IgE. Note that both c-kit^{-} cells (basophils) and c-kit^{+} cells (mast cells) express FcεRI but do not bear IgE, indicating that their FcεRI is empty. Representative of at least three (**a**) and two (**b**) independent experiments.



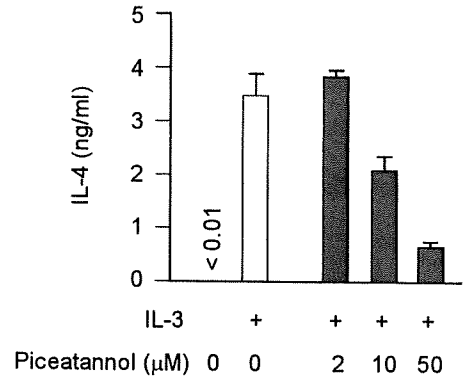
Supplementary figure 4. Impaired production of IL-6 by FcRγ-deficient basophils in response to IL-3. Basophil-enriched BM cells prepared from control or *Fcer1g*$^{-/-}$ mice (KO) were stimulated with IL-3 (20 ng/ml). The percentages of basophils in these cell preparations were 30-40%. The amounts of 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-FcRγ. Whole cell lysates (WCL) were also blotted with anti-FcRγ. Note that the amounts of FcRγ co-precipitated with βc from Y16-FcRγ were higher than those from Y16, as were those of total FcRγ. (**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 FcRγ (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 FcRγ in BM-derived basophils transduced with control or Syk-shRNA-expressing vectors. Representative of two independent experiments. (**e**) Cell surface staining for IL-3Rα, 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.