

Interleukin-3-dependent potentiation of IgE responsiveness in mouse basophils

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Abstract

Basophils produce interleukins (IL)-4 in response to various stimuli and may contribute to type 2 immune responses to various infections and allergens. We found that resting basophils freshly isolated from mice produce IL-4 in response to IL-3 but not to high-affinity Fc receptor (FcεRI) cross-linking (CL), yet both required the immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor Fc receptor γ-chain (FcRγ), while basophils activated in vitro by IL-3 become responsive to FcεRI CL. Acquisition of responsiveness to FcεRI CL occurred upon infection with *Trichinella spiralis* or administration of superantigen. Because cultured basophils return to a quiescent state upon starvation with IL-3 with surface FcεRI levels unchanged, this acquisition is reversible and probably reflects intracellular events requiring protein synthesis. Interestingly, similar activation-associated acquisition was observed for responsiveness to other stimuli, including CD200R3 CL, which is known to signal via DAP-12, and the allergen protease papain. This acquisition of responsiveness to FcεRI CL was inhibited by Jak inhibitor. Thus, the IL-3 signal bifurcates downstream of Jak, into two distinct pathway, one leading to IL-4 production and the other to render basophils competent to respond to stimuli dependent on ITAM-containing adaptors DAP12 and FcRγ for IL-4 production.

KEYWORDS

basophils, cytokine, helminth infection, IgE

1 | INTRODUCTION

It has been well known that differentiation from naive T cells into effector helper T cells is instructed by cytokines produced by various types of accessory cells (Abbas

et al., 1996; Reinhardt et al., 2006). Thus, T helper 1 (Th1) cell differentiation is initiated by interleukins (IL)-12 that is produced by dendritic cells upon stimulation through receptors for pathogen-associated molecules (Trinchieri, 2003). T helper 1 (Th17) cells are on the other

hand induced by transforming growth factor- β from unidentified cells and by dendritic cells (DC)-derived IL-6 and/or IL-23 (Stockinger & Veldhoen, 2007). In contrast, although IL-4 is well established as an essential cytokine for T helper 2 (Th2) differentiation, the cellular source(s) of this cytokine is still of debate.

Among several possible cellular sources of IL-4 and IL-6, basophils are recently attracting growing attention (Falcone et al., 2006; Min & Paul, 2008). Since the pioneering works by Paul's group on murine basophils (Ben-Sasson et al., 1990; Seder, Paul et al., 1991), these cells in mice and humans have been recognized as a cell type potentially capable of initiating type 2 immune responses by producing IL-4 in response to various stimuli. Such stimuli include IL-3, *Schistosoma mansoni* egg antigens, *Brugia malayi* antigens, *Echinococcus multilocularis* extracts and signals through cell surface molecules, such as high-affinity Immunoglobulin E (IgE) receptors (Fc ϵ RI) and CD200R3 (Aumuller et al., 2004; Falcone et al., 1996; Hida et al., 2005; Kojima et al., 2007; Mitre et al., 2004). Furthermore, basophils have recently been shown to accumulate in the spleen and liver in mice treated with Th2-inducing agents (Khodoun et al., 2004; Min et al., 2004; Voehringer et al., 2004). The allergen protease papain was reported to directly induce IL-4 production by basophils as well as to cause basophil accumulation in the regional lymph nodes where Th2 responses were assumed to be initiated (Sokol et al., 2008). We also observed previously that basophils could initiate Th2 differentiation in vitro in a manner dependent on IL-3, and elevation of basophil numbers in vivo due to the absence of negative regulator interferon-regulatory factor-2 resulted in relative Th2 dominance (Hida et al., 2005). Oh et al. (2007) reported that chronic treatment with IL-3 in vivo increased basophils numbers and accelerated Th2 immune responses. All these findings strongly supported the role of basophils in the initiation and promotion of Th2 responses. In addition, basophil-derived IL-6 is also important for Th17 differentiation and recruitment to inflammatory tissues (Denzel et al., 2008; Yuk et al., 2017).

Nature of the stimuli known to induce basophils to produce cytokines seems to be diverse; not to mention various pathogen-associated molecules including those molecularly not well defined, IL-3 is a local cytokine released rapidly upon antigen-activation of CD4⁺T cells, whereas IgE antibodies are a circulating product of class-switched B cells, generated much later as a result of Th2 responses. IL-3 signals are transduced through the intracellular pathway initiated upon binding to IL-3Rs, involving Janus kinases and STAT-5 (Geijsen et al., 2001), while IgE signals are mainly transduced through the immunoreceptor tyrosine-based activation motif (ITAM)-

containing adaptor Fc receptor γ -chain (FcR γ), a component of Fc ϵ RI as well as various immunoreceptors (Kinet, 1999). Some of pathogen-derived antigens required IgE for stimulating basophils (Aumuller et al., 2004; Mitre et al., 2004; Schramm et al., 2007), and hence likely signaled also through FcR γ . CD200R3 signals are mediated on the other hand by another molecule DAP-12, which is also an ITAM-containing adaptor (Kojima et al., 2007). Although the basophil "receptors" and downstream signals for many allergens and pathogens are to be identified, basophils seem to be a rather surprisingly versatile immune regulator that can produce IL-4 in response to such a wide variety of signals. It is not clear, however, whether cellular states of basophils responding to those diverse stimuli are identical or not, and the mechanisms controlling the responsiveness of basophils to those stimuli are totally unknown.

Here, we show that basophils respond differently to these stimuli depending on their activation state. In the "resting" state, basophils produced IL-4 and IL-6 in response to IL-3, but not to Fc ϵ RI or CD200R3 cross-linking and papain treatment. In contrast, upon "activation" by IL-3 in vitro or by nematode infection and T-cell-directed superantigen stimulation in vivo, basophils lose IL-3 responsiveness while acquired responsiveness to Fc ϵ RI CL, CD200R3 CL, and papain treatment. Thus, IL-3 switches intracellular signaling pathways through which basophils are induced to IL-4 and IL-6.

2 | RESULTS

2.1 | Activation of basophils for responsiveness to Fc ϵ RI signals

Bone marrow (BM) cells were isolated from untreated mice, which were kept in a strict clean room. Enriched basophils, removed with T, B, NK, macrophages, and erythrocytes from BM cells, were cultured for 24 h in vitro in the presence of IL-3 or IgE/anti-IgE immune complex. In BM, only 0.5% of cells were basophils (Fc ϵ RI α^+ DX5⁺), and when enriched up to >30% purity and stimulated with IL-3, substantial amounts of IL-4 were produced (Figure 1a), while basophils stimulated with IgE immune complexes produced less IL-4. Seder, Plaut, et al. (1991) reported that splenic Fc ϵ RI⁺ cells, the frequencies of which were increased several folds in mice infected with nematodes or treated with anti-IgD antibody, produced IL-4 upon stimulation ex vivo with IL-3 plus plate-bound anti-IgE antibodies. It was not directly examined, however, if these cells, most likely basophils, were activated in such settings. We also observed here that infection with a Th2-inducing nematode, *Trichinella*

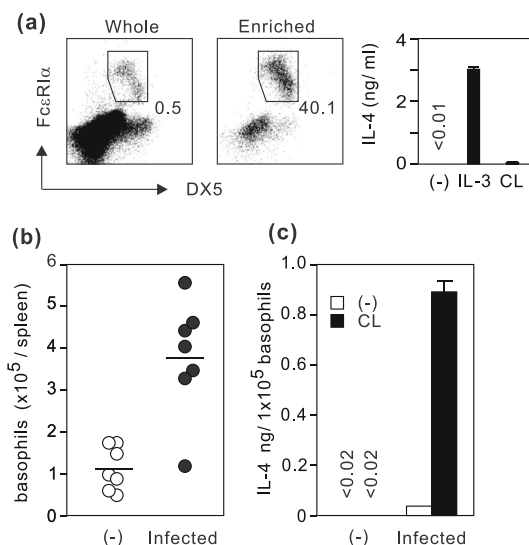


FIGURE 1 The acquisition of the responsiveness to FcεRI CL by basophils. (a) IL-4 production by fresh basophil-enriched populations in response to IL-3. Freshly isolated basophil preparations enriched as in each panel (1×10^5 /well) were stimulated with medium (–), IL-3 or FcεRI CL (CL) for 24 h, and the amounts of IL-4 produced are shown. (b) Numbers in the dot plots represent the frequencies of basophils as gated by the regions (DX5⁺FcεRI⁺). Numbers of basophils in the spleen in uninfected mice and mice infected with *Trichinella spiralis*. Each symbol represents an individual mouse with horizontal bars for the means ($n = 7$). (c) Splenic basophils enriched from uninfected or *T. spiralis*-infected mice were stimulated by FcεRI CL. IL-4 produced per 1×10^5 basophils is shown. Representative data of mice shown in (a). In panels (a) and (c), data for IL-4 production represent the means and SEMs of duplicated cultures and representative of more than three independent trials.

spiralis, caused not only the elevation of basophil numbers in the spleen (Figure 1b) but also the acquisition of the responsiveness to FcεRI CL (Figure 1c). As we calculated the amounts of IL-4 produced per 10^5 basophils present in the cell preparations, elevation in basophil numbers did not account for the upregulation of IL-4 production. This observation was consistent with many previous reports (Gessner et al., 2005; Mohrs et al., 2005; Schramm et al., 2007; Sokol et al., 2008) and indicated that activation of basophils rendered them responsive to FcεRI CL in terms of IL-4 production not only in vitro but also in vivo.

2.2 | T-cell activation results in the acquisition of IgE responsiveness in mouse basophils

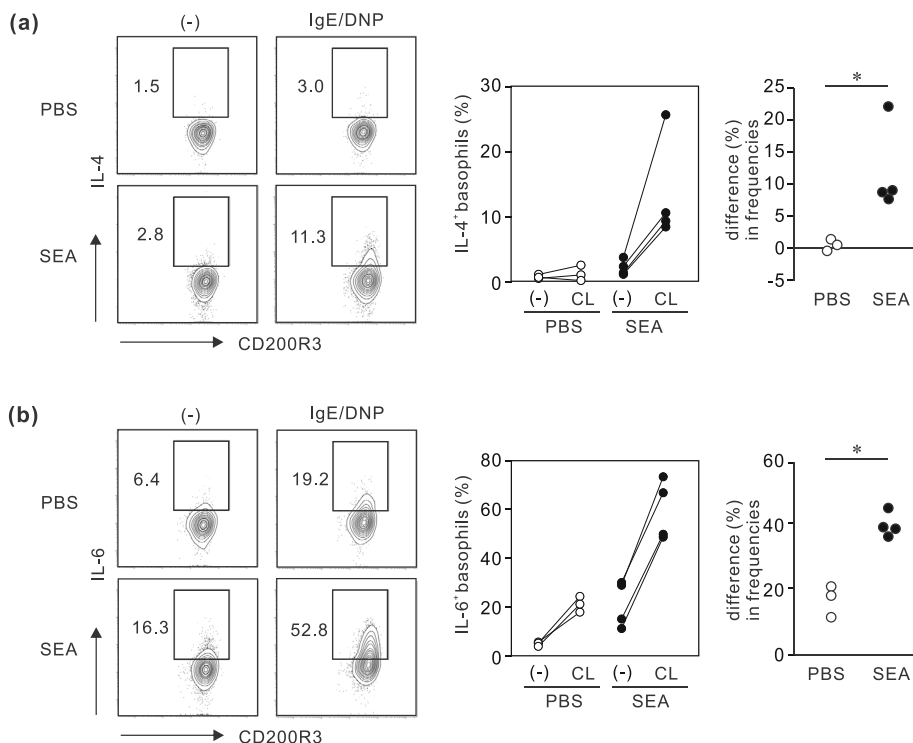
To examine IgE reactivity of basophils when T cells are activated in vivo, we administered the superantigen

recombinant Staphylococcal enterotoxin A (rSEA) intraperitoneally and examined IL-4 and IL-6 produced by splenic basophils by IgE and antigen stimulation ex vivo at 6 h. Compared to PBS-treated mice, splenic basophils from SEA-treated mice showed a marked increase in the percentage of IL-4 (Figure 2a) and IL-6 (Figure 2b)-producing cells. The difference in cytokine-producing cells compared between unstimulated and stimulated in each mouse was also predominantly increased (Figure 2a, b). In contrast to the parasite infection experiment in Figure 1, these experiments were done within a short time, arguing against the involvement of IgE in the serum that would be produced much later.

2.3 | IL-3-dependent enhancement of IgE responsiveness in mouse basophils

It is known that SEA activates peripheral T cells to produce large amounts of IL-3 in addition to other cytokines such as IL-2 and IFN-γ. Because mice infected with parasites, IL-3 is known to cause basophil proliferation (Lantz et al., 1998). We hence examined whether rSEA-induced enhancement of basophils IgE responses is mediated by IL-3 produced by T cells. Depletion of CD4⁺ T cells by negative sorting with anti-CD4 antibody abolished SEA-induced IL-3 production in vitro almost completely. CD8⁺ T-cell depletion also reduced IL-3 production substantially, while B-cell depletion with anti-B220 antibody rather augment IL-3 production (Figure 3a), perhaps through elevating T-cell frequencies. These results indicated that IL-3 was produced by T cells, mainly CD4⁺ and to a lesser extent CD8⁺ T cells. Next, mice were injected intraperitoneally with recombinant IL-3, and IgE reactivity of basophils was examined 6 h later. Compared to PBS treatment, IL-3 treatment significantly increased the frequency of basophils producing IL-4, but not IL-6, upon IgE plus antigen stimulation (Figure 3b). The essential role for T-cell-derived IL-3 in vivo was indicated by the observation that rSEA failed to enhance IgE plus antigen-induced cytokine production by basophils isolated from *Rag1*^{−/−} and *Il3*^{tm1Tyb/tm1Tyb} mice lacking entire T cells and IL-3, respectively (Figure 3c). Since basophils were present at normal frequencies in these mice, these results also indicate that rSEA neither act directly on peripheral basophils nor induce IL-3 production by cells other than T cells. In addition, IgE reactivity of basophils in *Stat6*^{−/−} mice treated with rSEA was comparable to that of wild-type basophils (Figure 3d), suggesting that SEA-induced enhancement of IgE reactivity did not require Th2 differentiation or serum IgE.

FIGURE 2 T-cell-activation results in the acquisition of IgE responsiveness in mouse basophil *in vivo*. (a, b) Splenic basophils enriched from mice treated with PBS or rSEA (50 μ g/mouse) were sensitized with IgE *in vitro* and then stimulated with DNP-BSA. The frequency of IL-4⁺ (a) or IL-6⁺ (b) among basophils (CD200R3⁺DX5⁺) in the cultures was measured by flow cytometry. Representative data for mice shown in (a and b, left). The frequency of IL-4⁺ (a, middle) or IL-6⁺ (b, middle) cells in basophils derived from spleen of mice treated with PBS or rSEA. (a, b right) Each plot represents the increased amount of IL-4 or IL-6 positive basophils upon Fc ϵ RI CL (control; $n = 3$, rSEA; $n = 4$) * $p < 0.05$ (Mann-Whitney's U test).



2.4 | The acquisition of Fc ϵ RI CL responsiveness was activation-associated and seemed to occur through multiple mechanisms

When expanded in the presence of IL-3 *in vitro*, basophils defined as c-kit⁺ cells still expressed surface Fc ϵ RI (Figure 4a). Unlike freshly isolated basophils, however, those cultured basophils, enriched to around 90% purity by removing c-kit⁺ mast cells as shown in Figure 4a, produced IL-4 when stimulated with Fc ϵ RI CL, but instead not with IL-3 (Figure 4b). Cultured, but not freshly isolated, basophils expressed the activation marker CD69, and were thus apparently in the activated state (Yoshimura et al., 2002), while those basophils returned to the resting state upon starvation as indicated by the reduction of CD69 expression (Figure 4c). Flow cytometry also revealed the unaltered surface Fc ϵ RI levels before and after starvation, indicating that the acquisition of the responsiveness to Fc ϵ RI CL took place without surface Fc ϵ RI modulation and likely through intracellular mechanisms. In contrast, the levels of the α - and β -chains of IL-3Rs were altered depending on the activation status of basophils. Thus, IL-3Rs on cultured, IL-3-nonresponsive basophils were reduced, yet not completely null, in comparison with those on freshly isolated or starved basophils, and IL-3R expression was almost fully restored on starved basophils (Figure 4c).

2.5 | Distinct intracellular signaling pathways for IL-4 production in fresh and cultured basophils

Loss of responsiveness to Fc ϵ RI CL occurred not immediately but gradually over several hours upon removal of IL-3 (Figure 5a). Furthermore, inhibition of *de novo* protein synthesis with cycloheximide rendered basophils unresponsive to Fc ϵ RI CL even in the continuous presence of IL-3 with the kinetics similar to that observed during IL-3 starvation (Figure 5b). Since cycloheximide was removed when those basophils were stimulated by Fc ϵ RI CL, it was unlikely that the synthesis of IL-4 proteins itself was inhibited by cycloheximide treatment. The activated state thus seemed to be ensured by a certain as yet unidentified molecule(s) involved in the signaling pathway downstream of Fc ϵ RI, the expression of which appeared to be dependent on IL-3.

2.6 | Switching of basophil responsiveness to other ITAM-mediated signals

In addition to IL-3 or Fc ϵ RI CL, IL-4 production by cultured basophils could be induced upon CD200R3 CL with anti-CD200R3 antibody (Ba91), in a fashion dependent on DAP-12 (Kojima et al., 2007). We found that IL-3-starved basophils produced much less IL-4 compared

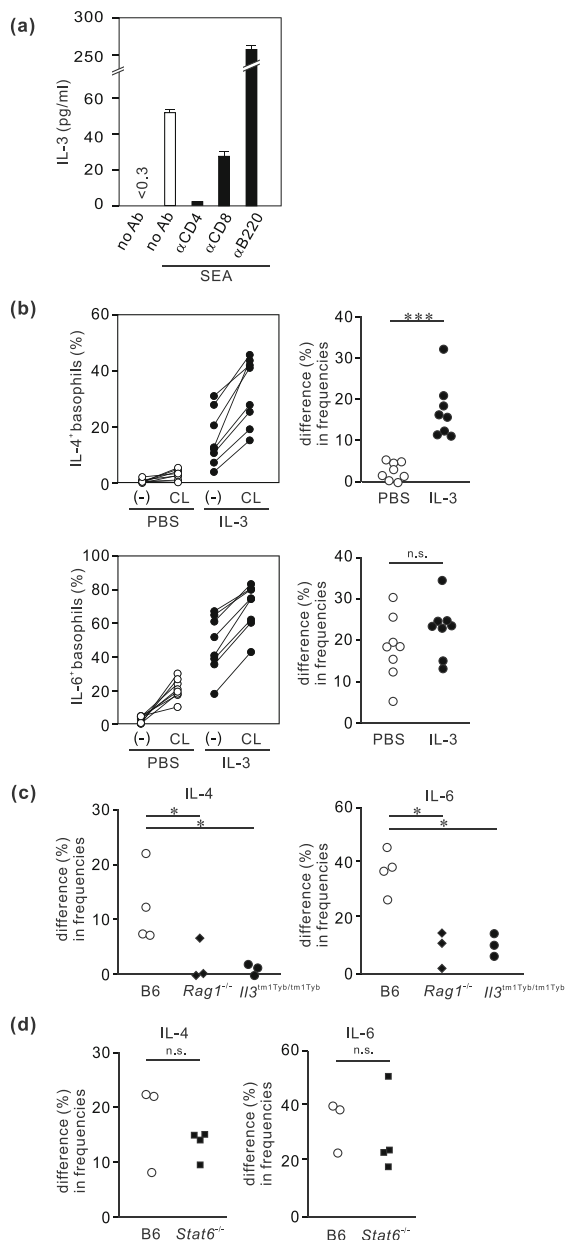


FIGURE 3 IL-3-dependent enhancement of IgE responsiveness in mouse basophils. (a) Mouse spleen cells were negatively sorted with anti-CD4, anti-CD8, or anti-B220 antibodies and stimulated with rSEA in vitro. Six hours later, IL-3 in the culture supernatant was measured by ELISA. (b) Mice were treated with PBS or rIL-3, then splenic basophils were enriched and stimulated with DNP-BSA after IgE sensitization; the frequency of IL-4⁺ (top) or IL-6⁺ (bottom) basophils was measured by flow cytometry ($n = 8$). (b, right) As in Figure 2, each plot represents the increased amount of cytokine-producing cells upon FcεRI CL stimulation of mouse basophils. (c) Increased frequency of IL-4 or IL-6 positivity cells upon FcεRI CL stimulation of splenic basophils when rSEA was administered to control mice, Rag1^{-/-} or Il3^{tm1Tyb/tm1Tyb} (control, $n = 4$; Rag1^{-/-}, $n = 3$; Il3^{tm1Tyb/tm1Tyb}, $n = 3$). (d) Increased frequency of IL-4 or IL-6 positivity cells upon FcεRI CL stimulation of splenic basophils when rSEA was administered to control mice, Stat6^{-/-} (control, $n = 3$; Stat6^{-/-}, $n = 4$). Data in (a) are representative of at least three independent experiments. * $p < 0.05$, *** $p < 0.001$. n.s., not significant (Mann–Whitney's U -test).

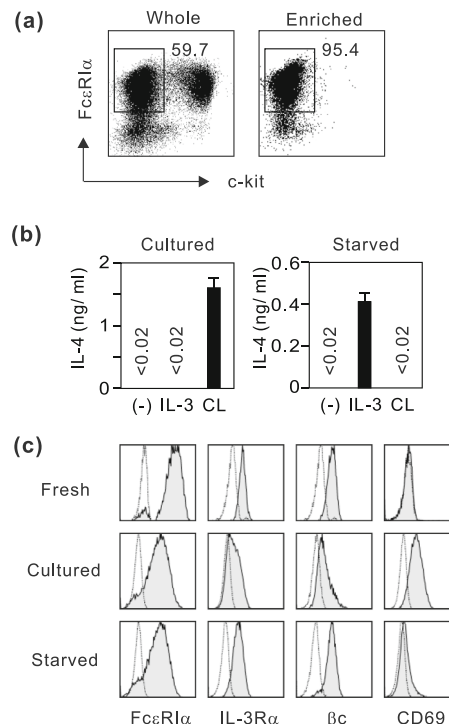


FIGURE 4 Surface marker profiles of resting and activated basophils. (a, b) Cultured basophil preparations enriched as in each panel (1×10^5 /well) were stimulated with medium (–), IL-3 or FcεRI CL (CL) for 24 h. (b) The amount of IL-4 produced by cultured or IL-3-starved basophils. (c) The expression of cell surface markers as indicated on fresh, cultured, and starved basophils. Histograms are shown for DX5⁺c-kit[–] cells for fresh basophils enriched as in Figure 1a and c-kit[–] cultured/starved basophils. Representative data for more than three independent experiments.

with those produced by cultured basophils upon CD200R3 CL (Figure 6a). As in the case for FcεRI (see Figure 4), the levels of surface CD200R3 were not different between cultured and starved basophils (Figure 6b), indicating that the failure of starved basophils to respond efficiently to CD200R3 CL was also likely due to the alteration of the intracellular state during starvation.

Consistent with what previously has been observed (Sokol et al., 2008), the allergen protease papain induced IL-4 production in cultured basophils albeit at the levels much lower than those observed upon CD200R3 CL or FcεRI CL but readily above control (Figure 6a). It was reported that papain-induced IL-4 production is dependent on the ITAM-containing adaptor protein FcRγ (Rosenstein et al., 2014). In order to confirm this result, we cultured BM cells from mice lacking the ITAM-bearing adaptor FcRγ in IL-3 and enriched c-kit[–] cells as above. FcRγ was essential for transport of FcεRI to the cell surface, basophils were negative for surface FcεRI expression and did not produce IL-4 upon FcεRI CL even in the activated state (Figure 6c). Notably, these basophils

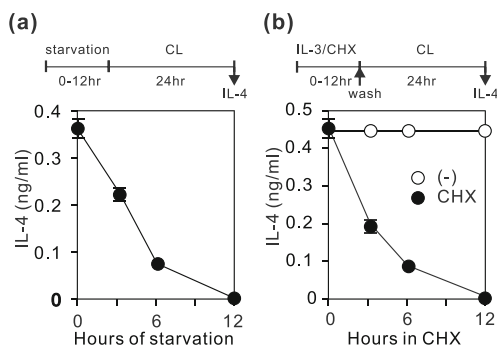


FIGURE 5 Distinct intracellular signaling pathways for IL-4 production in fresh and cultured basophils. (a) IL-4 production by cultured basophils starved for the indicated periods followed by stimulation with FcεRI CL for 24 h. (b) Basophils expanded in IL-3 for 13 days were further cultured with IL-3 for the indicated periods in the presence (CHX) or absence (–) of cycloheximide. Those cells were then washed extensively and stimulated with FcεRI CL for 24 h without cycloheximide, and IL-4 production by those basophils was measured. Data in panels represent the means, and error bars are for SEM of duplicated or triplicated cultures. Repeated at least three times with similar results.

failed to produce IL-4 upon treatment with papain (Figure 6d). In contrast, these Fcγ-deficient, activated basophils were fully capable of producing IL-4 in response to CD200R3 CL (Figure 6c). These observations indicated that the responsiveness to FcεRI CL, CD200R3 CL and papain was acquired and lost concurrently upon activation and starvation, respectively.

2.7 | Jak signaling is essential for the acquisition of FcεRI CL responsiveness

The IL-3 signaling pathway includes a Jak–Stat-mediated pathway that promotes cell proliferation and a pathway important for cytokine production via the Src family and Fcγ-Syk (Hida et al., 2009). Recently, Jak inhibitors have been developed and their indications have been expanded to autoimmune and allergic diseases. As shown in Figure 5, IL-3 starvation reduced time-dependent IL-4 production from cultured basophils. To investigate the involvement of Jak in this altered intracellular event, we examined IL-4 production from cultured basophils using AG490, an inhibitor of Jak2/3. Inhibitor was added for 3 or 6 h before FcεRI CL stimulation, and after removal of the inhibitor, stimulation with IgE/DNP immune complexes was performed (Figure 7a). The results showed that IL-4 and IL-6 productions were inhibited by Jak inhibitor in a concentration-dependent manner (Figure 7b, c). These results clearly indicate that Jak-dependent downstream signaling is important for the acquisition of responsiveness to FcεRI CL.

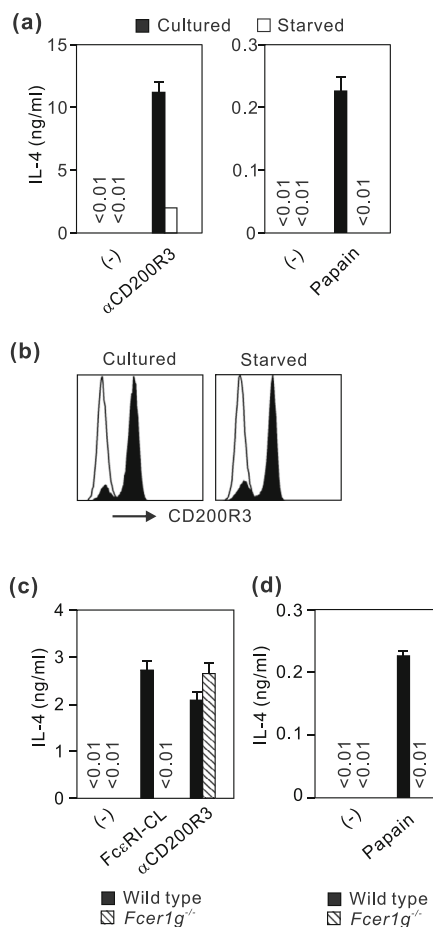


FIGURE 6 Similar switching for other ITAM-dependent stimulants. (a) Cultured and starved basophils were stimulated as indicated, and the amounts of IL-4 produced by 1×10^5 basophils are shown. (b) CD200R3 expression on cultured and starved basophils (c-kit⁺). Open histograms represent background staining. (c, d) IL-4 production by cultured basophils (1×10^5) from wild-type (black bar) or FcεRI^{-/-} (right hatched bar) mice stimulated as indicated. The means and SEMs of duplicated or triplicated cultures representative of at least three independent trials.

3 | DISCUSSION

In this study, we showed that murine basophils switched intracellular signaling pathways depending on their activation status, thereby responding to divergent stimuli that led to IL-4 and IL-6 production. Thus, basophils acquired responsiveness to FcεRI CL upon activation. Because IL-3 starvation reverted the basophil responsiveness to these stimuli, it was apparent that such a switching was not a result of basophil maturation, but instead activation-associated. It has become clear that there are Fcγ-dependent and Fcγ-independent signals downstream of the IL-3 signaling pathway. The Fcγ-dependent IL-3 signaling pathway is the pathway that induces IL-4 production in resting basophils, which we

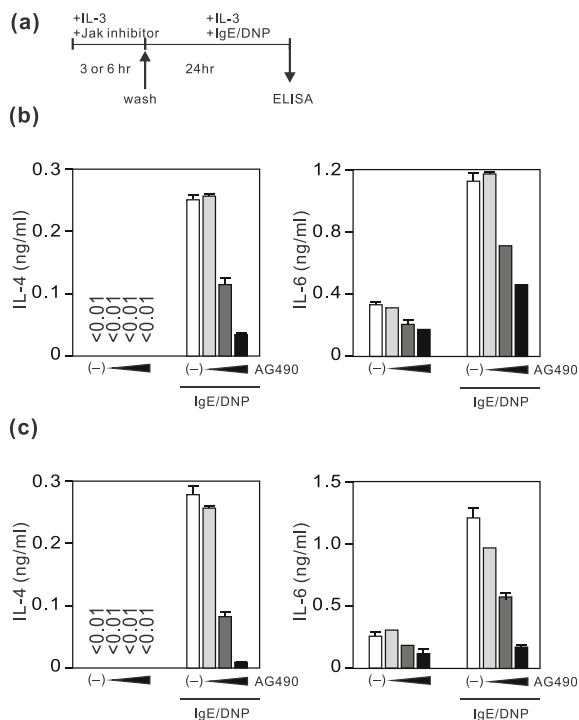


FIGURE 7 Jak signaling is essential for the potentiation of FcεRI signaling pathway. (a) Schedule of treatment with Jak2/3 inhibitor (AG490; 5 μM, 25 μM or 50 μM). (b, c) Cultured with Jak inhibitor for 3 h (b) or 6 h (c) before FcεRI CL stimulation. After cells were collected and inhibitors were removed, cells were stimulated with IgE/DNP-immune complexes. Twenty four hours of culture, IL-4 and IL-6 in the cell supernatant were measured by ELISA. Error bars in (b) and (c) denote mean ± SEM. Data in (b) and (c) are representative of at least three independent experiments.

identified in our previous study (Hida et al., 2009). On the other hand, FcRγ-independent IL-3 signaling is essential for survival and cell proliferation, known for example to induce Bcl-XL expression (Hida et al., 2009; Kohno et al., 2005). In this study, IL-3 also enhanced IL-4 production by CD200R3 CL in *FcεRIγ*^{-/-} basophils, as shown in Figure 6, suggesting that the potentiation of FcεRI signaling is an effect of IL-3 through FcRγ-independent signaling. The alteration of intracellular states seemed to be the major mechanism for the acquisition of the responsiveness to FcεRI and CD200R3 CL by activated basophils. A common feature was apparent among the IL-4 inducers for activated basophils examined here, in that the ITAM-bearing adaptors were utilized for the induction of IL-4; FcRγ by FcεRI CL and papain (Figure 6) and DAP-12 by CD200R3 CL (Kojima et al., 2007). Such a feature raised an interesting possibility that yet other pathogen-associated or host-derived substances could also induce IL-4 and IL-6 production in activated basophils through receptors associated with ITAM-bearing adaptors. Although the “receptor” on

basophils for papain is to be identified, such a receptor would likely be found among FcRγ-associated receptors (Nimmerjahn & Ravetch, 2006) as our present study indicated. The activation-associated, reversible intracellular alteration seemed to be controlled by a putative molecule(s), which was induced and sustained by IL-3 and lost gradually with a half-life of around several hours upon cessation of IL-3 signals or interruption of protein synthesis. Today, numerous signaling molecules have been placed downstream of FcRγ and DAP-12 (Abram & Lowell, 2007; Kambayashi & Koretzky, 2007), but it is not clear which of those are involved in the pathway connecting these ITAM-bearing adaptors to cytokine production in basophils. Identification of the putative activation-associated molecule(s) would shed new lights on the regulatory mechanism for the signaling pathways in basophils. In terms of cytokine production by basophils, IL-3 was unique in that this cytokine not only directly induced IL-4 and IL-6 production in resting basophils but also conferred them responsible to the activation-dependent cytokine inducers. Compared with FcεRI and CD200R3, however, even less is known for the IL-3 signaling pathways leading to IL-4 and IL-6 production in basophils. This is partly because studies of IL-3-triggered responses have been focused almost exclusively on those for proliferation and survival of hematopoietic cells (Geijsen et al., 2001), and partly because of a technically more serious reason, the paucity of basophils occupying only less than 1% of peripheral mononuclear cells. In this regard, our current culture protocol will be very useful as it can furnish us with substantial numbers of starved as well as activated basophils for biochemical and molecular biological analyses of the IL-3 signal transduction pathways for the basophil effector functions, namely IL-4 production and the acquisition of the responsiveness to the activation-dependent IL-4 inducers.

Activation-dependence distinguished at least two intracellular signaling pathways leading to cytokine production in murine basophils; one triggered by IL-3 in resting basophils and the other downstream of ITAM-bearing adaptors in activated basophils. Based on the current findings, we hypothesized that basophils would use either of these two signaling pathways in different stages of type 2 immune responses. Thus, basophils would be triggered to produce IL-4 by IL-3 released from antigen-activated CD4⁺ T cells, thereby playing roles in the initiation of Th2 differentiation at an early stage of immune responses. In later stages, when IgE antibodies were abundantly produced by class-switched B cells, basophils had presumably been activated by IL-3 and would produce IL-4 and perhaps other mediators upon CL of FcεRI IgE complexes with cognate antigens, thereby promoting type 2 immune responses further and contributing to the establishment of inflammatory lesions (Min et al., 2004; Mukai et al., 2005; Obata

et al., 2007; Voehringer et al., 2004). Once IL-3 production ceased as a result of antigen clearance, basophils would revert to the resting state gradually and become eventually unresponsive to FcεRI CL. At this moment, this scenario is in large measure speculative and awaits further studies to validate, but such a switching mechanism, if operated, would ensure the termination of type 2 immune responses, which may otherwise cause allergic disorders. We observed here that *T. spiralis* infection or superantigen rendered basophils competent for producing IL-4 in response to FcεRI CL. These observations together indicated that basophils required prior activation for the responsiveness to FcεRI CL also in vivo in terms of IL-4 production. Although IL-3 would presumably be produced by activated T cells in those immunized or infected animals as suggested by in vitro observations (Hida et al., 2005), it is currently unclear when IL-3 begins to be produced upon infection or immunization, and how long IL-3 production continues to maintain the activated status of basophils in vivo. Even, we have at this moment no direct evidence for the requirement of only IL-3 in the activation of basophils in *T. spiralis* infection and a possibility cannot be excluded that basophil activators other than IL-3 are responsible for the in vivo basophil activation.

Numbers of reports showed that human basophils released IL-4 in response to several parasite antigens in a manner dependent on IgE and hence FcεRI (Falcone et al., 2006), and IL-3 could “prime” human basophils for FcεRI CL-induced IL-4 production, yet the amounts of IL-4 produced in such settings seemed to vary among donors (Brunner et al., 1993; Falcone et al., 1996; MacGlashan, 2007). Furthermore, in one of these studies, either IL-3 or FcεRI CL alone was shown to induce little IL-4 production (Brunner et al., 1993). Another report claimed that pre-activation by IL-3 was dispensable to produce IL-4 upon FcεRI CL (Arock et al., 1993), and direct induction of IL-4 production by allergens was sometimes observed for human basophils (Kasaian et al., 1996; Phillips et al., 2003). Based on the findings in those previous reports and this study, we speculate that freshly prepared human basophils even from “healthy” donors have been “activated” to variable extents, conceivably reflecting difference in the personal history of infection, allergen exposure and allergic diseases as well as the genetic background of donors. Our study shows that environmental conditions such as infection and foreign antigens actually increase the cytokine-producing capacity of basophils. We imagine that our mouse basophils, a well-regulated experimental platform for studying what happens during the activation of basophils for the production of IL-4 and other mediators, will also contribute to a better understanding of parasitic infections and type I allergic diseases in humans.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animals

Rag1^{−/−}, *Stat6*^{−/−}, *FcεRIg*^{−/−} mice (Hida et al., 2009), on the C57BL/6 background, used in this study were bred and maintained in the animal facility in Shinshu University or Nagoya City University under strictly controlled specific pathogen-free (SPF) conditions, with regular monitoring of infection with agents including *Pasturella pneumotropica*, ectoparasites, intestinal protozoa and pinworms, and used at 8–12 weeks of age. Mice lacking IL-3 (Kohno et al., 2005) originally established and provided by Prof. V. Tybulewicz, The Francis Crick Institute, through Dr. Takashi Saito, RIKEN IMS, were backcrossed to the C57BL/6 background more than 10 times, maintained and used in the animal facility in Shinshu University. C57BL/6 mice were from SLC. All animal experiments were approved by the Committee for Animal Experimentation and Care of Shinshu University and Nagoya City University and conducted according to the guideline.

4.2 | Preparation of recombinant proteins

Recombinant superantigen SEA was amplified by PCR from genomic DNA of *S. aureus* (MRSA252), using forward primer 5'-GGG GGA TCC GAG AAA AGC GAA GAA ATA AAT GAA AAA GAT TTG-3' and reverse primer 5'-GGG AAG CTT AAC TTG TAT ATA AAT ATA TAT CAA TAT GCA TGT TTT-3'. Mouse IL-3 was amplified from the cDNA that synthesized with PrimeScript RT master mix (Takara) with C57BL/6 mouse spleen total RNA using forward primer 5'-GAG CTC GCT TCA ATC AGT GGC CGG GAT ACC-3' and reverse primer 5'-GAA TTC TTA ACA TTC CAC GGT TCC ACG GTT AGG AG-3'. The genes were cloned into prokaryotic expression plasmid pCold-II (Takara Bio) and then transformed into ClearColi™ (Lucigen, Middletown). The recombinant proteins were induced by cold shock in the presence of IPTG (1 mM, Nacalai tesque) as a N-terminal hexa-histidine tagged protein and affinity purified using Ni Sepharose 6 Fast Flow (GE Healthcare) in according to manufacturer's instruction. In the case of mIL-3, inclusion bodies were solubilized with denaturing buffer (20 mM Tris-HCl, 0.5 M NaCl, 8 M urea, pH 7.5) and proteins were refolded on column by gradually decreasing the urea concentration and eluted with 250 mM imidazole (Itoh et al., 2010). Purified proteins were dialyzed against PBS. The purities of them were estimated to be more than 95% by SDS-polyacrylamide electrophoresis and Coomassie Brilliant Blue staining.

(Figure S1). The concentration of rSEA was determined using a BCA reagent (Nacalai tesque) and the concentration of rmIL-3 was determined by ELISA.

4.3 | Recombinant SEA or mIL-3 administration

Mice were administered i.p. in a single shot with 50 μ g rSEA, or 2 μ g rmIL-3, each in 200 μ l PBS or with 200 μ l PBS alone. Six hours later, mice were sacrificed under anesthesia and spleens were collected. Enrichment of splenic basophils was carried out as described (Hida et al., 2005; Hida et al., 2009) through depletion of T, B, NK, NKT, mast, erythroid cells, and granulocytes using antibodies against TCR β , B220, CD19, Gr-1, TER119, c-kit, CD11c, and NKp46 (all biotin-antibodies were purchased from Biolegend) with IMag beads (BDBioscience). Basophils were sensitized with anti-DNP IgE (SPE7; Sigma) for 30 min, washed and cross-linked with DNP-BSA (100 μ g/mL). After stimulation, the cells were cultured in the presence of Berfeldin A for 2.5 h and the percentage of IL-4 or IL-6 positive cells within CD200R3⁺DX5⁺B220⁻ basophils were measured by flow cytometry.

4.4 | *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 (Tian et al., 2003). Ten days later, mice were sacrificed, and splenic basophils were enriched and stimulated as above.

4.5 | Surface and intracellular staining on basophils

Fluorochrome-conjugated and unlabeled antibodies were purchased from Biolegend. The recovered splenic basophils were enriched and stained with anti-mouse CD200R3, anti-mouse B220, and anti-mouse CD49b(DX5) mAb for 30 min at 4°C. The cells were fixed with 4% (w/v) paraformaldehyde and permeabilized by washing twice with buffer containing 0.5% (w/v) BSA, 0.1% (w/v) saponin and 0.05% (w/v) Na₃N. For intracellular cytokine staining, fixed/permeabilized cells were resuspended in fluorescent dye-labeled anti-mouse IL-4 Ab or anti-mouse IL-6 antibody at a given optimal concentration for 30 min at 4°C in the dark (Hida et al., 2009). After two washes, cells were immediately analyzed by flow cytometry. Analyses of stained cells were carried out on the Cytomics FC500 flowcytometer (Beckman-Coulter) or FACSverse

(BD Bioscience). KALUZA software (Beckman-Coulter) was used for data analyses.

4.6 | BM-derived basophil culture

BM-derived basophils were cultured as described (Hida et al., 2009). Briefly, whole BM cells (2×10^7) were cultured in 10 mL of 10% fetal calf serum-containing RPMI1640 medium (Nissui) supplemented with mIL-3 (5 ng/mL) for 10–12 days with medium changed every 3 days. Recombinant mIL-3 was produced by a cell line transfected with mIL-3 expression vector. For starvation, cultured basophils were washed extensively, depleted of c-kit⁺ mast cells, seeded at 2×10^6 /mL and starved for 1–12 h. Parallel non-starved cultures contained 5 ng/mL of IL-3. Cultured basophils were, in some experiments, treated with cycloheximide (FujifilmWako) at 10 μ M, washed and subjected to stimulation. For stimulation, cultured basophils were purified by depleting mast (c-kit⁺) cells using IMag beads. The regular purities of fresh and cultured/starved basophils subjected to stimulation were 20%–40% and higher than 85%, respectively.

4.7 | Stimulation of basophils for cytokine production

Fresh, cultured or starved basophils enriched as above (0.5 or 1×10^5 cells) were stimulated with IL-3 (5 ng/mL), IgE (2 μ g/mL, SPE7, Sigma Aldrich) + DNP-BSA (2 μ g/mL), Anti-CD200R3 antibody (0.5 μ g/mL, clone Ba91, kindly provided by H. Karasuyama) or papain (100 μ g/mL; Calbiochem) for 24 h, and the supernatants were collected to measure IL-4, IL-6 proteins using mouse IL-4 or IL-6 ELISA kit (Biolegend). In some experiments, basophils were incubated with IgE (1 μ g/mL) antibody, washed and cultured in the presence of anti-IgE antibody (2 μ g/mL) as above. Cells were surface-stained, fixed, permeabilized, and stained with PE-conjugated anti-IL-4 or IL-6 antibody (Biolegend).

4.8 | Statistical analysis

Statistical significance was determined by a Mann–Whitney *U*-test or Kruskal–Wallis test. * $p < 0.05$, *** $p < 0.001$ was considered significant. Values of $p > 0.05$ were regarded as nonsignificant (n.s.).

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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