CALCIUM SENSING

Pivotal role of STIM2, but not STIM1, in IL-4 production by IL-3–stimulated murine basophils

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Basophils have nonredundant roles in various immune responses that require Ca^{2+} influx. Here, we examined the role of two Ca^{2+} sensors, stromal interaction molecule 1 and 2 (STIM1 and STIM2), in basophil activation. We found that loss of STIM1, but not STIM2, impaired basophil IL-4 production after stimulation with immunoglobulin E (IgE)–containing immune complexes. In contrast, when basophils were stimulated with IL-3, loss of STIM2, but not STIM1, reduced basophil IL-4 production. This difference in STIM proteins was associated with distinct time courses of Ca^{2+} influx and transcription of the *Il4* gene that were elicited by each stimulus. Similarly, basophil-specific STIM1 expression was required for IgE-driven chronic allergic inflammation in vivo, whereas STIM2 was required for IL-4 production after combined IL-3 and IL-33 treatment in mice. These data indicate that STIM1 and STIM2 have differential roles in the production of IL-4, which are stimulus dependent. Furthermore, these results illustrate the vital role of STIM2 in basophils, which is often considered to be less important than STIM1.

INTRODUCTION

Ca^{2+} acts as a second messenger, and Ca^{2+} signaling is involved in a wide variety of effector functions of immune cells (*1*). The primary mechanism of Ca^{2+} entry into the cytosol in immune cells is thought to be the process of store-operated calcium entry (SOCE) (*1*). Ligand binding to receptors on the cell surface triggers the assembly of signaling complexes and activates phospholipase Cγ (PLCγ), which cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds IP_3 receptors expressed in the endoplasmic reticulum (ER), leading to release of Ca^{2+} from ER to the cytoplasm, where in unstimulated cells Ca^{2+} concentration is kept very low compared to that in ER and extracellular space. Stromal interaction molecule 1 and 2 (STIM1 and STIM2) are transmembrane proteins located in the ER membrane that function as Ca^{2+} sensors to detect decreases concentrations of ER Ca^{2+} (*2*, *3*). In response to depletion of Ca^{2+} stored in ER, the cytoplasmic domain of the STIM proteins unfolds and attaches to the plasma membrane, resulting in interaction of STIMs in the ER membrane with the plasma membrane Ca^{2+} channel Orai1. This interaction activates Orai1, leading to the entry of extracellular Ca^{2+} into the cytoplasm (*4*).

STIM1 and STIM2 are structurally similar molecules consisting of an N-terminal EF hand that binds Ca^{2+} in the ER lumen, a sterile α motif that mediates STIM oligomerization, and C-terminal cytosolic regions that regulate Ca^{2+} channel activity (*5*, *6*). STIM1 has a relatively high affinity for Ca^{2+} and is a strong activator of Ca^{2+} channel activity. In contrast, STIM2 has a relatively low affinity for Ca^{2+} and therefore can sense a smaller decrease in ER Ca^{2+} compared to STIM1, which is a weaker activator of Orai1 (*7*, *8*). In immune cells, including T cells, B cells, and mast cells, STIM1 is required for SOCE (*9*–*11*). Children with mutations of STIM1 manifest severe immunodeficiency, indicating an important role for STIM1 in the immune system (*12*). In contrast to STIM1, the pathophysiological role of STIM2 remains ill defined. Immune cells deficient for STIM2 have mild defects in SOCE and effector functions (*8*, *10*, *11*). Cells deficient for both STIM1 and STIM2 display more severe defects, when compared to those deficient for either of them (*10*, *11*), suggesting additive or synergistic action of both proteins.

Basophils are the least common granulocytes and account for less than 1% of peripheral blood leukocytes (*13*). They are phenotypically similar to mast cells and have long been neglected in immunological studies because of their low frequency in the blood. These cells express the high-affinity immunoglobulin E (IgE) receptor FcεRI on their surface and release of secretory granules containing histamine in response to allergens (*13*). However, basophils play crucial and nonredundant roles distinct from those played by mast cells in a variety of immune responses, including protective immunity against parasitic infections, allergic inflammation, autoimmune diseases, and regulation of other immune cells (*14*).

Depletion of extracellular Ca^{2+} abolishes degranulation and cytokine production by basophils (*15*, *16*). However, the molecular mechanism regulating Ca^{2+} influx in basophils remains to be investigated. In the present study, we sought to elucidate the role of STIM1 and STIM2 in the activation of basophils through two distinct stimuli. When we stimulated basophils through the FcεRI with IgE immune complex, we found that STIM1 was required. In contrast, basophils stimulated with interleukin-3 (IL-3) required STIM2, but not STIM1, for the production of IL-4. Thus, STIM1 and STIM2 are reciprocally required for FcεRI- and IL-3 receptor (IL-3R)–dependent basophil responses.

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RESULTS

STIM1/2-independent generation of BMBAs

Vav-iCre*Stim1*$^{fl/fl}$ and *Vav*-iCre*Stim2*$^{fl/fl}$ mice are deficient for STIM1 and STIM2, respectively, in hematopoietic cell lineages (*17*). To examine the role of STIM1 and STIM2 in basophils, we generated *Vav*-iCre*Stim1*$^{fl/fl}$ and *Vav*-iCre*Stim2*$^{fl/fl}$ bone marrow–derived basophils

(BMBAs), respectively, from bone marrow cells isolated from these mice. As a control, we also generated Fc receptor γ (FcRγ) chain–deficient (*Fcer1g*$^{-/-}$) BMBAs that lack the expression of FcεRI on the cell surface. The number of BMBAs generated in culture with IL-3 was comparable regardless of the presence or absence of STIM1, STIM2, or FcRγ (Fig. 1A). Western blot analysis confirmed the absence of STIM1 and STIM2 proteins in *Vav*-iCre*Stim1*$^{fl/fl}$ and *Vav*-iCre*Stim2*$^{fl/fl}$ BMBAs, respectively, whereas both proteins were normally detected in *Fcer1g*$^{-/-}$ BMBAs (Fig. 1, B and C). The expression of mRNAs encoding Ca^{2+} channels (*Orai1*, *Orai2*, and *Orai3*) was comparable in control (*Vav*-iCre), *Vav*-iCre*Stim1*$^{fl/fl}$, *Vav*-iCre*Stim2*$^{fl/fl}$, and *Fcer1g*$^{-/-}$ BMBAs (Fig. 1D). This was also the case in the surface expression of IL-3Rα chain (Fig. 1, E and F). The surface expression of FcεRI was normally detected in *Vav*-iCre*Stim1*$^{fl/fl}$ and *Vav*-iCre*Stim2*$^{fl/fl}$ BMBAs (Fig. 1, E and F). Thus, neither STIM1 nor STIM2 was essential for generation of BMBAs and their surface expression of FcεRI and IL-3R.

STIM1/2 differentially required for BMBA FcεRI and IL-3R responses

We examined the functional role of STIM1 and STIM2 in activation of basophils through either FcεRI or IL-3R. The FcRγ chain is essential not only for FcεRI expression but also for IL-4 production by IL-3–stimulated basophils, although it is dispensable for IL-3–mediated survival and proliferation of basophils (*18*). Therefore, we used *Fcer1g*$^{-/-}$ BMBAs as a negative control in stimulation through FcεRI or IL-3R. When BMBAs were sensitized with hapten 2,4,6-trinitrophenyl (TNP)–specific IgE and then stimulated with TNP-conjugated ovalbumin (TNP-OVA), the deficiency of STIM1, but not STIM2, greatly diminished the degranulation of BMBAs, as assessed by β-hexosaminidase assay (Fig. 2A and fig. S1). This was also the case in IL-4 production by IgE/allergen-stimulated BMBAs during the 16-hour culture (Fig. 2C). In contrast, loss of STIM2 slightly enhanced IL-4 production, when compared to *Vav*-iCre BMBAs (Fig. 2C). Unexpectedly, the stimulation of BMBAs with IL-3 did not follow this same pattern. The production of IL-4 by BMBAs in response to IL-3 stimulation was greatly diminished in BMBAs deficient for STIM2, but not STIM1, compared to *Vav*-iCre BMBAs (Fig. 2D). The amount of IL-4 produced by IL-3–stimulated *Vav*-iCre BMBAs was about half of that by IgE/allergen-stimulated *Vav*-iCre BMBAs (Fig. 2D). IL-3 stimulation induced little or no degranulation in any type of BMBAs (Fig. 2B), in contrast to the stimulation with IgE plus allergens (Fig. 2A). Thus, the FcεRI-mediated signaling mainly used STIM1 for IL-4 production, whereas the IL-3 signaling predominantly used STIM2 for it. As expected, BMBAs deficient for both STIM1 and STIM2 did not degranulate or produce IL-4 in response to either IgE/allergen or IL-3 (fig. S2). The analysis of time course of IL-4 accumulation in BMBA culture supernatants suggested that the IgE/allergen stimulation quickly induced IL-4 production, which ceased by 6 hours (Fig. 2E). In contrast, IL-4 production in response to IL-3 started later, around 6 hours after stimulation (Fig. 2F).

Distinct time course of Ca^{2+} influx, depending on the stimuli

We validated these results by investigating the transcription of *Il4* mRNA in BMBAs after IgE/allergen or IL-3 stimulated over time. We found that IgE/allergen stimulated *Il4* expression within 1 hour after in *Vav*-iCre and *Vav*-iCre*Stim2*$^{fl/fl}$, but not *Vav*-iCre*Stim1*$^{fl/fl}$ or *Fcer1g*$^{-/-}$, BMBAs (Fig. 3A), but was decreased by 6 hours (Fig. 3A). In sharp contrast, IL-3 stimulation induced little or no increase of *Il4* expression in BMBAs of any genotype at 1 hour. However, increased *Il4* expression was detected 6 hours after the IL-3 stimulation in *Vav*-iCre and *Vav*-iCre*Stim1*$^{fl/fl}$, but not *Vav*-iCre*Stim2*$^{fl/fl}$ or *Fcer1g*$^{-/-}$, BMBAs (Fig. 3B). These observations suggested that the IgE/allergen stimulation quickly boosted the *Il4* transcription in a STIM1-dependent manner, whereas the IL-3 stimulation induced it very slowly in a STIM2-dependent manner. This is well correlated with the time course of IL-4 secretion (Fig. 2, E and F).

The production of IL-4 by wild-type (WT) BMBAs was completely inhibited by treatment with an extracellular (EGTA) or an intracellular Ca^{2+} chelator [1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid–acetoxymethyl ester (BAPTA-AM)], irrespective of the stimuli (fig. S3). These results demonstrated that Ca^{2+} influx is required for IL-4 production. This prompted us to examine Ca^{2+} influx in activated BMBAs in the presence or absence of either STIM1 or STIM2. When stimulated with IgE/allergen, cytosolic Ca^{2+} concentration was increased in *Vav*-iCre and *Vav*-iCre*Stim2*$^{fl/fl}$ BMBAs within 15 min. In contrast, the amount of cytosolic Ca^{2+} did not increase in *Fcer1g*$^{-/-}$ and *Vav*-iCre*Stim1*$^{fl/fl}$ BMBAs (Fig. 3C and fig. S4). These data are consistent with the profile of the *Il4* expression at the early phase (Fig. 3A). At this same time point, we found that IL-3 stimulation did not increase intracellular Ca^{2+} concentration in BMBAs of any genotype (Fig. 3D and fig. S4), consistent with the profile of the *Il4* expression at the early phase (Fig. 3B).

The addition of EGTA to the culture of control *Vav*-iCre BMBAs 4 hours after the IL-3 stimulation almost completely abolished the IL-4 production (Fig. 3F), whereas the same treatment 4 hours after the IgE/allergen stimulation did not affect IL-4 secretion (Fig. 3E). These data suggest that the SOCE-mediated Ca^{2+} influx at the early, but not late, phase may be important for IL-4 production by IgE/allergen-stimulated BMBAs, whereas IL-4 production by IL-3–stimulated BMBAs may depend on late-phase Ca^{2+} influx. We then compared the concentration of cytosolic Ca^{2+} different times after the stimulation of BMBAs with either IgE/allergen or IL-3 (Fig. 3, G and H). In *Vav*-iCre BMBAs stimulated with IgE/allergen, intracellular Ca^{2+} concentration decreased during the 5 hours between the two time points (Fig. 3G). This was also true for that in *Vav*-iCre*Stim2*$^{fl/fl}$ BMBAs (Fig. 3G). In contrast, the IL-3 stimulation rather increased the Ca^{2+} concentration during the 5 hours between time points in *Vav*-iCre and *Vav*-iCre*Stim1*$^{fl/fl}$, but not *Vav*-iCre*Stim2*$^{fl/fl}$ and *Fcer1g*$^{-/-}$, BMBAs (Fig. 3H). Together, the IgE/allergen stimulation appeared to induce IL-4 production through STIM1-mediated quick and strong Ca^{2+} influx (Fig. 3C), whereas the IL-3 stimulation elicited IL-4 production through STIM2-mediated slow and weak increase of Ca^{2+} influx at the late phase (Fig. 3H).

The addition of IL-3–neutralizing monoclonal Ab (mAb) 6 hours after the start of IL-3 culture inhibited IL-4 production by basophils (Fig. 3I), suggesting that the prolonged stimulation through IL-3R is necessary for IL-3–elicited IL-4 production by basophils. Diphenyleneiodonium (DPI), a reactive oxygen species (ROS) inhibitor, almost completely inhibited IL-3–elicited IL-4 production in *Vav*-iCre and *Vav*-iCre*Stim1*$^{fl/fl}$ BMBAs (Fig. 3J), which suggests that a ROS-dependent signal may be involved in IL-4 production by IL-3–stimulated basophils.

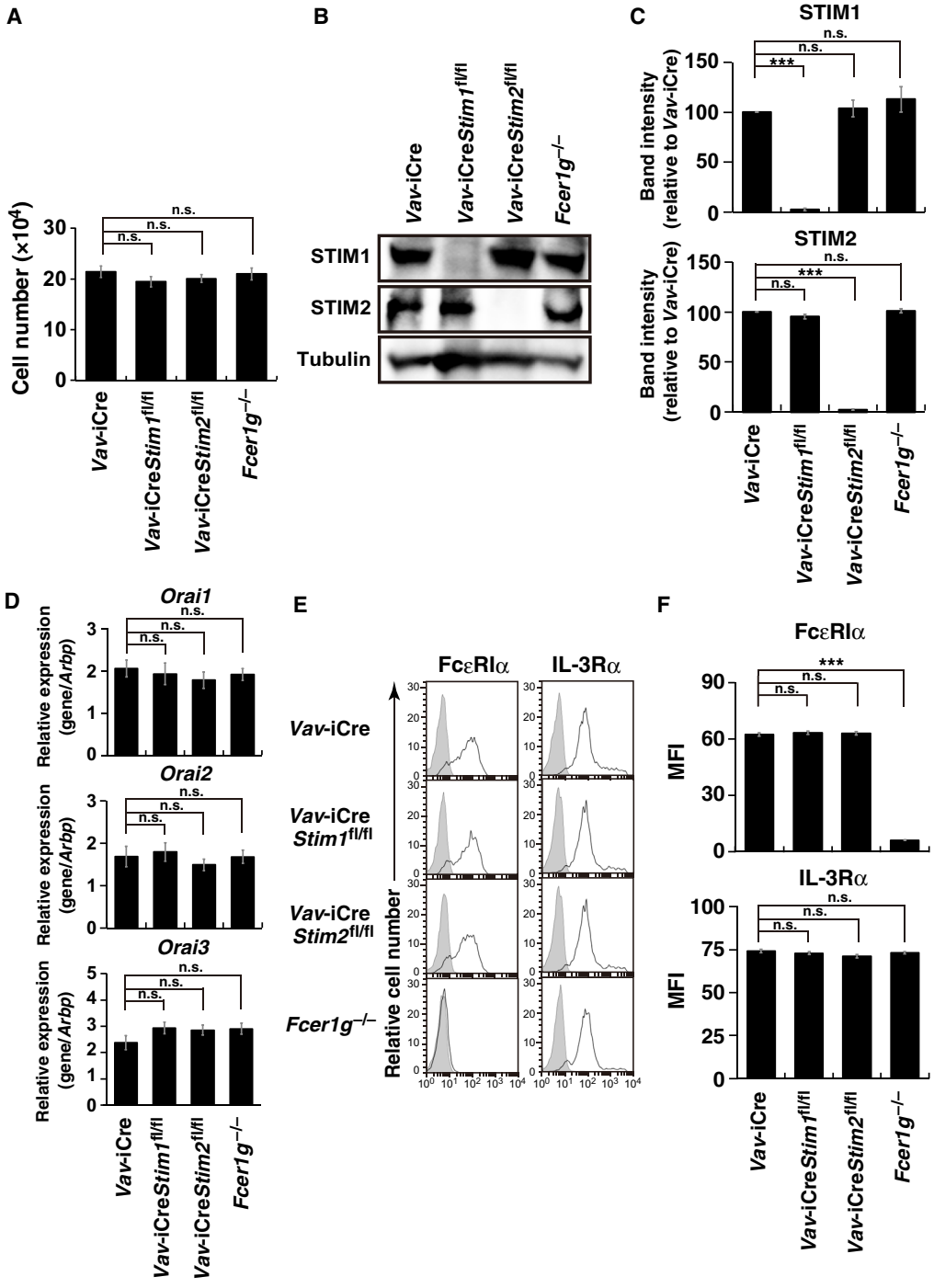

Fig. 1. Neither STIM1 nor STIM2 is essential for the generation of BMBAs and surface expression of FcεRI and IL-3R. (**A**) Total cell numbers of BMBAs generated from bone marrow cells that were isolated from *Vav*-iCre, *Vav*-iCre*Stim1*$^{fl/fl}$, *Vav*-iCre*Stim2*$^{fl/fl}$, or *Fcer1g*$^{-/-}$ mice and cultured in the presence of IL-3 for 6 days. Data are means ± SEM from three independent experiments performed in triplicate. (**B** and **C**) Western blot analysis of STIM1, STIM2, and control protein abundance in lysates from the indicated BMBAs. Blots (B) are representative of three independent experiments. Quantified band values (C) are means ± SEM from all experiments. (**D**) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of the expression of *Orai1*, *Orai2*, and *Orai3* mRNAs in the indicated BMBAs. Data are means ± SEM from three independent experiments performed in triplicate. (**E** and **F**) Flow cytometry analysis of cell surface FcεRIα and IL-3Rα abundance on the indicated BMBAs. Shaded histograms indicate control staining with isotype-matched antibodies (Abs). Histograms (E) are representative of at least three independent experiments. Quantified mean fluorescence intensity (MFI) values are means ± SEM from all experiments performed in triplicate. n.s., not significant. ***$P < 0.001$ by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

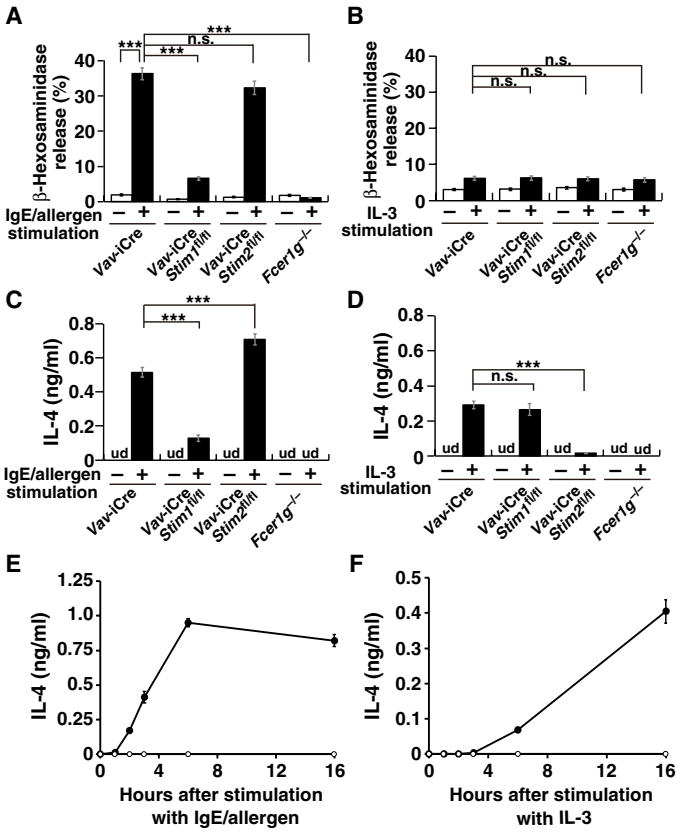

Fig. 2. BMBA FcεRI response requires STIM1, but IL-3R response requires STIM2. (**A** and **B**) β-Hexosaminidase release assay of degranulation in *Vav*-iCre, *Vav*-iCre*Stim1*$^{fl/fl}$, *Vav*-iCre*Stim2*$^{fl/fl}$, or *Fcer1g*$^{-/-}$ BMBAs stimulated with IgE/allergen (A) or IL-3 (B) for 1 hour. Data are means ± SEM from three independent experiments performed in triplicate. (**C** and **D**) Enzyme-linked immunosorbent assay (ELISA) analysis of IL-4 production by *Vav*-iCre, *Vav*-iCre*Stim1*$^{fl/fl}$, *Vav*-iCre*Stim2*$^{fl/fl}$, or *Fcer1g*$^{-/-}$ BMBAs stimulated with IgE/allergen (A) or IL-3 (B) for 16 hours. Data are means ± SEM from three independent experiments performed in triplicate. ud, undetectable. (**E** and **F**) ELISA analysis of IL-4 concentration in supernatants by WT BMBAs stimulated with IgE/allergen (E) or IL-3 (F) for the indicated periods. Data are means ± SEM from three independent experiments performed in triplicate. ***$P < 0.001$ by one-way ANOVA followed by Tukey's multiple comparison test.

Establishment of engineered mice that lack STIM1/2 only in basophils

We examined the in vivo relevance of our findings on basophil activation in *Mcpt8*^{iCreERT2} transgenic mice, which express the improved Cre fused with human estrogen receptor (iCreER^{T2}) under the control of basophil-specific promotor *Mcpt8*. In these mice, Cre recombinase activity is induced only in basophils by means of treatment of mice with tamoxifen (TAM) (fig. S5A). When *Mcpt8*^{iCreERT2} mice were crossed with *Rosa26*^{YFP} mice that express yellow fluorescent protein (YFP) in a Cre activity–dependent manner (*19*), about 90% of basophils in the spleen, bone marrow, and peripheral blood expressed YFP within 3 days after the TAM treatment. However, about 20% of basophils also expressed YFP even without the TAM treatment, possibly due to the background leakage of Cre activity (fig. S5B). The TAM treatment induced the appearance of YFP$^+$ basophils in the peripheral blood at the high frequency for 5 days (fig. S5C). Less than 3% of cell lineages other than basophils expressed YFP after the TAM treatment (fig. S5B), indicating that TAM preferentially induced Cre activity in basophils.

We crossed *Mcpt8*^{iCreERT2} mice with *Stim1*$^{fl/fl}$ and *Stim2*$^{fl/fl}$ mice to obtain *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$ and *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$ mice, respectively. Western blot analysis of basophils isolated from the spleen of *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$ and *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$ mice treated with TAM confirmed that abundance of STIM1 and STIM2 was reduced, as expected (Fig. 4, A and B). The TAM treatment showed little or no deleterious effect on the number of basophils and the surface expression of FcεR1α and IL-3Rα in these mice (Fig. 4, C to E). When splenic basophils isolated from those TAM-treated mice were stimulated in vitro with IgE/allergen, IL-4 production was impaired in *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$, but not *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$, basophils (Fig. 4F). By contrast, when stimulated with IL-3, IL-4 production was deficient in *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$, but not *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$, basophils (Fig. 4G). These results were consistent with those observed in STIM1- or STIM2-deficient BMBAs (Fig. 2, C and D), indicating that the TAM treatment of the mice efficiently ablated the *Stim* genes in basophils.

Differential physiological functions of STIM1/2 in basophil

The role of STIM1 and STIM2 in basophils in vivo was analyzed by using *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$ and *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$ mice, as well as control *Mcpt8*^{iCreERT2} mice. We first examined the response of these mice in IgE-mediated chronic allergic inflammation (IgE-CAI), which is a delayed-onset allergic response in the skin. This response can be induced in mice sensitized with TNP-specific IgE by an intradermal administration of TNP-OVA as an allergen (*20*). Basophil depletion before the allergen challenge completely abolishes the IgE-CAI response, demonstrating that basophils are essential for the development of IgE-CAI (*20*). In addition, IgE is essential for the development of IgE-CAI (*20*), whereas IL-3 is dispensable for it (fig. S6). When we treated mice with TAM before the allergen challenge, we found that *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$, but not *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$, mice developed IgE-CAI, as characterized by ear swelling (Fig. 5A), microvascular hyperpermeability (Fig. 5B), infiltration of leukocytes (Fig. 5C), and increased *Il4* transcription (Fig. 5D), compared to control *Mcpt8*^{iCreERT2} mice. Thus, STIM1, but not STIM2, in basophils plays an important role in the development of IgE-CAI.

We next examined the role of STIM1 and STIM2 in the IL-4 production by basophils stimulated with IL-3 in vivo. A single intraperitoneal administration of IL-3 in *Vav*-iCre mice failed to increase IL-4 concentration in serum at detectable levels, and even four consecutive daily administration of IL-3 for 4 days failed to do so (Fig. 6A). However, IL-33 stimulation through MyD88 enhances IL-3–induced IL-4 production by basophils in vitro (*21*). In accordance with this, the addition of IL-33 together with IL-3 to the culture of *Vav*-iCre BMBAs enhanced IL-4 production, whereas IL-33 alone failed to stimulate IL-4 production (fig. S7). When stimulated with IL-3 + IL-33, *Vav*-iCre*Stim1*$^{fl/fl}$ BMBAs produced IL-4 as much as *Vav*-iCre BMBAs did, whereas *Vav*-iCre*Stim2*$^{fl/fl}$ BMBAs produced much smaller amounts of IL-4 (fig. S7) as observed in BMBAs when stimulated with IL-3 alone (Fig. 2D).

When WT (C57BL/6) mice were treated with four consecutive administration of IL-3 + IL-33, an increased amount of IL-4 in the serum was detected (Fig. 6A). This increase was severely impaired by diphtheria toxin (DT)–mediated basophil ablation in *Mcpt8*^{DTR} mice (Fig. 6B), indicating that basophils were the major source of increased IL-4. To examine the role of STIM1 and STIM2 in the

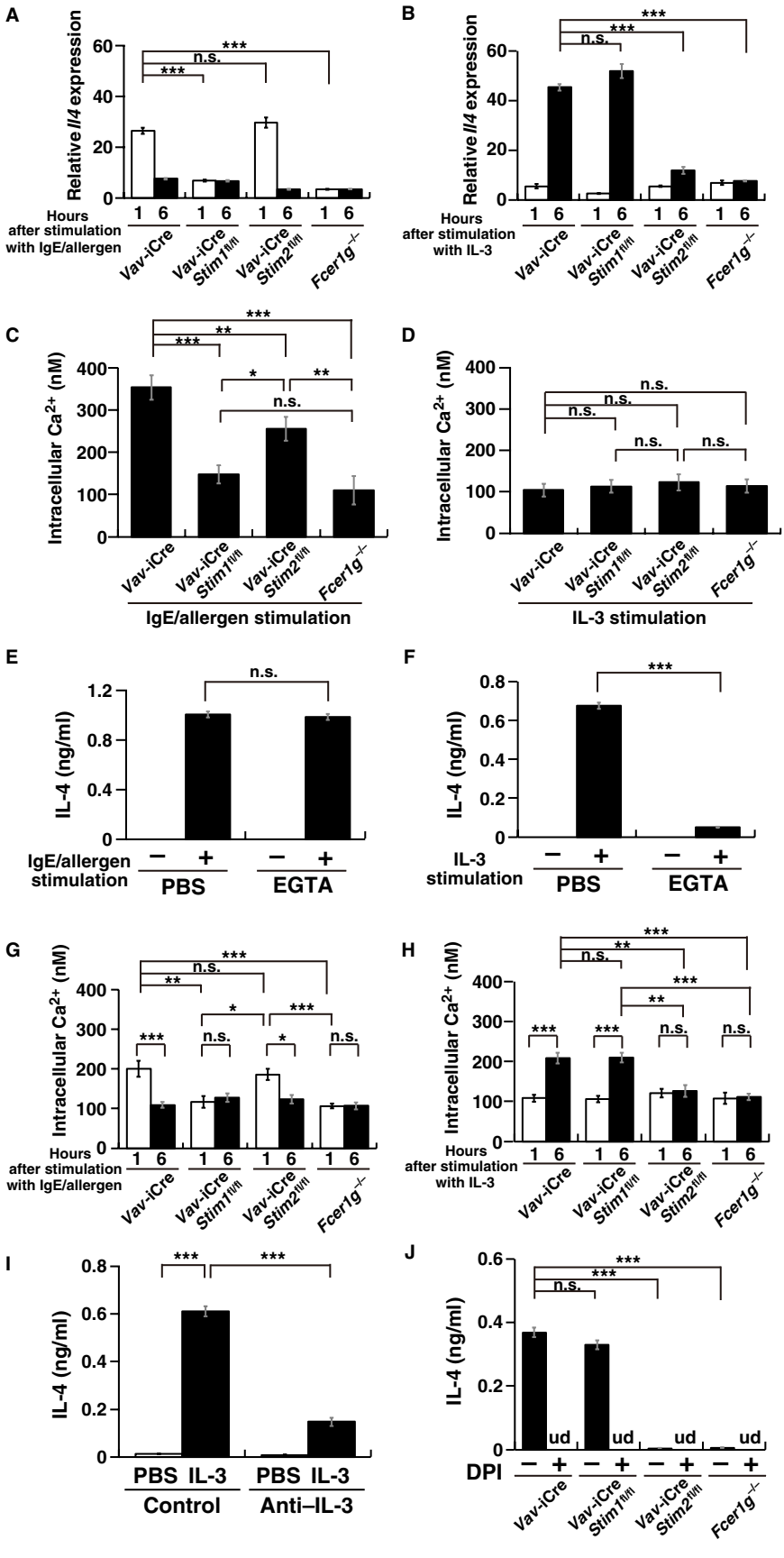

Fig. 3. Distinct time course of *Il4* expression and Ca^{2+} influx in BMBAs, depending on the stimuli. (**A** and **B**) qRT-PCR analysis of *Il4* mRNA expression in *Vav*-iCre, *Vav*-iCre*Stim1*$^{fl/fl}$, *Vav*-iCre*Stim2*$^{fl/fl}$, or *Fcer1g*$^{-/-}$ BMBAs stimulated with IgE/allergen (A) and IL-3 (B) for the indicated times. Data are means ± SEM from three independent experiments performed in triplicate. (**C** and **D**) Florescence microscopy analysis of peak of cytosolic Ca^{2+} concentration in BMBAs of the indicated genotype that were stimulated with IgE/allergen (C) or IL-3 (D). Data are means ± SEM from >150 single cells from three independent experiments. (**E** and **F**) ELISA analysis of IL-4 production by WT BMBAs stimulated with either IgE/allergen [+ in (E)] or IL-3 [+ in (F)] for 16 hours with or without EGTA, as indicated. Data are means ± SEM from three independent experiments performed in triplicate. (**G** and **H**) Florescence microscopy analysis of cytosolic Ca^{2+} concentration in BMBAs of the indicated genotype stimulated with either IgE/allergen (G) or IL-3 (H) for the indicated times. Data are means ± SEM from >150 single cells from three independent experiments. (**I**) ELISA analysis of IL-4 production by WT BMBAs cultured with or without IL-3 in the presence or absence of IL-3–neutralizing mAb. Data are means ± SEM from three independent experiments performed in triplicate. ***$P < 0.001$ by two-way ANOVA followed by Tukey's multiple comparison test. (**J**) ELISA analysis of IL-4 production by BMBAs of the indicated genotype that were preincubated with control DMSO or DPI and stimulated with IL-3 for 16 hours. Data are means ± SEM from three independent experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by two-way ANOVA followed by Tukey's multiple comparison test.

IL-4 production by basophils stimulated with IL-3 + IL-33 in vivo, *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$, *Mcpt8*^{iCreERT2}*Stim2*$^{fl/fl}$, and control *Mcpt8*^{iCreERT2} mice were first treated with TAM and then repeatedly treated with IL-3 + IL-33. Treatment with IL-3 + IL-33 increased serum IL-4 concentration in *Mcpt8*^{iCreERT2}*Stim1*$^{fl/fl}$ mice similar to control *Mcpt8*^{iCreERT2} mice (Fig. 6C). In contrast, very little serum IL-4 was found in *Mcpt8*^{iCreERT2}-*Stim2*$^{fl/fl}$ mice treated with IL-3 + IL-33 (Fig. 6C), although the number of basophils in the spleen was comparable in all three types of mice (Fig. 6D). These results indicated that STIM2, but not STIM1, plays the major role in the IL-4 production by basophils in vivo in response to the stimulation with IL-3 + IL-33.

DISCUSSION

Basophils readily produce large quantities of IL-4 in response to various stimuli, such as allergen immune complexes through FcεRI and IL-3 through IL-3R (*22*), and therefore, they are an important source of IL-4. Basophil-derived IL-4 acts on a variety of immune cells, including B cells, T cells, monocytes, macrophages, group 2 innate lymphoid cells, as well as nonhematopoietic cells such as fibroblasts and endothelial cells, to both promote and inhibit immune responses

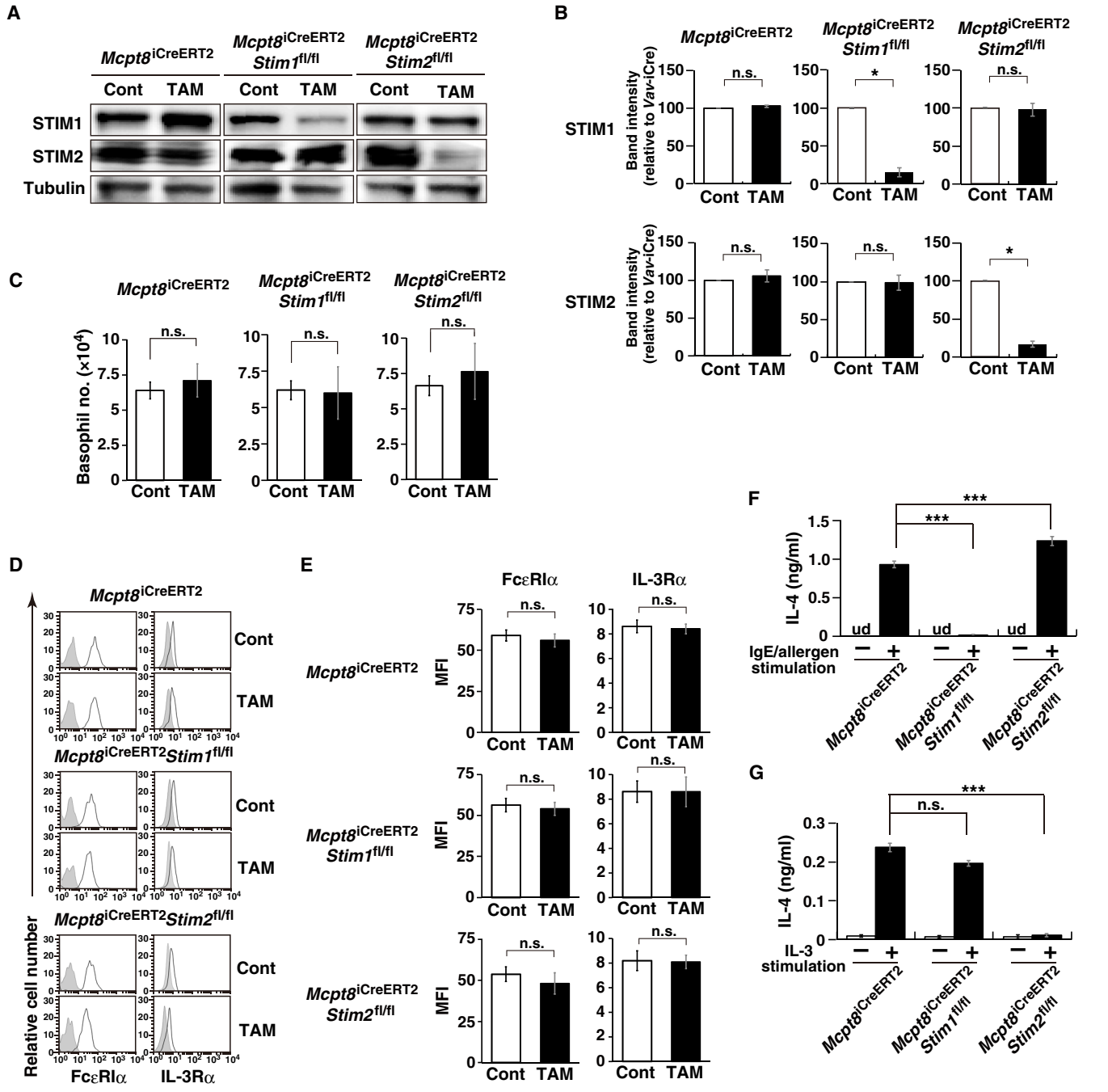

Fig. 4. Conditional loss of STIM1 or STIM2 in basophils impairs either FcεRI- or IL-3R–dependent responses. (**A** and **B**) Western blot analysis of STIM1, STIM2, and control protein abundance in lysates from splenic basophils of the indicated genotype after TAM treatment. Blots (A) are representative of four independent experiments. Quantified band intensity values (B) are means ± SEM from all experiments. (**C**) Number of splenic basophils in the indicated mice. Data are means ± SEM of nine mice per group from three independent experiments (**D** and **E**) Flow cytometry analysis of cell surface FcεRIα and IL-3Rα abundance on splenic basophils of the indicated genotype. Shaded histograms indicate control staining with isotype-matched Abs. Histograms (D) are representative of three independent experiments. Quantified MFI values (E) are means ± SEM from all experiments performed in triplicate. (**F** and **G**) ELISA analysis of IL-4 production by splenic basophils isolated from the indicated mice 3 days after the last TAM treatment and stimulated with IgE/allergen (F) or IL-3 (G) for 16 hours. Data are means ± SEM from three independent experiments performed in triplicate. *$P < 0.05$ and ***$P < 0.001$ by t test (B, C, and E) or two-way ANOVA followed by Tukey's multiple comparison test (F and G).

(*23*). Although Ca^{2+} influx is required for basophil activation (*15*, *16*), the precise mechanism controlling Ca^{2+} influx in basophils remained ill defined. The present study demonstrated both in vitro and in vivo that STIM1 is necessary for IL-4 production downstream of FcεRI engagement in basophils, whereas IL-3R–mediated stimulation required STIM2 to induce IL-4 production. Thus, STIM1 and STIM2 differentially function in IL-4 production by basophils, depending on the stimuli.

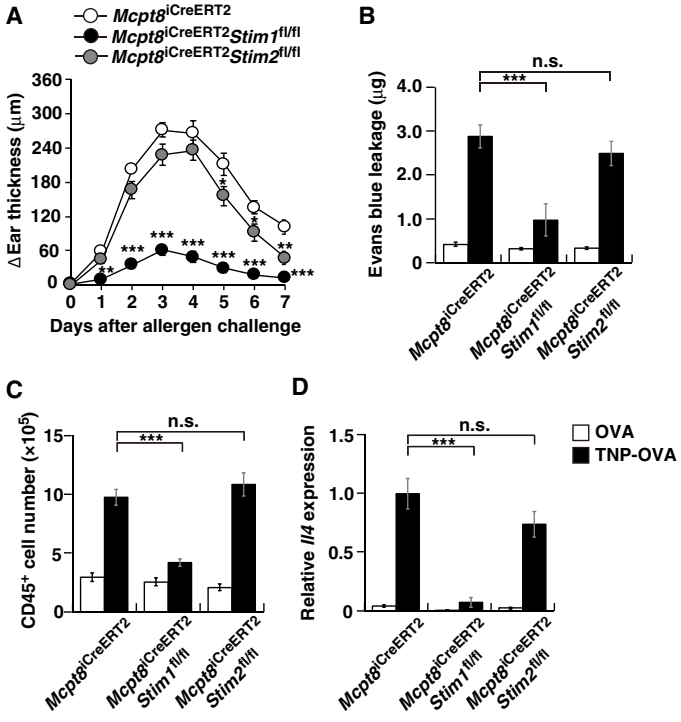

Fig. 5. STIM1, but not STIM2, in basophils is required for the development of IgE-CAI. (**A**) Change in ear thickness in mice of the indicated genotype passively sensitized with TNP-specific IgE and challenged with an intradermal TNP-OVA or control OVA injection. Data are means ± SEM of 13 mice per group from three independent experiments. (**B**) Extravascular leakage of Evans blue dye injected into the ear skin was examined 3 days after the allergen challenge in mice treated as in (A). Data are means ± SEM of 12 mice per group from three independent experiments. (**C**) Number of $CD45^+$ leukocytes accumulating in the ear skin lesions 3 days after allergen challenge in mice treated as in (A). Data are means ± SEM of nine mice per group from three independent experiments. (**D**) qRT-PCR analysis of *Il4* expression in the skin lesions 3 days after allergen challenge in mice treated as in (A). Data are means ± SEM of 12 mice per group from three independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by two-way ANOVA followed by Tukey's multiple comparison test.

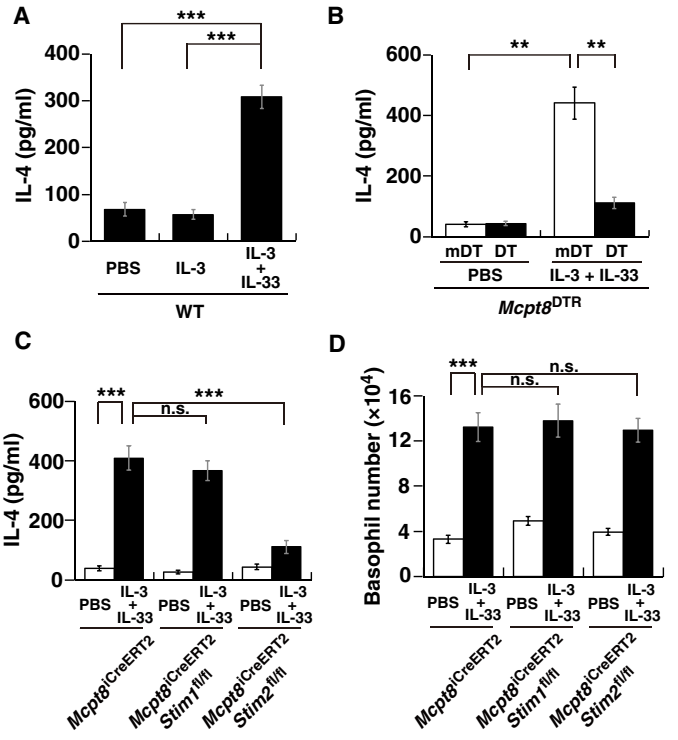

Fig. 6. STIM2, but not STIM1, promotes IL-4 production by IL-3/IL-33–stimulated basophils in vivo. (**A**) ELISA analysis of IL-4 concentration in serum of WT mice 6 hours after treatment with PBS, IL-3, or IL-3 + IL-33. Data are means ± SEM of 15 mice per group from three independent experiments. (**B**) ELISA analysis of IL-4 concentration in serum of DT- or mutant DT (mDT)–treated *Mcpt8*^{DTR} mice given PBS or IL-3 + IL-33. Data are means ± SEM of at least nine mice per group from three independent experiments. (**C** and **D**) ELISA analysis of IL-4 concentration in the serum (C) or total number of splenic basophils (D) in TAM-treated mice of the indicated genotype given PBS or IL-3 + IL-33. Data are means ± SEM of at least nine mice per group from three independent experiments. $**P < 0.01$ and $***P < 0.001$ by two-way ANOVA followed by Tukey's multiple comparison test.

Mice deficient for STIM1 and STIM2 die perinatally and at 4 to 5 weeks of age (*9*, *10*), respectively, indicating that both STIMs play key roles in vivo, although the exact cause of death remains to be clarified. In T cells, STIM1, but not STIM2, is a predominant effector of SOCE and production of cytokine IL-2 when stimulated with cross-linking of T cell receptors (*10*). Similarly, in B cells stimulated with cross-linking of B cell receptors, the deficiency of STIM1 greatly impairs SOCE, cellular proliferation, and production of cytokine IL-10, whereas STIM2 deficiency shows much milder impairment (*11*). Mast cells deficient for STIM1 display severe reduction in SOCE, degranulation, and cytokine production (*9*). Thus, STIM1, but not STIM2, appears to play the major role in these cells under experimental conditions used. STIM2 functions as a feedback regulator that stabilizes basal cytosolic and ER Ca^{2+} concentration (*24*, *25*). In addition, STIM2 has a unique role in the activation of STIM1 and STIM1/Orai1 coupling in cells, where the ER Ca^{2+} concentration is not low enough to stimulate STIM1 activation (*26*).

In the present study, we demonstrated the vital role of STIM2 in IL-3–elicited basophil activation, independently of STIM1. Although basophils deficient for FcRγ proliferate normally in response to IL-3, they fail to produce IL-4 in contrast to WT basophils (*18*). FcRγ constitutively associates with common β (βc) chain of IL-3R through an intramembrane interaction and transduces signals for IL-4 production by recruiting Syk (*18*). FcRγ is also an essential component of FcεRI, and cross-linking of FcεRI with IgE plus allergens induces recruitment of Syk to FcRγ. In both cases, recruited Syk activates PLCγ, leading to SOCE. Nevertheless, we found that the FcεRI-mediated signal mainly uses STIM1 for the induction of IL-4 production, whereas the IL-3R–mediated signal predominantly uses STIM2 for it. Although it remains to be investigated what makes this difference, it is notable that FcεRI and IL-3R have FcεRIβ and βc chains (*27*, *28*), respectively, as another component for signal transduction. These molecules may differentially modify the signaling through FcRγ in FcεRI and IL-3R. Alternatively, the differences in the time course and the receptor abundance of signals through FcεRI versus IL-3R may account for the differential usage of STIM1 and STIM2. Cross-linking of FcεRI with IgE plus allergens induced prompt and efficient internalization of FcεRI (*29*), suggesting that FcεRI-mediated signaling may occur in a short period. In contrast, the prolonged stimulation via IL-3R is

necessary for IL-3–elicited IL-4 production (Fig. 3I). Considering that STIM2 has a relatively low affinity for Ca^{2+} and therefore can sense a smaller decrease in ER Ca^{2+} concentration compared to STIM1, one may assume that the prolonged, maybe weak signaling by IL-3R elicits a mild drop in the ER Ca^{2+} concentration and therefore activates STIM2 rather than STIM1. By contrast, the short-term, maybe stronger signaling by FcεRI can induce a sharp fall in the ER Ca^{2+} concentration, resulting in activation of STIM1.

In neutrophils, STIM1, but not STIM2, plays a predominant role in ROS production, phagocytosis, and degranulation when stimulated with various stimuli, including *N*-formyl-Met-Leu-Phe (*f*MLP) and zymosan (*30*, *31*). Nevertheless, STIM2-deficient, but not STIM1-deficient, neutrophils display a marked defect in production of cytokines such as tumor necrosis factor–α (TNFα), suggesting that cytokine production requires STIM2 rather than STIM1 (*30*). Although Ca^{2+} influx is decreased in STIM1-deficient neutrophils, cytokine production is nearly normal. Because STIM1, but not STIM2, is required for ROS production in neutrophils, and ROS inhibits cytokine production in neutrophils, compensatory mechanisms may normalize cytokine production in STIM1-deficient neutrophils. In STIM2-deficient neutrophils, inhibition of cytokine production through STIM1-mediated ROS production may suppress cytokine production. Thus, identifying the independent effects of specific Ca^{2+} channels on cytokine production by neutrophils and mast cells may be complicated by ROS-dependent signals (*32*, *33*). In the present study, we found that IL-4 production by basophils is distinct from TNFα production by neutrophils in terms of the involvement of ROS. DPI almost completely inhibited IL-3–elicited IL-4 production by basophils (Fig. 3H), indicating that a ROS-dependent signal promotes, rather than inhibits, IL-4 production in basophils. Together, our data suggest that STIM2-mediated signaling may elicit the production of ROS that, in turn, promotes IL-4 production in basophils when stimulated with IL-3. This mechanism is quite distinct from the regulation of cytokine production in other granulocytes.

In conclusion, we demonstrated in the present study the important role for STIM2, rather than STIM1, in the production of IL-4 when basophils are stimulated with the cytokine IL-3. In contrast, STIM1, but not STIM2, is a predominant player in IL-4 production by basophils when stimulated with IgE/allergen immune complexes. Thus, STIM1 and STIM2 differentially function in the IL-4 production by basophils, depending on the stimuli, through FcεRI and IL-3R, respectively. Our findings have shed light on the vital role of STIM2 that had often been paid less attention compared to STIM1, although further studies are needed to clarify whether the finding in mice can be applied to other animal species and humans.

MATERIALS AND METHODS

Mice

Stim1$^{fl/fl}$, *Stim2*$^{fl/fl}$, *Mcpt8*^{DTR}, and *Il3*$^{-/-}$ mice were previously described (*10*, *34*, *35*). *Vav*-iCre transgenic (*36*) and *Fcer1g*-deficient (*Fcer1g*$^{-/-}$) (*37*) mice were provided by D. Kioussis and T. Takai, respectively. *Rosa26*^{YFP} mice (*19*) were obtained from the Jackson Laboratory. All mice were maintained under specific pathogen–free conditions in our animal facilities, and all animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (permit number: 0170087A).

Generation of *Mcpt8*^{iCreERT2} transgenic mice

A bacterial artificial chromosome (BAC; RP23-16G10) harboring the *Mcpt8* gene was purchased from the Children's Hospital Oakland Research Institute. To generate the *Mcpt8*^{iCreERT2} BAC, the first exon of *Mcpt8* was replaced with the iCreER^{T2} gene, TAM-inducible Cre recombinase generated by fusing the ER^{T2} (*38*) gene to the C terminus of improved Cre (*39*) gene with deletion of the stop codon, by the Red/ET recombination system in *Escherichia coli* (Gene Bridges). The 20-kb fragment, ranging from the 15 kb upstream to the 5 kb downstream of the iCreER^{T2} insertion site, was isolated from the *Mcpt8*^{iCreERT2} BAC and inserted into pBR322 to obtain p*Mcpt8*^{iCreERT2}. The *Mcpt8*^{iCreERT2} transgene was prepared from p*Mcpt8*^{iCreERT2} by Sac II/Swa I enzyme digestion, gel-purified using the QIAquick Gel Extraction Kit (Qiagen), and then microinjected to fertilized eggs of C57BL/6J mice.

Abs and reagents

The following Abs were purchased from BD Pharmingen: allophycocyanin (APC)–conjugated anti-CD49b (HM2α), phycoerythrin (PE)–conjugated anti–Siglec-F (E50-2440), and anti-NK1.1 (PK136). Unlabeled anti–IL-3 (MP2-8F8) and rat IgG1κ isotype (RTK2071); biotin-conjugated anti-CD49b (DX5), anti-CD123 (IL-3Rα, 5B11), anti-FcεRIα (MAR-1), anti–I-A/I-E (M5/114.15.2), American hamster IgG isotype (HTK888), and rat IgG2aκ isotype (RTK2758); PE-conjugated CD200R3 (Ba13), anti-IgE (RME-1), and anti-CD4 (RM4.5); APC-conjugated anti–Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), and anti-F4/80 (BM8); PE/Cy7-conjugated anti–c-kit (2B8) and anti-CD45 (30-F11); Brilliant Violet 421–conjugated anti-CD3 (145-2C11) and anti-CD11c (N418); and APC/Cy7-conjugated streptavidin were from BioLegend. PE-conjugated CD11b Abs (M1/70) were obtained from eBioscience. Anti-STIM1 (D88E10), anti-STIM2 (4917), and anti–α-tubulin (2144) were from Cell Signaling Technology. TNP-specific IgE (IGEL-b4) and anti-CD16/32 (2.4G2) were prepared in our laboratory. TAM (T5648), DT (D0564), inactive mDT (D2189), EGTA, BAPTA-AM, and DPI were purchased from Sigma-Aldrich. Recombinant mouse IL-3 and IL-33 were obtained from BioLegend.

Preparation and activation of BMBAs

BMBAs were generated as described previously (*40*). They were first sensitized with TNP-specific IgE (1 μg/ml) overnight in the absence of IL-3 and then incubated at 37°C with TNP-OVA or control OVA (30 ng/ml) or IL-3 or control phosphate-buffered saline (PBS) (1 μg/ml) for 1 hour in the β-hexosaminidase release assay (*40*) or for 16 or indicated hours in the IL-4 production assay using Mouse ELISA MAX Standard (BioLegend). For Ca^{2+} chelation, cells were preincubated with 2 mM EGTA or 100 μM BAPTA-AM and then incubated with the indicated stimulants for 16 hours. For ROS inhibition, cells were preincubated with 5 μM DPI or control vehicle [dimethyl sulfoxide (DMSO)] for 30 min and then incubated with the indicated stimulants for 16 hours. For IL-3 neutralization, anti–IL-3 (1 μg/ml) or isotype-matched control Ab was added to the culture 6 hours after the start of culture.

Western blotting

Cells were lysed with radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Roche), and total cell lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis with indicated Abs. The intensity of blot bands was quantified using ImageJ software.

RNA preparation and qRT-PCR

Total mRNAs from BMBAs were prepared using the RNeasy Mini Kit (Qiagen). Complementary DNAs were synthesized using reverse transcription using ReverTra Ace (TOYOBO) and oligo(dT) primers. Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems) using the Fast SYBR Green Master Mix (Applied Biosystems) and the following primer sets: *Arbp* (sense-GGCCCTCGACTCTCGCTTTC and antisense-TGCCAGGACGCGCTTGT), *Il4* (sense-GGCATTTTGAACGAGGTCAC and antisense-AAATATGCGAAGCACCTTGG), *Orai1* (sense-GATCGGCCAGAGTTACTCCG and antisense-TGGGTAGTCATGGTCTGTGTC), *Orai2* (sense-GGCCACAAGGGCATGGATTA and antisense-TGAGGGTACTGGTACTTGGTC), and *Orai3* (sense-GGCTACCTGGACCTTATGGG and antisense-GCAGGCACTAAATGCCACC). The expression of these genes was normalized using *Arbp* expression as a reference.

Flow cytometry

Single-cell suspensions were prepared from the bone marrow, spleen, and peripheral blood. For isolation of cells from the ear skin, excised ears were treated with collagenase (125 U/ml, Wako) in RPMI 1640 complete medium at 37°C for 2 hours. After preincubation with anti-CD16/32 and normal rat serum on ice for 30 min to prevent the nonspecific binding of irrelevant Abs, cells were stained with indicated combination of Abs and analyzed with FACSCanto II (BD Biosciences) and FlowJo (TreeStar). Each cell lineage was identified as follows: hematopoietic cells ($CD45^+$), basophils ($CD49b^+$c-$kit^-CD200R3^+$), skin-resident mast cells ($CD49b^+$c-$kit^+CD200R3^+$), neutrophils (Gr-1^{high}Siglec-F^-), eosinophils (Gr-1^{int}Siglec-F^+SSC^{high}), B cells ($CD3^-B220^+$), natural killer (NK) cells ($CD49b^+NK1.1^+CD3^-$), NKT cells ($CD49b^+NK1.1^+CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), $CD8^+$ T cells ($CD3^+CD4^-CD8^+$), dendritic cells ($CD11c^+$I-A/I-E^{high}), and monocyte-macrophages ($F4/80^+CD11b^+SSC^{low}$).

Measurement of Ca^{2+} influx

For immediate phase of Ca^{2+} influx analysis, BMBAs were sensitized with TNP-specific IgE, and then cells (2×10^6 cells/ml) were loaded with 2 μM Fura-2 AM (TEFLabs) in a loading buffer at 37°C for 30 min. Cells were washed and resuspended in Ca^{2+}-free Hanks' balanced salt solution (HBSS) and were attached for 10 min to collagen-coated glass-bottom dishes. After acquiring the baseline of cytosolic Ca^{2+} concentration for 2 min, cells were stimulated with TNP-OVA (30 ng/ml) or IL-3 (1 ng/ml) in Ca^{2+}-free HBSS to monitor the intracellular Ca^{2+} store release. Six minutes later, cells were treated with 2 mM $CaCl_2$ to monitor the dynamics of SOCE. The fluorescence intensity of Ca^{2+} indicator was monitored with a fluorescence microscope (Nikon), and the cytosolic Ca^{2+} concentration was calculated from the Fura-2 fluorescence according to the formula of Grynkiewicz *et al.* (*41*): $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)] \times (F_{380max}/F_{380min})$, with $K_d = 227$ nM. For measurement of Ca^{2+} influx at late phase, BMBAs sensitized with TNP-specific IgE were incubated at 37°C with TNP-OVA (30 ng/ml) or IL-3 (1 ng/ml) for 1 or 6 hours in RPMI 1640 plus 10% fetal calf serum (complete medium) before loading the indicator. After washing out the indicator, cells were attached to the glass-bottom dish in Ca^{2+}-sufficient HBSS containing indicated stimulators, and the fluorescence intensity of Ca^{2+} indicator was monitored with a fluorescence microscope. Data were analyzed using AquaCosmos 2.0 software.

Induction of IgE-CAI and extravascular leakage analysis

Induction of IgE-CAI and the analysis of extravascular leakage were performed as described previously (*40*). The value of Δear thickness, the differences in ear thickness (right − left), was calculated for the evaluation of inflammation. The extravascular leakage analysis was performed on day 3 after challenge in the IgE-CAI response.

Treatment of mice with cytokines

Mice were treated daily with intraperitoneal administration of IL-3, IL-33, IL-3 plus IL-33 (2 μg each time), or control PBS for 4 days. Serum samples were collected from the mice 6 hours after the last treatment to determine IL-4 concentration.

Treatment of mice with TAM and DT

For turning on Cre activity, mice were treated twice with intragastric administration of TAM solved in corn oil, 150 mg/kg on day −1 and 50 mg/kg on day 0 of the allergen challenge or cytokine administration. For depletion of basophils in vivo, $Mcpt8^{DTR}$ mice were treated once with intravenous administration of 500 ng of DT or control mDT 1 day before allergen challenge.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/12/576/eaav2060/DC1

Fig. S1. Loss of STIM1, STIM2, or FcRγ does not alter the abundance of β-hexosaminidase in BMBAs.
Fig. S2. Loss of both STIM1 and STIM2 inhibits both FcεRI- or IL-3R–dependent responses of BMBAs.
Fig. S3. IL-4 production by activated BMBAs requires intracellular and extracellular Ca^{2+}.
Fig. S4. Stimulation of BMBAs with IgE/allergen, but not IL-3, elicits the Ca^{2+} influx.
Fig. S5. Generation of transgenic mice expressing a TAM-inducible improved Cre recombinase under the control of *Mcpt8* promoter.
Fig. S6. IL-3 is dispensable for the development of IgE-CAI.
Fig. S7. Loss of STIM1, but not STIM2, impairs BMBA IL-4 production stimulated by IL-3 + IL-33.

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Pivotal role of STIM2, but not STIM1, in IL-4 production by IL-3–stimulated murine basophils

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STIMulating basophils

The ER-resident Ca^{2+} sensors stromal interaction molecule 1 (STIM1) and STIM2 promote store-operated Ca^{2+} entry, which is required for immune cell function (see the Focus by Alansary and Niemeyer). Using basophils from conditional knockout mice, Yoshikawa *et al*. found that STIM2 was required for production of the cytokine IL-4 in vitro in response to IL-3, whereas STIM1 was necessary for IL-4 production in response to antigen-antibody complexes. In mice, the development of antigen-dependent allergic inflammation required STIM1 expression in basophils, but the response to a combination of IL-3 and IL-33 required basophil expression of STIM2. These results suggest that the STIM proteins in basophils have distinct roles in mediating responses to antigens or cytokines.

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