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Establishment of a Reporter System to Monitor FcRγ-Dependent Activation of NFAT

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Basophils are recognized as effectors of type 2 immune responses, producing IL-4 in response to various stimuli such as IL-3 and papain in addition to IgE. In this study, we have established a novel cell-based reporter system that can monitor the activation of the transcription factor NFAT using retroviral vectors. Using this system, we examined whether papain, which is known to induce IL-4 in an FcRγ-dependent manner, could be used to identify its receptor. We created a chimeric receptor in which the extracellular and transmembrane portions are CD8 and the cytoplasmic domain is FcRγ. This chimeric receptor was able to induce GFP in RBL by cross-linking with anti-CD8 antibody. Furthermore, we found that this chimeric receptor can function as a papain receptor, as GFP was upregulated by papain stimulation. On the other hand, statins were able to suppress the expression of GFP by IgE crosslinking. This reporter system can be used to transduce candidate receptors and examine their papain receptor activity using GFP expression as an indicator, and is therefore a useful system that can be used for expression-cloning of unknown papain receptors and for the study of various inhibitors.

Key words cytokine, NFAT, reporter

INTRODUCTION

Basophils are innate immune cells that are present in only about 0.1% of peripheral blood. Basophils function as effector cells of inflammation in allergic inflammation, releasing potent inflammatory mediators such as histamine and PAF in an immunoglobulin E (IgE)-dependent manner.[1)] IgE receptor FcεRI is expressed as a tetramer consisting of specific FcεRIα, FcεRIβ, and FcRγ homodimers.[2)] FcRγ has immunoreceptor tyrosine-based activation motifs (ITAM) within its cytoplasmic domain. Cross-linking of FcεRI with IgE and multivalent antigen initiates an activation signaling cascade via tyrosine phosphorylation of this ITAM by Src family kinases, followed by the recruitment and activation of the spleen tyrosine kinase (Syk). Syk then activates a signaling cascade leading to downstream activation of NF-κB and calcium signaling-dependent NFAT.[3)] FcRγ binds to immune receptors, such as PirA and mincle, and signals as their adapter molecules.[4)]

Basophils produce large amounts of cytokines such as IL-4 and IL-6 by IL-3.[5,6)] Furthermore, it was reported that papain, a plant allergen protease, directly induces IL-4 production by basophils and causes basophils to accumulate in draining lymph nodes where Th2 responses are expected to begin.[7)] Interestingly, both IL-3 and papain are IgE-independent and mediated by FcRγ.[6,8,9)] Basophils can produce IL-4 in response to diverse signals, and the role of IL-4 in the pathogenesis of allergic diseases is of importance due to its potent biological activity.[10)] In addition, IL-4 has been established as an essential cytokine for Th2 differentiation, but the cellular source of this cytokine is still under controversy. Basophils have been recognized as a potential cell type capable of producing IL-4 in response to various stimuli and initiating type 2 immune responses.[11-13)] However, basophils regulate the *Il4* gene in a STAT6-independent manner through a molecular mechanism that differs from that of Th2 cells, the details of which are not yet clear.[6)] NFAT, C/EBPα, and GATA family have been reported as transcription factors important in the regulation of IL-4 production.[14-16)] NFAT2 is predominantly expressed in human basophils compared to NFAT1 and NFAT4, and NFAT2 binds to the promoter region of IL-4 through cytoplasmic to nuclear translocation in a FcεRI receptor stimulation dependent manner.[16)] In human basophils, NFAT2, which is important for IL-4 production by T cells and a transcription factor essential for myeloid cell differentiation, and C/EBPα can selectively activate IL-4 promoter-reporter gene transcription in response to IgE cross-linking.[14)] It is possible that C/EBPα, in cooperation with NFAT, directly regulates *Il4* gene transcription. We have established stable reporter cells expressing NFAT-depend-

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ent GFP in various cell types using retroviral vectors. Basophil "receptors" and downstream signals for many allergens and pathogens will be identified in the future, and we expect that this system will contribute to various aspects of immune response and inflammation.

MATERIALS AND METHODS

Mice C57BL/6 mice were from SLC (Shizuoka, Japan). All mice used in this study were maintained in the animal facilities in Nagoya city University under strictly controlled specific pathogen-free conditions, with regular monitoring of infection, and used at 8-14 weeks of age. All animal experiments were approved by the Committee for Animal Experimentation and Care of Nagoya City University and Shinshu University and conducted according to the guideline.

Cell Culture BM-derived basophils (BMBs) were obtained as described.[17] Briefly, whole BM cells (2 x10^7) were cultured in 10 ml of 10% fetal bovine serum (FBS)-containing RPMI1640 medium (Nissui) supplemented with recombinant murine IL-3 (5 ng/ml) for 10-12 days with medium changed every 3 days. BM-derived basophils were purified by depleting mast (c-kit$^+$) cells using IMag-DM beads (BD Biosciences), and used for stimulation. BMBs were stimulated with IL-3 (5 ng/ml), anti-DNP IgE mAb (SPE-7, SIGMA or Nihon biotest, 1 μg/ml) + anti-DNP IgE mAb (1 μg/ml) +DNP-BSA (1 μg/ml), papain (Calbiochem, 100 μg/ml), LPS (BD Biosciences and SIGMA-Aldrich, 50 ng/ml) and Zymosan (Fujifilm, 100 μg/ml). The supernatants were collected to measure IL-4 proteins using Mouse IL-4 ELISA antibodies set (Biolegend). RBL-2H3 cell were cultured in 10% FBS-containing RPMI1640 medium (Nissui) with medium changed every 3 days.

Plasmid Construction A reporter plasmid containing the NFAT-binding region in the promoter sequence of human *Il2* was kindly provided by Drs. Takashi Saito and Sho Yamazaki.[18] The multicloning site in pBluescript contains three NFAT binding regions, GFP, and polyA signals. The plasmid was blunted after digesting with Sac I, and further digested with Cla I. This fragment was ligated into the retroviral vector pSINsi-mU6 digested with Cla I and Hinc II. Clones were then confirmed by sequencing.

Retroviral Infection Retroviral vector (pSINsi-mU6) were used for transfection of reporter gene. These retroviral constructs were transfected into the packaging cell line Phoenix using GeneJuice reagent (Merck Millipore). Retrovirus-containing supernatants were collected 48 h after transfection, concentrated about 10-fold by centrifugation and added to 12-well plates, which were processed by treatment with RetroNectin solution (20 μg/ml, Takara Bio) in PBS.[6] After incubating the plates with the virus supernatants for 3 h at room temperature, RBL-2H3 or RBL-pNFAT-GFP reporter cells (1 x 10^5 cells/ml) were infected for 2 days. To establish reporter cells, retrovirus-infected RBL-2H3 cells were cultured in the presence of G418 (Roche, 0.5 mg/ml) for 1 week. Wild type FcRγ and chimeric protein consisting of the intracellular portions of FcRγ fused to the extracellular and transmembrane portions of CD8, CD8/FcRγ, inserted into the retroviral vectors (pMX-IRES-ratCD2). Cells stably expressing ratCD2 were sorted with IMag-DM beads, and used for stimulation.[6,9]

Statistical Analysis All data are presented as means ± S.D. Statistical significance of differences between groups was assessed using Student's t-test. *$p < 0.05$, **$p < 0.01$ were considered significant.

RESULTS

Establishment of a Reporter Cell Line to Monitor NFAT Activity The rat basophil cell line, RBL-2H3 cell, expresses the high-affinity IgE receptor FcεRI, and we will establish a reporter cell line using these cells. FcεRI is composed of three subunits: FcεRIα, FcεRIβ and FcRγ. The FcεRIβ and FcRγ subunits are essential for receptor expression and IgE signaling. The amino acid homology between rat and mouse FcεRIα, FcεRIβ, and FcRγ is approximately 70%, 80%, and 90%, respectively, and the binding of mouse IgE to RBL-2H3 cells was also confirmed by flow cytometry (data not shown). In research on the C-type lectin receptor, mincle, a receptor that binds to FcRγ, lymphocyte-based reporter cells were used to activate NFAT.[19]

In this study, we attempted to establish basophilic reporter cells that could quantitatively evaluate FcRγ signaling using activation of NFAT as an indicator. To establish a stable cell line, we constructed a retrovirus vector pSINsi with the NFAT-binding region derived from the human IL-2 promoter and GFP. This retrovirus vector plasmid lacks promoter activity in the 3'LTR U3 region. Therefore, after reverse transcription, the promoter activity of the 5'LTR is lost in the state of the provirus inserted into the chromosome, and mRNA derived from the provirus is not transcribed. In addition, GFP was inserted as shown in Fig. 1A, except for the promoter region of the pol III system, which is important for shRNA synthesis. After transfection of the retroviral vector into packaging cells, culture supernatant containing retrovirus was obtained. After this retrovirus was infected with RBL-2H3, the cells were cultured in RPMI containing 0.5 mg/ml of G418 for 1 week, and G418-resistant clones were isolated. The reporter cells were designated RBL-pNFAT-GFP cells.

The ability of the reporter cell line to monitor NFAT activation was then evaluated based on increased expression of GFP. Flow cytometry was applied to evaluate the mean fluorescence intensity (MFI). After sensitization with 1 μg/ml IgE for 12 h, the MFI of GFP increased in a concentration-dependent manner with antigen when stimulated with the multivalent antigen DNP-BSA (Fig.1B). Examined whether there is a difference in the time of GFP expression by IgE sensitization method and IgE immune complex stimulation. As shown in Fig. 1C and 1D, these methods induced GFP expression after 2 h of stimulation and reached its highest value about 12 h later. Even after 24 h, the MFI of GFP remained at the same fluorescence intensity as that at 12 h. Based on these results, we decided to evaluate the fluorescence intensity of GFP after 24 h of stimulation in our experiments.

Activation of NFAT by Various Stimuli Previously, we have examined cytokine production, including IL-4, using cultured basophils derived from mouse bone marrow (BMBs).[6,17] We have shown that cultured BMBs produce large amounts of IL-4 by IgE-IC and papain. LPS, a ligand for Toll-like receptor (TLR) 4, did not produce any IL-4. A small amount of IL-4 production was observed when stimulated with Zymosan. (Fig. 2A). We next examined whether RBL-pNFAT-GFP cells responds to these stimuli. In NFAT reporter cells, compared to no stimulation (MFI: 0.4 x 10^3 ± 5.1), the MFI of GFP was significantly increased by IgE-IC (MFI: 3.2 x 10^3 ± 56.7).

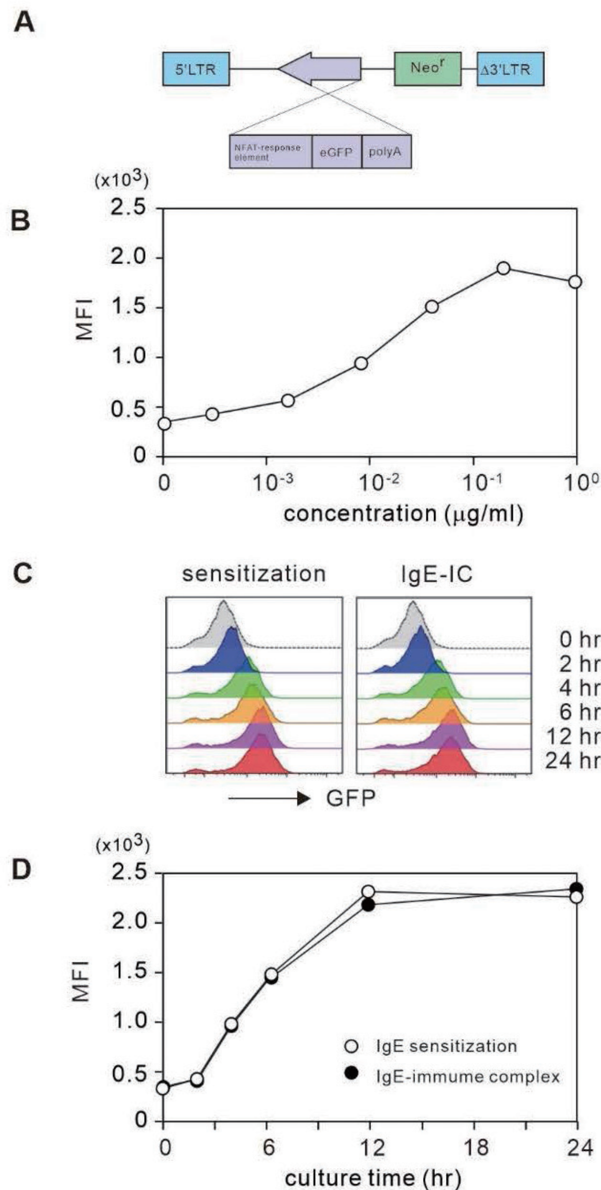

Fig. 1. Generation of GFP-Expressing Reporter Cell Lines to Monitor FcRγ-Mediated Activation of NFAT

(A) Schematic of NFAT reporter retroviral vectors. This insert fragment has three copies of the NFAT site cloned upstream of the minimal IL-2 promoter and GFP reporter. (B) RBL-pNFAT-GFP reporter cell lines were sensitized with IgE (1 μg/ml) for 12 h and then stimulated with DNP-BSA at the indicated concentrations. GFP expression was analyzed by flow cytometry and the mean fluorescence intensity (MFI) was measured. (C) Comparison of IgE crosslinking methods. The left panel showed a histogram of GFP fluorescence intensity when reporter cells were stimulated with the multivalent antigen DNP-BSA (1 μg/ml) after 12 h of IgE (1 μg/ml) sensitization. The right panel showed histograms of fluorescence intensity when cells were stimulated with the immune complex (IC) after 30 minutes of incubation at room temperature to form IC of IgE (1 μg/ml) and DNP-BSA (1 μg/ml). (D) The time course of MFI in C was shown in the graph. Data shown are representative of two independent experiments.

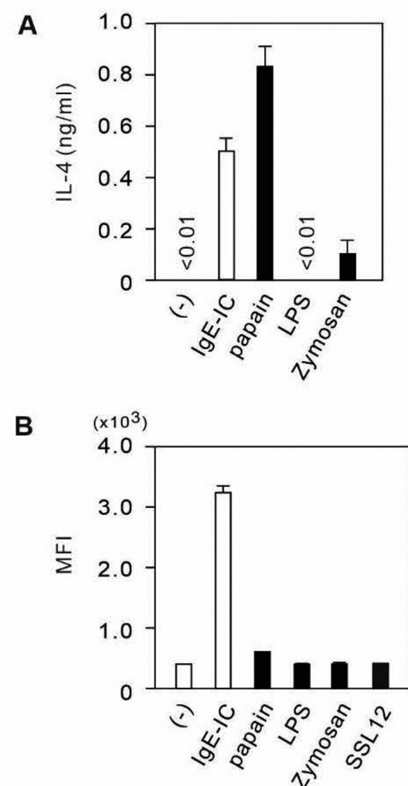

Fig. 2. Activation of NFAT by Various Stimuli

(A) Enriched BMBs were stimulated with IgE-IC (IgE; 1 μg/ml + DNP-BSA; 1 μg/ml), papain (100 μg/ml), LPS (50 ng/ml) or Zymosan (100 μg/ml) in the presence of IL-3 (5 ng/ml) for 24 h, and the amount of IL-4 produced. (B) RBL-pNFAT-GFP reporter cells were stimulated with IgE-IC, papain, LPS, zymosan, or SSL12 (10 μg/ml) for 24 h, Mean values of triplicate determinations ± S.D. of cytokine concentrations were measured in the supernatants using specific ELISAs. Error bars represent the mean ± S.D., and representative of more than three independent trials.

Papain slightly increased the fluorescence intensity of GFP (MFI: 0.6 x 10^3 ± 5.3). However, neither LPS nor zymosan increased GFP fluorescence intensity in NFAT reporter cells (MFI: 0.4 x 10^3 ± 9.2, 0.4 x 10^3 ± 14.0). BMBs produce IL-4 by the Staphylococcus aureus toxin SSL12,[20] but SSL12 did not increased the MFI of GFP in NFAT reporter cells (MFI: 0.4 x 10^3 ± 1.5) (Fig. 2B). These results indicate that NFAT was not activated by stimulation of pattern recognition receptors such as TLRs. Furthermore, the sensor molecules that recognize papain, LPS, zymosan, and SSL12, may be poorly expressed in RBL cells.

Evaluation of Receptor Recognizing Cysteine Protease, Papain We previously reported that BMBs produce IL-4 when stimulated with papain *in vitro*; BMBs from mice lacking FcRγ (*Fcer1g* $^{-/-}$ mice) cannot produce IL-4 when stimulated with papain.[9] Based on previous observations, we believe that BMBs "senses" the protease activity of papain and specific FcRγ-related surface molecules to produce IL-4. One molecule that is cleaved by papain is the CD8 molecule; CD8 is a receptor that forms dimers on T cells that recognize MHC class I. A chimeric protein fusing the extracellular and plasma membrane portions of CD8, a sensitive cell surface molecule, with the intracellular portion of FcRγ was transduced into RBL-pNFAT-GFP cells (Fig. 3A). When RBL-pNFAT-GFP cells were stimulated with anti-CD8 antibody, GFP fluorescence intensity increased to the same level as that of IgE-IC in the reporter cells expressing CD8/FcRγ but not wild-type FcRγ (Fig. 3B). This suggests that when the CD8/FcRγ fusion molecule was expressed in the cells, the ITAM of FcRγ was phosphorylated by cross-linking CD8 and transmitted the signal. Furthermore, when this BMBs was treated with papain, IL-4 was produced, suggesting that papain activates NFAT via this CD8/FcRγ molecule.[9] Consistent with this observation,

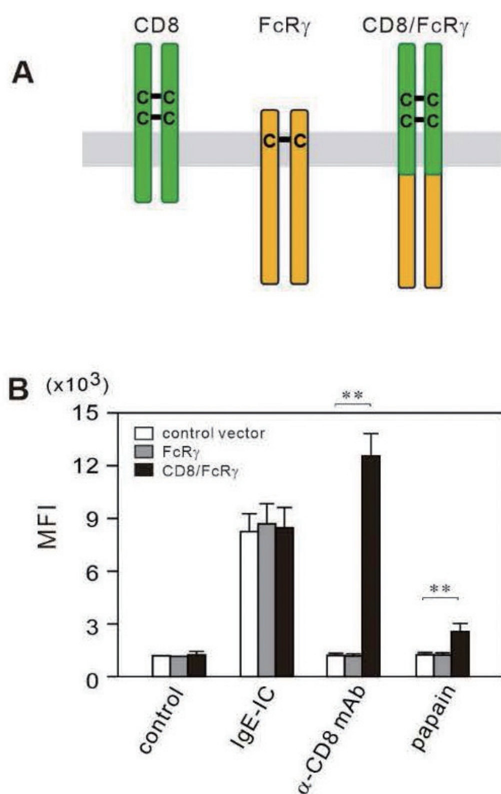

Fig. 3. The Extracellular Region of CD8 Molecules Fused with FcRγ Could Transduce Papain Signals

(A) Schematic representation of mouse CD8, FcRγ and CD8/FcRγ chimera protein. (B) Establishment of RLB-pNFAT-GFP reporter cells expressing FcRγ or CD8/FcRγ. These reporter cells were transduced with pMX-IRES-ratCD2 retrovirus and purified as described in "Materials and methods". The reporter cells expressing FcRγ or the CD8/FcRγ chimera were stimulated with IgE-IC (IgE; 1 μg/ml + DNP-BSA; 1 μg/ml), soluble anti-CD8 mAb (0.5 μg/ml) or papain (100 μg/ml) for 24 h. Results are mean fluorescence intensity (MFI); representative data (mean ± S.D.; n = 6) are shown. ***p* < 0.01 compared with IgE-IC-treated cells.

RBL-pNFAT-GFP cells showed a slight detectable GFP fluorescence enhancement when stimulated with papain (Fig. 4B). Furthermore, inhibitors of cysteine proteases suppressed papain-dependent GFP expression, indicating that GFP expression is dependent on protease activity (data not shown).

In the absence of IL-3, the amount of IL-4 induced by papain is about one-tenth of that produced in response to cross-linking of CD200R3 and FcεRI.[9,21] Similarly, when RBL-pNFAT-GFP cells were stimulated with papain, the mean fluorescence intensity of GFP was lower than that of IgE-IC stimulation (Fig. 3B). These results indicate that the extracellular portion of the CD8 molecule may act as a "sensor" to transmit papain signals to the intracellular portion of FcRγ and activate NFAT. Therefore, it may be possible to identify the sensor molecule of papain by transducing cDNA of FcRγ-related molecules derived from basophils using these reporter cells.

Statins Suppress IgE-Mediated NFAT Activation in Report Cells IgE-mediated cytokine production is affected by a variety of chemicals. Statins act by competitively inhibiting 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), resulting in cholesterol reduction. Statins have also been reported to exert immunomodulatory effects.[22] Kolawole *et al.* and colleagues recently tested statin effects on IgE-mediated activation of mast cells and basophils. Fluvastatin showed a marked inhibitory effect on IgE-induced cytokine secretion by mast cells and basophils in mice.[23] We used the NFAT reporter cells to investigate if the inhibitory effect of several statin families on IgE-mediated cytokine production could also be evaluated. RBL-pNFAT-GFP cells treated for 24 h before IgE-IC stimulation showed a decrease in GFP MFI of propidium iodide (PI)-negative viable cells in all statin treatments (Fig. 4A). Lovastatin reduced GFP fluorescence intensity predominantly by about 50% at 1 μM. Fluvastatin and sinvastatin reduced their MFI by about 70% at 1 μM. At 5 μM of statin, all statins reduced GFP fluorescence intensity to about 50%. Cell viability also showed that treatment with statins other than atrovastatin increased the number of PI-positive cells, and PI-negative viable cells decreased to 10-30% at 5 μM. In particular, fluvastatin induced cell death predominantly at 1 μM or higher. On the other hand, atorvastatin induced almost no cell death even above 1 μM. Statins not only inhibit FcRγ -mediated activation of NFAT, but also affect cell survival. These results indicate that statins differ in their effects on signaling molecules involved in NFAT activation and in their sensitivity to various molecules involved in cell survival. This difference in the inhibitory effect of statins on NFAT activation may be due to differences in compound structure, which affected absorption, metabolism. In the future, clarification of the differences in the sensitivity of statins to their target molecules may provide a system that can be used for risk assessment when statins are used as therapeutic agents in clinical practice.

DISCUSSION

The search for sensor molecules of cysteine protease and other exogenous molecules expressed on basophils may be useful in elucidating the defense mechanisms against parasites and the pathogenesis of allergic inflammation. Basophils account for less than 1% of peripheral blood leukocytes and are thought to have a lifespan of only a few days.[24] Cultured basophils by IL-3 from mouse bone marrow have a short lifespan, with cell death occurring in approximately 14 days.[17] Primary cultured basophils are not suitable for analysis of molecular mechanisms of cytokine production because they are inefficient for gene expression.

Basophils produce large amounts of IL-4 when activated by stimuli that elicit a calcium response, such as FcεRI-mediated activation. Since basophils are only present in peripheral blood in small quantities, it is difficult to search for endogenous and exogenous ligands and their sensor molecules that induce IL-4 production. In addition, bone marrow-derived mast cells from C57BL/6 mice have low IL-4 production capacity, making them unsuitable for analysis of the IL-4 production mechanism by papain. In this study, we aimed to establish various stable reporter cells using retroviral vectors and considered the rat basophil-like cell line RBL-2H3 as a candidate for cultured cells equipped with a signal transduction pathway from FcRγ to IL-4 production. We also established cells in which the GFP gene was introduced downstream of the NFAT-binding sequence cloned from the human IL-2 promoter as a reporter gene (RBL-pNFAT-GFP cells). In this reporter cell, IgE-IC stimulation caused NFAT to bind to its promoter binding sequence and its activation increased GFP fluorescence inten-

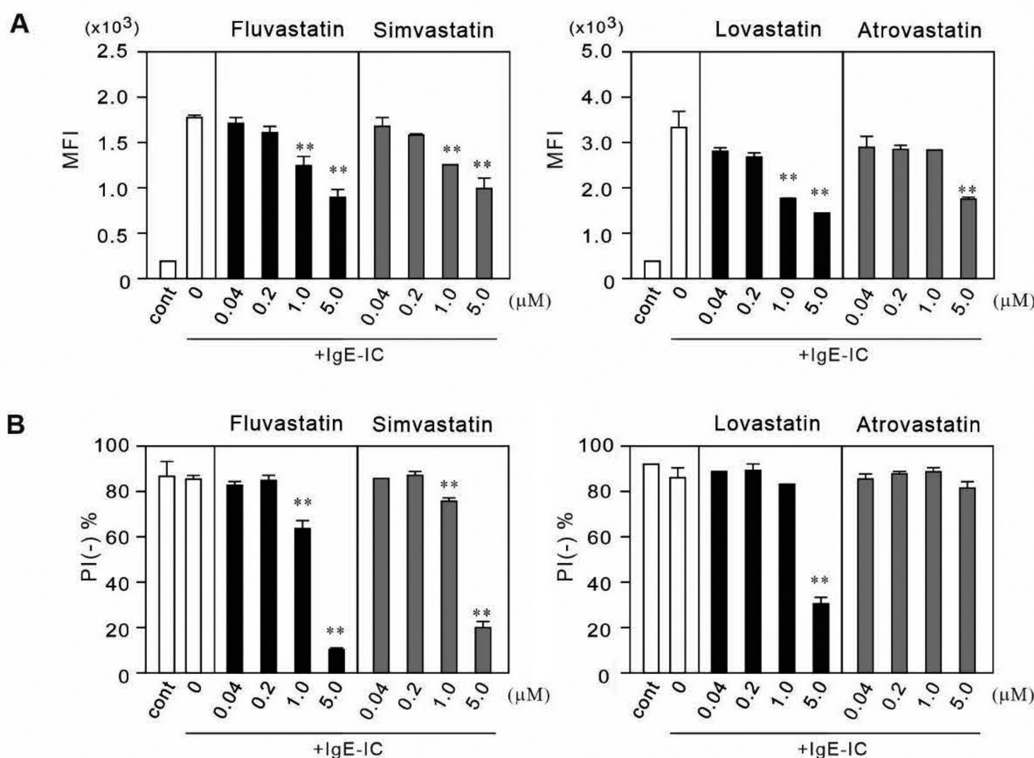

Fig. 4. Statins Suppress IgE-Mediated NFAT Activation in Report Cells

(A) RBL-pNFAT-GFP cells were cultured with DMSO or each statin at the indicated concentrations for 24 h and then stimulated with IgE-IC (IgE; 1 μg/ml + DNP-BSA; 1 μg/ml). Results are shown as mean fluorescence intensity. (B) After RBL-pNFAT-GFP cells were cultured as in A, the percentage of viable cells (PI-negative cells) was measured by flow cytometry. A representative of one of three independent experiments done in duplicate is shown (mean ± S.D.). $**p < 0.01$ compared with the results of IgE-IC alone stimulation.

sity. NFAT2, but not NFAT1, has been reported to be selectively expressed on human basophils,[16] and the correlation between NFAT2 localization and IL-4 production suggests that this molecule is important for IL-4 production.

We attempted to construct an artificial sensor molecule that activates FcRγ by the protease activity of papain. We searched for membrane molecules that are sensitive to cleavage by papain using splenocytes, and CD8 was considered as a candidate. Since this chimeric molecule has the extracellular region of CD8 cleaved by papain and an intracellular region of FcRγ, it is expected to activate the FcRγ-mediated signaling pathway, since the CD8 molecule can also mediate papain stimulation by fusing with the FcRγ cytoplasmic portion. Papain "sensing" may not require a specific structure. Indeed, by transducing the CD8/FcRγ molecule into reporter cells, we observed a weak increase in GFP fluorescence intensity. This increase of MFI was suppressed by cysteine protease inhibitors, suggesting that it is dependent on protease activity; we postulate ITAM phosphorylation-Syk activation via activation of phosphorylated molecules such as Src family kinases, but the detailed mechanism is not clear. In the future, when the sensor molecules are identified, we would like to discuss the detailed signaling pathway and molecular mechanism.

Statins are also widely prescribed for hypercholesterolemia and cardiovascular disease. However, apart from their effects on lipids, it has been suggested that statins have anti-inflammatory and Th2 response-promoting effects. The immunomodulatory effects of statins are not due to large-scale changes in lipid rafts, including cholesterol geranylgeranylation has been suggested to affect cytokine production via IgE.[23] Two types of statins exist, lipophilic and hydrophilic, depending on their structure. Interestingly, different statins have different immunomodulatory effects, but the molecular mechanisms are not clear. In our study with reporter cells, we found differences in the inhibition of NFAT activation and cell viability in response to IgE-IC stimulation (Fig. 4). Since fluvastatin suppresses Akt and Erk phosphorylation in bone marrow-derived mast cells, it may also affect signaling and transcription factors related to cell proliferation and survival. It is very interesting that sensitivity to each statin may depend on species differences and genetic background.[23]

The reporter cells established in this study could be used not only for cysteine proteases, but also for the search for various endogenous and exogenous ligands and their receptors. Moreover, they could also be used to screen for inhibitors of immune cell activation by known allergens and chemicals. In conclusion, we have developed a reporter system using retroviral vectors to monitor NFAT activation. This system can be applied to a variety of cells and is expected to be a useful tool for elucidating the molecular mechanisms involved in cytokine production, thereby contributing to immunological research and drug discovery.

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Conflict of Interest The authors declare that they have no competing financial interests or personal relationships that would affect the research reported in this paper.

REFERENCES

1) Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, Kawano Y, Minegishi Y, Shimizu T, Karasuyama H. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity*, **28**, 581–589 (2008).
2) Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.*, **15**, 639–646 (2003).
3) Ivashkiv LB. Cross-regulation of signaling by ITAM-associated receptors. *Nat. Immunol.*, **10**, 340–347 (2009).
4) Hamerman JA, Ni M, Killebrew JR, Chu CL, Lowell CA. The expanding roles of ITAM adapters FcRgamma and DAP12 in myeloid cells. *Immunol. Rev.*, **232**, 42–58 (2009).
5) Min B, Prout M, Hu-Li J, Zhu J, Jankovic D, Morgan ES, Urban JF Jr, Dvorak AM, Finkelman FD, LeGros G, Paul WE. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.*, **200**, 507–517 (2004).
6) Hida S, Yamasaki S, Sakamoto Y, Takamoto M, Obata K, Takai T, Karasuyama H, Sugane K, Saito T, Taki S. Fc receptor gamma-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. *Nat. Immunol.*, **10**, 214–222 (2009).
7) Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.*, **9**, 310–318 (2008).
8) Rosenstein RK, Bezbradica JS, Yu S, Medzhitov R. Signaling pathways activated by a protease allergen in basophils. *Proc. Natl. Acad. Sci. USA*, **111**, E4963–E4971 (2014).
9) Morikawa A, Matsui Y, Kitano T, Itoh S, Taki S, Hida S. Interleukin-3 potentiates murine basophils for protease allergen-induced interleukin-4 production. *BPB Reports*, **5**, 88–93 (2022).
10) Gubernatorova EO, Namakanova OA, Gorshkova EA, Medvedovskaya AD, Nedospasov SA, Drutskaya MS. Novel anti-cytokine strategies for prevention and treatment of respiratory allergic diseases. *Front. Immunol.*, **12**, 601842 (2021).
11) Eglite S, Pluss K, Dahinden CA. Requirements for C5a receptor-mediated IL-4 and IL-13 production and leukotriene C4 generation in human basophils. *J. Immunol.*, **165**, 2183–2189 (2000).
12) Kojima T, Obata K, Mukai K, Sato S, Takai T, Minegishi Y, Karasuyama H. Mast cells and basophils are selectively activated *in vitro* and *in vivo* through CD200R3 in an IgE-independent manner. *J. Immunol.*, **179**, 7093–7100 (2007).
13) Aumuller E, Schramm G, Gronow A, Brehm K, Gibbs BF, Doenhoff MJ, Haas H. Echinococcus multilocularis metacestode extract triggers human basophils to release interleukin-4. *Parasite Immunol.*, **26**, 387–395 (2004).
14) Qi X, Nishida J, Chaves L, Ohmori K, Huang H. CCAAT/enhancer-binding protein alpha (C/EBPalpha) is critical for interleukin-4 expression in response to FcepsilonRI receptor cross-linking. *J. Biol. Chem.*, **286**, 16063–16073 (2011).
15) Nei Y, Obata-Ninomiya K, Tsutsui H, Ishiwata K, Miyasaka M, Matsumoto K, Nakae S, Kanuka H, Inase N, Karasuyama H. GATA-1 regulates the generation and function of basophils. *Proc. Natl. Acad. Sci. USA*, **110**, 18620–18625 (2013).
16) Schroeder JT, Miura K, Kim HH, Sin A, Cianferoni A, Casolaro V. Selective expression of nuclear factor of activated T cells 2/c1 in human basophils: evidence for involvement in IgE-mediated IL-4 generation. *J. Allergy Clin. Immunol.*, **109**, 507–513 (2002).
17) Hida S, Tadachi M, Saito T, Taki S. Negative control of basophil expansion by IRF-2 critical for the regulation of Th1/Th2 balance. *Blood*, **106**, 2011–2017 (2005).
18) Ohtsuka M, Arase H, Takeuchi A, Yamasaki S, Shiina R, Suenaga T, Sakurai D, Yokosuka T, Arase N, Iwashima M, Kitamura T, Moriya H, Saito T. NFAM1, an immunoreceptor tyrosine-based activation motif-bearing molecule that regulates B cell development and signaling. *Proc. Natl. Acad. Sci. USA*, **101**, 8126–8131 (2004).
19) Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.*, **9**, 1179–1188 (2008).
20) Nishiyama S, Urabe A, Morikawa A, Kobayashi M, Onozaki K, Itoh S, Hida S. Staphylococcal superantigen-like 12 induces the production of interleukin 4 in murine basophils. *Biochem. Biophys. Res. Commun.*, **532**, 200–204 (2020).
21) Kitano T, Togawa K, Takemori J, Motoki Y, Kishida K, Itoh S, Takamoto M, Taki S, Hida S. Interleukin-3-dependent potentiation of IgE responsiveness in mouse basophils. *Genes Cells*, **28**, 226–236 (2023).
22) Liao JK, Laufs U. Pleiotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol.*, **45**, 89–118 (2005).
23) Kolawole EM, McLeod JJ, Ndaw V, Abebayehu D, Barnstein BO, Faber T, Spence AJ, Taruselli M, Paranjape A, Haque TT, Qayum AA, Kazmi QA, Wijesinghe DS, Sturgill JL, Chalfant CE, Straus DB, Oskeritzian CA, Ryan JJ. Fluvastatin suppresses mast cell and basophil IgE responses: genotype-dependent effects. *J. Immunol.*, **196**, 1461–1470 (2016).
24) Karasuyama H, Yamanishi Y. Basophils have emerged as a key player in immunity. *Curr. Opin. Immunol.*, **31**, 1–7 (2014).