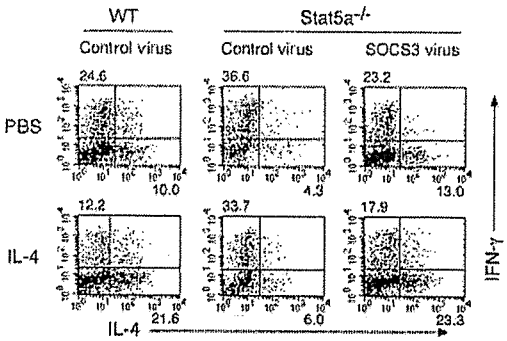

FIGURE 7. Retrovirus-mediated gene transduction of SOCS3 restores Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml) and then infected with retroviruses of pMX-SOCS3-IRES-GFP or pMX-IRES-GFP (as a control) as described in *Materials and Methods*. Cells were cultured with IL-2 in the presence or absence of IL-4 for another 72 h. Cells were restimulated with plate-bound anti-CD3 mAb for 6 h and intracellular cytokine profiles for IL-4 vs IFN-γ were evaluated on infected CD4$^+$ T cells (GFP$^+$ CD4$^+$ cells). In these conditions, we found that the levels of SOCS3 evaluated by intracellular FACS analysis were ~1.5-fold higher in SOCS3 retrovirus-infected Stat5a$^{-/-}$ CD4$^+$ T cells as compared with those in WT CD4$^+$ T cells cultured in Th2 condition. Shown is a representative intracellular cytokine staining from four independent experiments.

Discussion

In this study, we show that Stat5a regulates IL-12-induced Th1 cell differentiation through SOCS3 induction. We found that enhanced Th1 cell differentiation and the IFN-γ-mediated suppression were a principal reason for the decreased Th2 cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells (Fig. 1). We then found that IL-12-induced Th1 cell differentiation (Fig. 2) and Stat4 phosphorylation (Fig. 3, *a* and *b*) were enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. Moreover, SOCS3, a potent inhibitor of IL-12/Stat4 signaling (14), was decreased in Stat5a$^{-/-}$ CD4$^+$ T cells (Fig. 4). Furthermore, the reporter assay showed that Stat5a but not Stat6 directly activated the SOCS3 promoter (Fig. 5) and ChIP assay revealed that Stat5a bound to the SOCS3 promoter in CD4$^+$ T cells (Fig. 6). Finally, the retrovirus-mediated expression of SOCS3 restored the altered Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells (Fig. 7). These results suggest that Stat5a induces SOCS3 expression in CD4$^+$ T cells and thus inhibits IL-12-induced Th1 cell differentiation, resulting in the increase in Th2 cell differentiation.

We show that IL-12/Stat4 signaling and subsequent IL-12-induced Th1 cell differentiation are up-regulated in Stat5a$^{-/-}$ CD4$^+$ T cells. We found that Stat5a$^{-/-}$ CD4$^+$ T cells but not WT CD4$^+$ T cells differentiated into Th1 cells in response to a low concentration of IL-12 (Fig. 2). We also found that IL-12-induced Stat4 phosphorylation was enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells (Fig. 3, *a* and *b*). In contrast, IL-12 production from APCs was not significantly altered in Stat5a$^{-/-}$ mice (data not shown). These results suggest that the increased sensitivity to IL-12/Stat4 signaling is responsible in part for the enhanced Th1 cell differentiation and subsequent Th2 cell suppression in Stat5a$^{-/-}$ CD4$^+$ T cells. However, even in the presence of anti-IFN-γ Ab, Th2 cell differentiation was still decreased in Stat5a$^{-/-}$ CD4$^+$ T cells as compared with that in WT CD4$^+$ T cells (Fig. 1), suggesting that the increased IFN-γ production cannot account for all of the impairment in Th2 cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. Direct induction of the IL-4 gene by Stat5a (12) may account for the difference between WT CD4$^+$ T cells and Stat5a$^{-/-}$ CD4$^+$ T cells in Th2 cell differentiation in the presence of an anti-IFN-γ Ab.

We also show that Stat5a regulates the expression of SOCS3 in CD4$^+$ T cells. Increasing evidence has revealed that SOCS family proteins are involved in a negative feedback loop of JAK/STAT signaling (28–30). Among SOCS family proteins, SOCS3 has been shown to be preferentially expressed in Th2-polarized cells and to prevent IL-12-induced Th1 cell differentiation (13, 14). In this study, we found that the expression of SOCS3 was decreased not only in freshly isolated CD4$^+$ T cells but also in Th2-polarized CD4$^+$ T cells in Stat5a$^{-/-}$ mice (Fig. 4). We also found that Stat5a bound to the SOCS3 promoter in CD4$^+$ T cells upon IL-2 stimulation (Fig. 6). In addition, we found that a constitutively active form of Stat5a but not a constitutively active form of Stat6 could activate the SOCS3 promoter in a STAT-binding sequence-dependent fashion (Fig. 5*b*), which is in agreement with a previous finding that Stat5a preferentially recognizes TTC-N3-GAA STAT-binding sequence, whereas Stat6 preferentially recognizes TTC-N4-GAA STAT-binding sequence (31). Taken together, these results suggest that Stat5a but not Stat6 induces SOCS3 expression in the developing Th2 cells. Moreover, because the retrovirus-mediated expression of SOCS3 restored the altered Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells (Fig. 7), the reduced expression of SOCS3 is likely to be involved in the disregulated Th1/Th2 balance in Stat5a$^{-/-}$ CD4$^+$ T cells.

Accumulating evidence suggests that the Stat5a-induced SOCS3 expression is also involved in the regulation of Th2 cell-mediated allergic inflammation in vivo. First, we have previously shown that Th2 cell-mediated allergic airway inflammation is decreased in Stat5a$^{-/-}$ mice (10), indicating that Stat5a is involved in the induction of in vivo Th2 cell-mediated immune responses. Second, a recent study has shown that SOCS3 expression is increased in peripheral T cells in asthma patients and that the constitutive expression of SOCS3 within T cells results in the enhanced airway hyperreactivity in a mouse model of asthma (14), suggesting that SOCS3 also plays an important role in the induction of Th2 cell-mediated allergic airway inflammation. Third, in the present study, we show that Stat5a is essential for the appropriate expression of SOCS3 in CD4$^+$ T cells, especially in developing Th2 cells (Figs. 4–6). Therefore, although further studies are required, it is suggested that the Stat5a-mediated SOCS3 induction participates in the induction of Th2 cell-mediated allergic airway inflammation.

Because Stat5 has been shown to up-regulate a number of SOCS family proteins (28) and because it is suggested that, in addition to SOCS3, some of SOCS family proteins may regulate Th1 cell and Th2 cell differentiation (30), it is possible that other SOCS family proteins are also involved in Stat5a-mediated Th cell differentiation. For example, SOCS1, an important negative regulator of IFN-γ signaling (32, 33), has been shown to be induced by Stat5 activation (23). However, we found that IFN-γ-induced Stat1 phosphorylation was not enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells (data not shown), suggesting that SOCS1 may not be involved in Stat5a-mediated suppression of Th1 cell differentiation. The possible involvement of other SOCS family proteins in Stat5a-induced Th cell differentiation needs to be determined in future.

Recently, progress has been made on an upstream cytokine for Stat5a activation during Th cell differentiation. Among a number of cytokines that activate Stat5a, it has been demonstrated that blocking of IL-2, either by the neutralization of IL-2 itself or the blocking of IL-2R, decreases Th2 cell differentiation (12, 34, 35). It has also been shown that the developing Th2 cells express higher levels of the IL-2R α-chain and exhibit stronger Stat5 activation than the developing Th1 cells (34), consistent with a previous finding that Stat5a functions as an amplifier of IL-2 signaling by inducing the expression of the IL-2R α-chain (36). Moreover, it has recently been demonstrated that IL-2 but not IL-9, IL-15, or IL-21

induces Stat5 phosphorylation and IL-4 production in activated $CD4^+$ T cells (37). Therefore, it is suggested that IL-2 is most likely to be a cytokine responsible for Stat5a activation during Th cell differentiation.

It is well recognized that Stat5a regulates the expression of CD25 by directly binding to the 5′ regulatory region of the CD25 gene (38, 39). Consistent with this observation, we have previously shown that the number of $CD4^+$ T cells that express CD25 ($CD25^+CD4^+$ T cells) is decreased in $Stat5a^{-/-}$ mice and we have suggested that the decreased number of $CD25^+CD4^+$ T cells may account for the altered Th cell differentiation of $Stat5a^{-/-}$ $CD4^+$ T cells to some extent (11). In addition, it has been demonstrated that Stat5a directly induces IL-4 production by regulating the accessibility of the IL-4 gene (12). Moreover, we show in this study that the induction of SOCS3 expression by Stat5a in conventional $CD4^+$ T cells is important for Stat5a-mediated Th cell differentiation. Therefore, it is suggested that Stat5a regulates Th cell differentiation in multiple pathways. Further studies are required for the understanding of the relative importance of these pathways in Stat5a-mediated Th cell differentiation.

Because Stat5b is highly homologous to Stat5a (40) and because the mice lacking both Stat5a and Stat5b exhibit a severe defect in T cell responses as compared with $Stat5a^{-/-}$ mice or $Stat5b^{-/-}$ mice (41), it is suggested that the function of Stat5a and Stat5b is somehow overlapped. However, the different phenotypes of $Stat5a^{-/-}$ mice and $Stat5b^{-/-}$ mice underscore the distinctive roles of Stat5a and Stat5b. For example, it has been demonstrated that, although $Stat5a^{-/-}$ T cells exhibit no detectable defects in anti-CD3-induced proliferation, $Stat5b^{-/-}$ T cells are defective in anti-CD3-induced proliferation (10, 36, 42), suggesting that Stat5b is likely to play a role in the proliferation and/or survival of activated T cells and that this function of Stat5b may not be shared with Stat5a.

Regarding Th cell differentiation, we have recently found that Stat5a is essential for the residual Th2 cell differentiation in $Stat6^{-/-}$ $CD4^+$ T cells by comparing $Stat6^{-/-}$ mice to Stat5a- and Stat6-double deficient mice in the same genetic background (44). Because Stat5b is normally expressed and activated in response to IL-2 even in the absence of Stat5a (36, 42), the results suggest that Stat5b cannot compensate the role of Stat5a in Stat6-independent Th2 cell differentiation. In contrast, we have previously shown that in addition to Th2 cell differentiation, Th1 cell differentiation is also decreased in $Stat5b^{-/-}$ mice (10). Because it has recently been shown that Stat5 activates the distal region of the human IFN-γ promoter (43), Stat5b may be involved in the induction of IFN-γ production during Th1 cell differentiation.

In conclusion, we show that Stat5a forces the Th1/Th2 balance toward a Th2-type by preventing IL-12-induced Th1 cell differentiation through the induction of SOCS3. Because it has been demonstrated that SOCS3 regulates the onset and maintenance of Th2 cell-mediated allergic diseases such as asthma and atopic dermatitis (14), it is suggested that Stat5a-mediated SOCS3 induction could be a target for the treatment of Th2 cell-mediated allergic diseases.

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Disclosures

The authors have no financial conflict of interest.

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Stat5a Is Essential for the Proliferation and Survival of Murine Mast Cells

Kei Ikeda Hiroshi Nakajima Kotaro Suzuki Norihiko Watanabe
Shin-ichiro Kagami Itsuo Iwamoto

Department of Allergy and Clinical Immunology, Clinical Cell Biology, Graduate School of Medicine, Chiba University, Chiba, Japan

Key Words
Stat5a · Mast cell · Bcl-x(L) · Apoptosis

Abstract
The regulatory role of signal transducer and activator of transcription (Stat) 5a in the proliferation and survival of mast cells was determined using Stat5a-deficient ($Stat5a^{-/-}$) mice. First, although the mast cells in $Stat5a^{-/-}$ mice were morphologically indistinguishable from those in wild-type (WT) mice, the number of peritoneal mast cells was significantly decreased in $Stat5a^{-/-}$ mice as compared with that in WT mice. Furthermore, the interleukin-3 (IL-3)-dependent development of bone marrow-derived mast cells (BMMCs) was markedly decreased in $Stat5a^{-/-}$ mice. Second, IL-3-induced but not stem cell factor (SCF)-induced proliferation of BMMCs was significantly diminished in $Stat5a^{-/-}$ mice as compared with that in WT mice. Moreover, survival rates of both peritoneal mast cells and BMMCs were significantly decreased with increased apoptotic cells in $Stat5a^{-/-}$ mice as compared with those in WT mice. Finally, mRNA of Bcl-x(L) was induced after IL-3 stimulation in WT BMMCs but not in $Stat5a^{-/-}$ BMMCs, which may account for the accelerated apoptosis in $Stat5a^{-/-}$ mast cells. These results indicate that Stat5a plays an important role in mast cell development, proliferation, and survival.

Introduction

Mast cells are recognized as the major effector cells of the type I hypersensitivity reactions by virtue of their possessing high-affinity receptors for immunoglobulin E (IgE). They are known to play a pivotal role in allergic diseases, such as asthma, atopic rhinitis, and atopic dermatitis, and are also known to play an essential role in parasite infection in mice [1, 2]. Mature mast cells are distributed throughout all vascularized tissues, and the development and proliferation of mast cells require proper signaling from several cytokines, among which the c-kit/stem cell factor (SCF) system and interleukin-3 (IL-3) are the best studied [1–4].

Although IL-3 is not essential for the generation of murine mast cells under physiological conditions, it does contribute to increased numbers of tissue mast cells and enhanced immunity in mice infected with the nematode *Strongyloides venezuelensis* [5]. IL-3 is also known to play a central role in the development of bone marrow-derived mast cells (BMMCs) in mice [1].

Signal transducer and activator of transcription (Stat) 5a and Stat5b are cytosolic latent transcription factors that are activated by a very wide range of cytokines, including IL-3 [6, 7]. Under IL-3/Jak2 activation, Stat5a and Stat5b directly regulate the gene expression of a number of important genes. Among Stat5-inducible genes, pim-1 is essential for proliferation, Bcl-x(L) is essential

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Correspondence to: Dr. Hiroshi Nakajima
Department of Allergy and Clinical Immunology
Clinical Cell Biology, Graduate School of Medicine, Chiba University
1-8-1 Inohana, Chiba City, Chiba 260-8670 (Japan)
Tel. +81 43 226 2093, Fax +81 43 226 2095, E-Mail nakajimh@faculty.chiba-u.jp

for survival, and CIS and SOCS-1 play an important role in termination of cytokine signaling in proB cell lines [8]. Although Stat5a and Stat5b are highly homologous, the different phenotypes of Stat5a-deficient ($Stat5a^{-/-}$) mice and $Stat5b^{-/-}$ mice underscore the distinctive roles of Stat5a and Stat5b [9]. Recently, the study of Stat5a/b-deficent mice showed that Stat5 expression is critical for mast cell development [10]. However, the distinctive role of either Stat5a or Stat5b in mast cell development and survival remains to be determined.

In this study, in order to determine the importance of Stat5a in mast cell development, survival, and proliferation, we analyzed the differentiation and expansion of mast cells in $Stat5a^{-/-}$ mice. We present the data that demonstrate an important role of Stat5a in the proliferation and survival of murine mast cells. Our data also suggest the role of Bcl-x(L), which is induced by Stat5a, in the survival of murine mast cells against apoptosis.

Materials and Methods

Mice and Genetic Analysis

Stat5a-deficient ($Stat5a^{-/-}$) mice [11] were backcrossed to BALB/c mice (Charles River Laboratories, Kanagawa, Japan) for at least 8 generations and littermate wild-type (WT) mice were used as controls. Mice were housed in microisolator cages under pathogen-free conditions. All animal experiments were performed under the guidelines approved by the Chiba University.

Culture of BMMCs

Primary culture of IL-3-dependent BMMCs was prepared from 8- to 10-week-old WT or $Stat5a^{-/-}$ mice and maintained as described previously [12]. Briefly, the mice were sacrificed and the bone marrow was aseptically flushed from femurs and tibias into RPMI 1640 medium containing 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, antibiotics, and 10% (vol/vol) of murine IL-3 transfectant X63 cell-conditioned medium [13] (X63-IL-3, a kind gift from Dr. H. Karasuyama, Tokyo Medical and Dental University) as a source of IL-3. The nonadherent bone marrow cells were then maintained at 37°C at a density of 2–5 × 10^5 cells/ml in the same medium, with biweekly replacement of old media with fresh ones. BMMCs were used for experiments at 4–5 weeks of culture unless otherwise stated.

Peritoneal Lavage Cells

Peritoneal lavage was performed by injecting 10 ml of ice-cold PBS into the peritoneal cavity of the mouse. After cells were centrifuged (×400 *g*), resuspended in 1 ml of PBS, and counted using a hemocytometer, differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution. A fraction of the cells was subjected to flow-cytometric analysis as described below.

Flow-Cytometric Analysis

Cells from the peritoneal cavity and BMMCs were stained and analyzed on a FACScalibur (Becton Dickinson, San Jose, Calif., USA) using CellQuest software. FACS analysis was performed with anti-CD117 (c-kit) FITC (2B8, BD PharMingen, San Diego, Calif., USA) and anti-CD16/32 (FcγR II/III) PE (2.4G2, BD PharMingen). Before anti-CD117 staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BD PharMingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific antibodies (BD PharMingen).

IgE Receptors on Mast Cells

To quantify the levels of IgE receptors expressed on the cell surface, cells were first incubated with mouse anti-TNP IgE (IgE3, BD PharMingen) at 4°C for 60 min to saturate IgE receptors, and were then labeled with anti-IgE FITC (R35-72, BD PharMingen).

Cell Survival Assay

BMMCs were washed 3 times with PBS and cultured at 1 × 10^6 cells/ml in triplicate at 37°C for 48 h in RPMI 1640 medium without IL-3. Peritoneal lavage cells were cultured at 1 × 10^6 cells/ml in triplicate at 37°C for 24 h in RPMI 1640 medium without IL-3. The viability of those cells was determined by FACS with anti-CD117 FITC and 5 μg/ml of propidium iodide (PI; Boehringer Mannheim, Indianapolis, Ind., USA) [14].

Annexin V Staining of BMMCs

BMMCs were washed twice with PBS containing 1% BSA, stained with Annexin V FITC (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions, and analyzed on a FACScalibur with 5 μg/ml of PI.

Proliferation Assay

BMMCs (2 × 10^5/well) were cultured in triplicate at 37°C for 36 h in 96-well plates in RPMI 1640 medium containing the indicated amounts of murine IL-3 (10^{-5} to 1 μg/ml; R&D Systems) or SCF (10^{-5} to 1 ng/ml; R&D Systems) with 0.5 μCi of [^{3}H]-thymidine added for the final 12 h.

Cell Division Assay

BMMCs were incubated with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, 10 μM, Molecular Probes, Eugene, Oreg., USA) in PBS at 37°C for 10 min, and then washed with RPMI 1640 medium. CFSE-labeled BMMCs were cultured at 37°C for 72 h with IL-3. Cells were harvested and analyzed by FACS.

RT-PCR Assay

BMMCs were washed twice with PBS and total RNA was extracted using Isogen reagent (Nippon Gene Co., Tokyo, Japan). The first-strand complementary DNA (cDNA) was then synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT) primers (Pharmacia Biotech, Buckinghamshire, UK). cDNAs encoding Bcl-x(L) [8] and β-actin (as a control) were amplified by PCR.

Data Analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t test. p values <0.05 were considered significant.

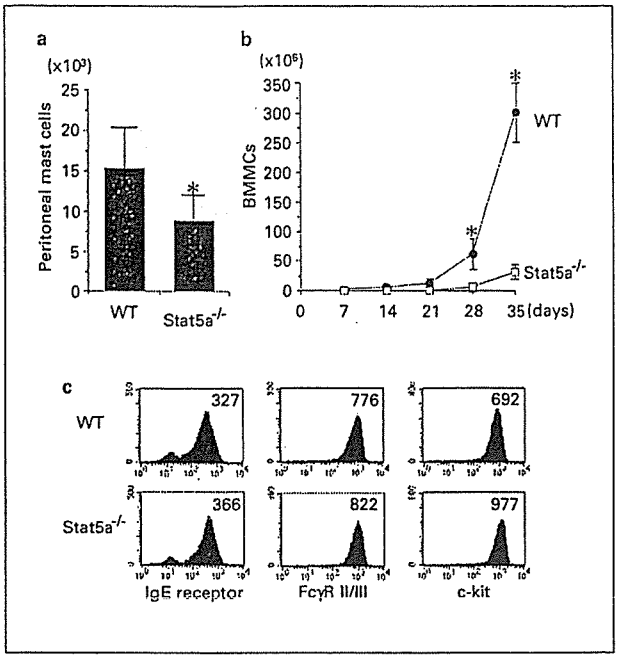

Fig. 1. Stat5a is required for the development of mast cells. **a** The number of peritoneal mast cells is decreased in Stat5a$^{-/-}$ mice. Peritoneal cells were recovered by the lavage in 8- to 10-week-old WT and Stat5a$^{-/-}$ mice and the number of mast cells in the lavage was evaluated. Peritoneal mast cells were identified morphologically on cytospin cell preparations stained with Wright-Giemsa solution. Data are means ± SD for 6–8 mice in each group. The mean value of Stat5a$^{-/-}$ mice is significantly different from that of WT mice. * $p < 0.01$. **b** Development of IL-3-dependent BMMCs is decreased in Stat5a$^{-/-}$ mice. Bone marrow cells from WT mice and Stat5a$^{-/-}$ mice were cultured in the presence of IL-3 at 37°C and the number of mast cells was determined at day 7, day 14, day 21, day 28, and day 35 using a hemocytometer and cytospin cell preparations stained with Wright-Giemsa solution. Data are means ± SD for 5 mice in each group. * $p < 0.001$. **c** BMMCs normally develop in Stat5a$^{-/-}$ mice. Expression of IgE receptors, FcγR II/III, and c-kit on WT and Stat5a$^{-/-}$ BMMCs was determined by FACS using anti-IgE FITC, anti-FcγR II/III PE, and anti-c-kit FITC, respectively, as described in the Materials and Methods. Representative FACS profiles and the mean fluorescence intensities from four independent experiments are shown.

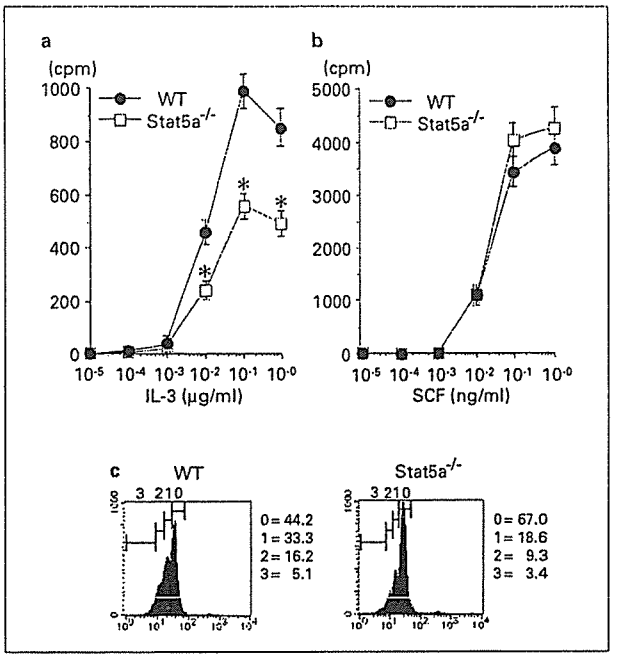

Fig. 2. Stat5a is crucial for IL-3-induced but not SCF-induced proliferation of BMMCs. **a**, **b** IL-3-induced but not SCF-induced proliferation of BMMCs is decreased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured in the presence of either IL-3 (10^{-5} to 1 μg/ml) or SCF (10^{-5} to 1 ng/ml) at 37°C for 48 h and the proliferative responses were evaluated by the addition of [^{3}H]-thymidine for the final 12 h. Data are means ± SD for 5 mice in each group. * $p < 0.001$. **c** IL-3-induced cell division of BMMCs is decreased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were labeled with CFSE. These cells were cultured with IL-3 at 37°C for 48 h and then analyzed by FACS. Representative FACS profiles for the intensity of CFSE (n = 4) are shown. The figures indicate percent of cell numbers after 0–3 cell divisions.

Results

Stat5a Is Required for the Development of Mast Cells

To determine whether Stat5a is essential for mast cell development in vivo, we first analyzed the number of peritoneal mast cells in Stat5a$^{-/-}$ mice. The number of mast cells recovered from the peritoneal cavity was significantly decreased in Stat5a$^{-/-}$ mice as compared with that in WT mice (n = 6–8 mice, $p < 0.01$) (fig. 1a). We also found reduced numbers of mast cells in tissue sections of ear and stomach in Stat5a$^{-/-}$ mice (data not shown). Furthermore, IL-3-dependent development of BMMCs was markedly decreased in Stat5a$^{-/-}$ mice (n = 5, $p < 0.001$) (fig. 1b), suggesting that Stat5a plays an important role in the development of mast cells both in vivo and in vitro. However, Stat5a$^{-/-}$ BMMCs normally expressed IgE receptors, FcγR II/III, and c-kit (fig. 1c), and Stat5a$^{-/-}$ BMMCs were morphologically indistinguishable from WT BMMCs (data not shown). These results suggest that mast cells mature normally, but the expansion of mast cells is impaired in Stat5a$^{-/-}$ mice.

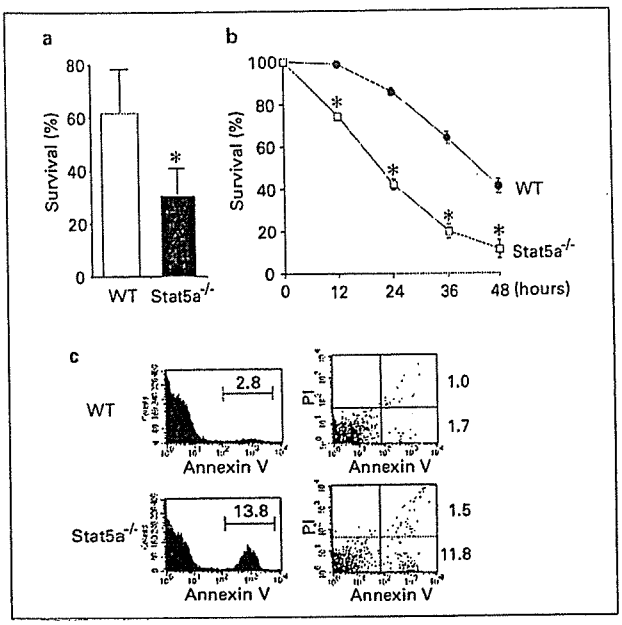

Fig. 3. The survival of mast cells is diminished in Stat5a$^{-/-}$ mice. **a** The survival of peritoneal mast cells is diminished in Stat5a$^{-/-}$ mice. Freshly isolated peritoneal cells from WT mice or Stat5a$^{-/-}$ mice were cultured for 24 h in RPMI 1640 medium without IL-3. The viability of c-kit+ cells was determined by FACS using anti-c-kit FITC and 5 μg/ml of PI. Data are means ± SD of the percent survival (n = 4 mice in each group). The mean value of Stat5a$^{-/-}$ mice is significantly different from the mean value of WT mice. * $p < 0.01$. **b** The survival of BMMCs is diminished in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured at 1×10^6 cells/ml at 37°C for 48 h in RPMI 1640 medium without IL-3. Cells were harvested and the viability was determined by FACS using 5 μg/ml of PI. n = 4 experiments, * $p < 0.001$. **c** Apoptosis of BMMCs is increased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured in the presence of IL-3 and stained with Annexin V and 5 μg/ml of PI. Representative FACS profiles from five independent experiments are shown.

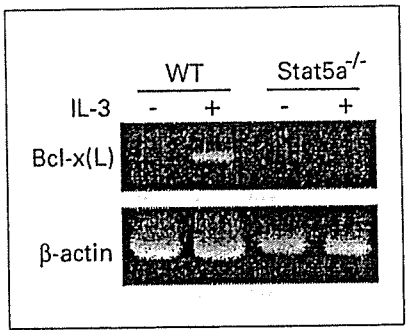

Fig. 4. Bcl-x(L) expression is decreased in Stat5a$^{-/-}$ BMMCs. BMMCs from WT mice or Stat5a$^{-/-}$ mice were washed with PBS and total RNA was extracted using Isogen reagent. The first strand complementary DNA (cDNA) was then synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. cDNAs encoding Bcl-x(L) and β-actin (as a control) were amplified by PCR.

Stat5a Is Crucial for IL-3-Induced but Not SCF-Induced Proliferation of Mast Cells

To clarify the mechanism of the reduced number of mast cells in Stat5a$^{-/-}$ mice, we examined the proliferation of BMMCs. IL-3-induced but not SCF-induced proliferation of BMMCs was significantly diminished in Stat5a$^{-/-}$ mice as compared with that in WT mice (n = 5, at 0.01–1 μg/ml of IL-3, $p < 0.001$) (fig. 2a, b). In addition, IL-3-induced cell division of Stat5a$^{-/-}$ BMMCs was less frequent than that of WT BMMCs (fig. 2c). These results indicate that IL-3-induced proliferation of mast cells is decreased in Stat5a$^{-/-}$ mice.

Apoptosis Is Increased in Stat5a$^{-/-}$ Mast Cells

We next examined the survival and apoptosis of mast cells in Stat5a$^{-/-}$ mice. Survival rates of both peritoneal mast cells and BMMCs were significantly decreased in Stat5a$^{-/-}$ mice as compared with those in WT mice (n = 4 experiments each, $p < 0.01$ and $p < 0.001$, respectively) (fig. 3a, b). Interestingly, apoptotic cells were significantly increased in Stat5a$^{-/-}$ BMMCs even when they were cultured with IL-3 (n = 5, $p < 0.001$) (fig. 3c). Furthermore, mRNA of Bcl-x(L), an antiapoptotic molecule [15–17], was expressed in WT BMMCs but not in Stat5a$^{-/-}$ BMMCs (fig. 4), suggesting that Stat5a may suppress apoptosis of mast cells by inducing the expression of the antiapoptotic gene, Bcl-x(L).

Discussion

In this study, we show that Stat5a plays an important role in the development, proliferation, and survival of murine mast cells. We found that the lack of Stat5a resulted in reduced numbers of peritoneal mast cells in vivo and impaired development of BMMCs (fig. 1). We also found that IL-3-induced but not SCF-induced proliferation was decreased in Stat5a$^{-/-}$ BMMCs (fig. 2). Finally, we found that apoptosis was increased and the expression of an antiapoptotic molecule Bcl-x(L) was diminished in Stat5a$^{-/-}$ BMMCs (fig. 3, 4). These results indicate that Stat5a is crucial for IL-3-induced proliferation and survival of murine mast cells.

We show that Stat5a mediates IL-3-induced proliferation of murine mast cells. IL-3 has been shown to be an important growth factor for murine mast cells [3, 4]. IL-3 promotes the growth of multipotential mast cell progenitors, whereas SCF induces unipotential mast cell progenitors and also supports the growth of them [3, 4]. IL-3 has also been shown to be required for mast cell expansion in the tissues during immune responses to parasitic infection in mice [5]. Because it has been shown that IL-3, but not SCF, leads to activation of Jak2 and Stat5 and induces pim-1 expression in mast cells [18], Stat5a-mediated pim-1 expression is possibly involved in their proliferation [8].

SCF is also a critical growth factor for the proliferation and suppression of apoptosis in mast cells. Activation of the receptor tyrosine kinase c-kit by SCF induces receptor autophosphorylation and association with various signaling molecules including phosphatidylinositol 3-kinase (PI 3-kinase) and Src kinases. Timokhina et al. [19] have shown that the activation of PI 3-kinase and Src kinases contribute to c-kit-mediated proliferation and suppression of apoptosis induced by factor deprivation in BMMCs. Furthermore, the Rac1/JNK pathway has been shown to be critical for SCF-induced proliferation of mast cells [19]. Although it has been reported that SCF-induced proliferation of Stat5a/b-deficient BMMCs is impaired [10], our findings indicate that SCF normally induces the proliferation of Stat5a$^{-/-}$ BMMCs (fig. 2b), suggesting that Stat5a is not involved in SCF-induced proliferation of mast cells.

We also show that Stat5a is essential for the induction of Bcl-x(L) transcript after IL-3 stimulation in murine BMMCs (fig. 4). Bcl-x is a gene of the Bcl family and its longer isoform, Bcl-x(L), is known to have an antiapoptotic function [15–17]. Thus, diminished expression of Bcl-x(L) can account for the increased apoptosis in Stat5a$^{-/-}$ BMMCs in our study. On the other hand, we found normal expression levels of Bcl-2 gene in Stat5a$^{-/-}$ BMMCs as compared with that in WT BMMCs (data not shown). Our findings are in agreement with the observation that in the bone marrow-derived Ba/F3 cell line, IL-3-induced Stat5 activation induces Bcl-x(L) expression and IL-3-dependent suppression of apoptosis [20].

We found that the IL-3-induced proliferation of BMMCs in Stat5a$^{-/-}$ mice was reduced to about half of that in WT mice (fig. 2a). We also found that the number of apoptotic BMMCs was increased 4-fold in Stat5a$^{-/-}$ mice as compared with that in WT mice even in the presence of IL-3 (fig. 3c). As the results of the decreased proliferation and the increased apoptosis, we found that IL-3-induced mast cell development from bone marrow was strikingly impaired in Stat5a$^{-/-}$ mice (fig. 1b). On the other hand, we found that the decrease of mast cells in the peritoneal cavity and in ear and stomach was not so severe in Stat5a$^{-/-}$ mice (fig. 1a and data not shown), suggesting that Stat5a-independent pathways also participate in the in vivo development of mast cells in mice.

Acknowledgments

We thank Dr. L. Hennighausen for Stat5a$^{-/-}$ mice and Dr. H. Karasuyama for X63-IL-3 cells. This work was supported in part by grants from Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

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IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses

Michishige Harada,[1] Kumiko Magara-Koyanagi,[5] Hiroshi Watarai,[1] Yuko Nagata,[1] Yasuyuki Ishii,[2] Satoshi Kojo,[1] Shigetoshi Horiguchi,[6] Yoshitaka Okamoto,[6] Toshinori Nakayama,[5] Nobutaka Suzuki,[3] Wen-Chen Yeh,[7] Shizuo Akira,[8] Hiroshi Kitamura,[4] Osamu Ohara,[4] Ken-ichiro Seino,[1] and Masaru Taniguchi[1]

[1]Laboratory for Immune Regulation, [2]Laboratory for Vaccine Design, [3]Laboratory for Cell Signaling, and [4]Laboratory for Immunogenomics, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan
[5]Department of Molecular Immunology and [6]Otorhinolaryngology/Head and Neck Surgery, Chiba University School of Medicine, Chiba-City, Chiba 260-8670, Japan
[7]Amgen Institute, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2C1, Canada
[8]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita Osaka 565-0871, Japan

Epidemiological studies have suggested that the recent increase in the incidence and severity of immunoglobulin (Ig)E-mediated allergic disorders is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination; however, the underlying mechanisms remain uncertain. Here, we demonstrate that natural killer T (NKT) cells in mice and humans play a crucial role in the BCG-induced suppression of IgE responses. BCG-activated murine Vα14 NKT cells, but not conventional CD4 T cells, selectively express high levels of interleukin (IL)-21, which preferentially induces apoptosis in Bε cells. Signaling from the IL-21 receptor increases the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor, resulting in Bε cell apoptosis. Similarly, BCG vaccination induces IL-21 expression by human peripheral blood mononuclear cells (PBMCs) in a partially NKT cell–dependent fashion. BCG-activated PBMCs significantly reduce IgE production by human B cells. These findings provide new insight into the therapeutic effect of BCG in allergic diseases.

CORRESPONDENCE
Masaru Taniguchi:
taniguti@rcai.riken.jp

Abbreviations used: α-GalCer, α-galactosylceramide; BCG, *Mycobacterium bovis* bacillus Calmette Guerin; BM-DC, BM-derived DC; Bmf, Bcl-2–modifying factor; γc, common γ-chain; IRAK, IL-1R–associated kinase; MNC, mononuclear cell; MyD88, myeloid differentiation factor 88; PGN, peptidoglycan; TLR, Toll-like receptor.

The prevalence of IgE-mediated allergic diseases such as asthma, hay fever, and atopic dermatitis has increased dramatically over the past two decades, especially in industrialized countries (1). For example, the incidence of asthma has nearly doubled since 1980 in the United States as well as in Japan (1, 2). However, the precise mechanisms underlying the increased incidence of allergic diseases are not fully understood. One possible explanation has been termed "the hygiene hypothesis," which proposes that improved hygiene combined with the excessive use of antibiotics in industrial countries has markedly reduced the incidence of infections, particularly in children. This lack of early exposure to infectious agents is associated with accelerated IgE production and an increased incidence of allergic disorders (1–3). Epidemiological studies support this hypothesis (4–6), and bacterial and viral products have been proposed as therapeutic strategies to suppress the development of allergic responses. For example, vaccination with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) has been reported to suppress IgE production and inhibit the development of allergic diseases in mouse models (7–9) and in humans (10). Furthermore, injection of CpG oligodeoxynucleotides, bacterial DNA surrogates recognized by Toll-like receptor (TLR)9, reduces serum IgE levels in mice (11).

It has been widely accepted that IgE production is totally dependent on Th2 cells, whose functions are reciprocally inhibited by Th1 cells. Mechanistically, therefore, the hygiene hypothesis is based on an imbalance in

The online version of this article contains supplemental material.

the Th1/Th2 ratio because bacterial components stimulate Th1 responses that in turn inhibit Th2 responses and IgE production (12). On the other hand, recent findings have indicated that a spectrum of T cells with immunoregulatory properties is involved in the regulation of IgE production and the pathophysiology of allergic diseases (13). For example, $CD4^+CD25^+$ regulatory T cells inhibit Th2 responses by producing immunosuppressive cytokines that can directly inhibit B cell activation (14, 15). Furthermore, NKT cells expressing an invariant antigen receptor (Vα14-Jα281 for mice and Vα24-JαQ for humans; reference 16) suppress Th2 and IgE responses via their production of IFN-γ (17).

In addition to these cellular mechanisms, it has also been reported that IL-21 is involved in the suppression of IgE production in both mice and humans (18, 19). IL-21 is a type I cytokine produced by activated $CD4^+$ T cells and has a broad capacity to regulate lymphoid cell functions (20–22). Among these functions, IL-21 directly inhibits antibody production by IgE-bearing B (Bε) cells induced by CD40L and IL-4 (18). Conversely, IL-21R–deficient mice exhibit enhanced IgE production (23). IL-21 has been shown to specifically inhibit germ line transcription of the IgE constant region (Cε) gene but not of other isotype genes (18). However, there is no direct evidence that this inhibition of germ line transcription is responsible for the suppression of IgE production, as class switch recombination of Ig genes and subsequent antibody secretion are differentially regulated events (24). IL-21 also induces apoptosis in B cells (25, 26), which could partially explain the reduction of IgE production; however, this effect was not shown to be specific for IgE. Hence, the mechanism by which IL-21 specifically inhibits IgE production is not yet fully understood.

Here, we have investigated BCG-mediated IgE suppression and found that NKT cells specifically induced apoptosis in Bε cells through the production of IL-21, resulting in a dramatic decrease in IgE production. IL-21 increased the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor (Bimf), which is selectively expressed in Bε cells and counteracts the antiapoptotic activity of Bcl-2. We have found that similar mechanisms are operative in humans. This is the first report demonstrating that IL-21 produced by Vα14 NKT cells plays an important role in the regulation of IgE responses in both mouse and human immune systems.

RESULTS

Vα14 NKT cell–dependent IgE suppression by BCG treatment

We used an OVA-patched sensitization protocol (27) to determine if BCG activates Vα14 NKT cells. Vα14 NKT cells were detected by α-galactosylceramide (α-GalCer)–loaded CD1d tetramer staining. In control mice treated with PBS or OVA without BCG, ~15% of the liver mononuclear cells (MNCs) were Vα14 NKT cells (Fig. 1 A, left and middle). However, BCG treatment significantly increased the frequency of Vα14 NKT cells to >25% (Fig. 1 A, right). BCG treatment also increased the absolute number of Vα14 NKT cells because the total number of liver MNCs was also increased by 50–80% (not depicted). Sera were collected from these mice 1 wk after the last sensitization, and IgE levels were evaluated. In WT mice, both total and OVA-specific IgE levels were suppressed by BCG treatment (Fig. 1 B). In mice lacking the Jα18 gene (Vα14 NKT KO), there was no significant BCG-induced suppression of IgE responses, suggesting that suppression requires Vα14 NKT cells.

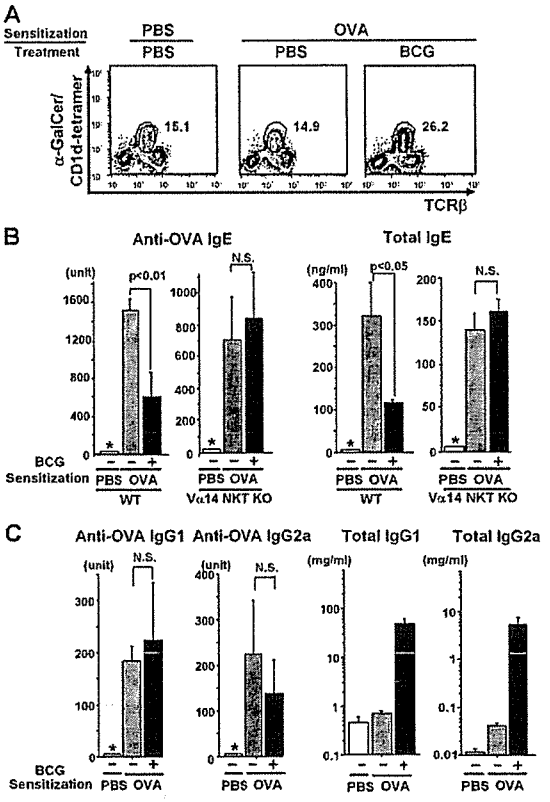

Figure 1. Requirement of Vα14 NKT cells in BCG-mediated IgE suppression. (A) FACS profiles of liver MNCs. The liver MNCs obtained 1 wk after the last immunization were stained with α-GalCer/CD1d tetramer and anti-TCRβ mAb. Three mice per each group were analyzed and representative data are shown. (B and C) Effects of BCG on antibody responses in WT and Vα14 NKT KO mice. Total and OVA-specific serum IgE (B), IgG1, and IgG2a (C) were assayed by ELISA. Five mice were used in each group. Values are expressed as mean ± SD. The asterisks (*) indicate that the amount of IgE was below the detection level for anti-OVA IgE (<31.2 U/ml), anti-OVA IgG1 (<0.002 U/ml), or anti-OVA IgG2a (<1.25 U/ml). N.S., not significant. All experiments were repeated three times with similar results.

The effect of BCG administration on Th1/Th2 responses

It is well known that the isotype commitment of B cells during Ig class switching is tightly regulated by Th1/Th2 cell cytokines (28) and that Vα14 NKT cells play a regulatory role in T cell differentiation (17, 29, 30). Therefore, we measured serum IgG2a (Th1) and IgG1 (Th2) levels to assess any changes in the Th1/Th2 balance. BCG administration did

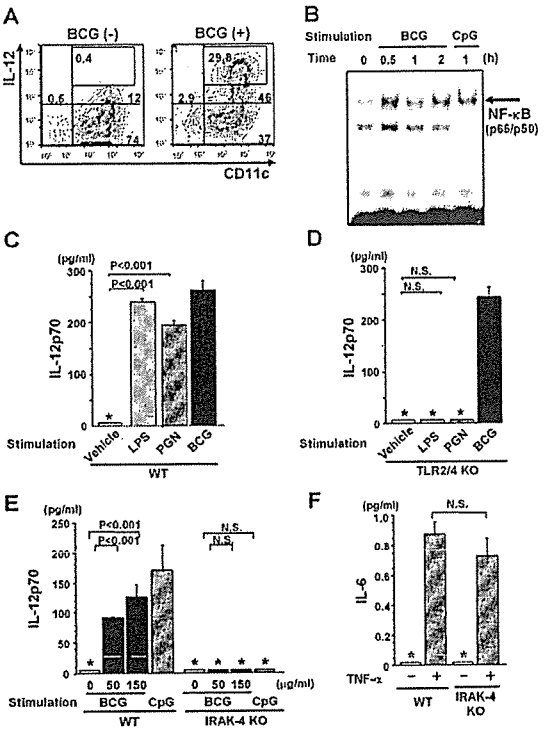

Figure 2. Activation of DCs by BCG. IL-12 production (A) and NF-κB activation (B). (A) Intracellular staining of BM-DCs with anti-IL-12p40/p70 and anti-CD11c mAbs with or without in vitro BCG (50 μg/ml) treatment for 12 h. BCG-treated BM-DCs (10,000 cells) were analyzed by FACS, and the number in each panel indicates the percentage of total cells. (B) NF-κB activation. 2 × 10^5 BM-DCs were stimulated with or without 50 μg/ml BCG or 1 μM CpG in vitro. NF-κB activity was determined by EMSA. (C and D) No requirement of TLR2 and TLR4 in BCG-mediated IL-12 production. 2 × 10^5 BM-DCs derived from WT (C) or TLR2/4 double KO (D) mice were stimulated in vitro with or without 10 μg/ml LPS, 10 μg/ml PGN, or 150 μg/ml BCG for 48 h, and IL-12p70 levels were measured by ELISA. (E and F) Requirement of IRAK-4 for IL-12 production. 2 × 10^5 BM-DCs were assayed for IL-12p70 by ELISA after stimulation with 0, 50, or 150 μg/ml BCG or 1 μM CpG (E), and for IL-6 with 10 ng/ml TNF-α stimulation for 48 h (F). In C–F, values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the levels were below the detection limits for IL-12p70 (<62.5 pg/ml) and IL-6 (<15.6 pg/ml). N.S., not significant. All experiments were repeated twice with similar results.

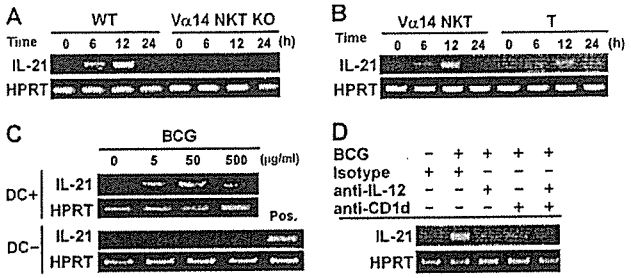

Figure 3. IL-21 expression. (A) Vα14 NKT cell–dependent IL-21 production. Liver MNCs were obtained after BCG injection (500 μg/mouse) and examined for IL-21 mRNA expression. (B) Identification of the source of IL-21. Vα14 NKT and conventional T cells were sorted from liver MNCs and examined for IL-21 mRNA expression. (C) Requirement of DCs for BCG-induced IL-21 expression by Vα14 NKT cells. Liver TCRβ$^+$ cells were cultivated in the presence of 50 μg/ml BCG with (top) or without (bottom) BM-DCs for 24 h and analyzed for IL-21 mRNA expression. Liver TCRβ$^+$ cells stimulated with 10 μg/ml anti-CD3 mAb were used as a positive (Pos.) control. (D) Requirement of IL-12– and CD1d-mediated signals for IL-21 mRNA expression upon BCG stimulation. An isotype control, anti–IL-12p40/p70, or anti-CD1d mAb (20 μg/ml) was added to the cultures of liver TCRβ$^+$ cells and BM-DCs as described in C. All experiments were repeated twice with similar results.

not significantly alter the levels of OVA-specific IgG1 or IgG2a, although total levels of both isotypes were significantly enhanced (Fig. 1 C).

Innate signaling pathway for BCG-mediated IL-12 production

During microbial infection, both CD1d- and IL-12–mediated signals are required for the rapid activation of Vα14 NKT cells (31). Thus, we assessed IL-12 production after BCG treatment. BM-derived DCs (BM-DCs) were stimulated in vitro with 50 μg/ml BCG and examined for IL-12 production by intracellular cytokine staining using an IL-12p40/p70 mAb. Upon BCG stimulation, a large fraction of CD11c^{high} cells produced IL-12 (Fig. 2 A). NF-κB activation is crucial for IL-12 production, and BCG treatment activated NF-κB to the same extent as treatment with the positive control CpG, as demonstrated by electrophoretic mobility shift assay (Fig. 2 B). These results indicate that BCG directly induces IL-12 production in DCs by activating NF-κB.

It has been reported that mycobacterial cell wall antigens such as peptidoglycan (PGN) or lipoarabinomannan induce proinflammatory gene transcription through TLR2 and TLR4 (32). However, when we compared IL-12p70 production by BCG-stimulated WT and TLR2/TLR4 double KO BM-DCs, there was no difference (Fig. 2 C). As expected, however, the TLR2/4-deficient cells failed to respond to LPS or PGN (Fig. 2 D). These results indicate that receptor(s) other than TLR2 and TLR4 are responsible for the recognition of whole BCG organisms.

To analyze intracellular signaling pathways activated by BCG, we measured IL-12p70 production by BM-DCs from WT and IL-1R–associated kinase (IRAK)-4 KO mice. BM-DCs from IRAK-4 KO mice produced less IL-12p70 than those from WT mice in response to both BCG and CpG (Fig. 2 E), whereas they produced comparable levels of IL-6 in response to TNF-α stimulation (Fig. 2 F). Similarly, BM-DCs from myeloid differentiation factor 88 (MyD88) KO mice produced nearly undetectable IL-12p70 upon BCG stimulation, whereas IL-6 production remained unchanged (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). Therefore, the recognition of BCG organisms is mediated by innate receptors other than TLR2 and TLR4 that signal through both IRAK-4 and MyD88.

BCG-induced IL-21 expression in Vα14 NKT cells

The recently identified IL-21 and its receptor (IL-21R), members of the common γ-chain (γc)-dependent cytokine family, have been shown to regulate IgE production without influencing Th2 cell differentiation (18, 20, 23). Thus, we examined the possibility that IL-21 might be induced by BCG stimulation and might suppress IgE responses in a Vα14 NKT cell–dependent manner. We first measured IL-21 mRNA expression in TCRβ$^+$ liver MNCs by a RT-PCR. IL-21 mRNA was detected in liver TCRβ$^+$ liver MNCs of WT mice within 6 h after BCG injection (Fig. 3 A). In contrast, no IL-21 mRNA was detected in the Vα14 NKT KO mice (Fig. 3 A), suggesting that Vα14 NKT cells are the source of IL-21 in response to BCG. To test this hypothesis, we separated conventional T cells and Vα14 NKT cells and found that IL-21 mRNA was more abundant in the Vα14 NKT cells after BCG injection (Fig. 3 B). Similarly, after stimulation with anti-CD3, IL-21 mRNA levels in Vα14 NKT cells were more than seven times higher than in CD4 T cells, confirming that these cells are the major source of IL-21 in this model (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1).

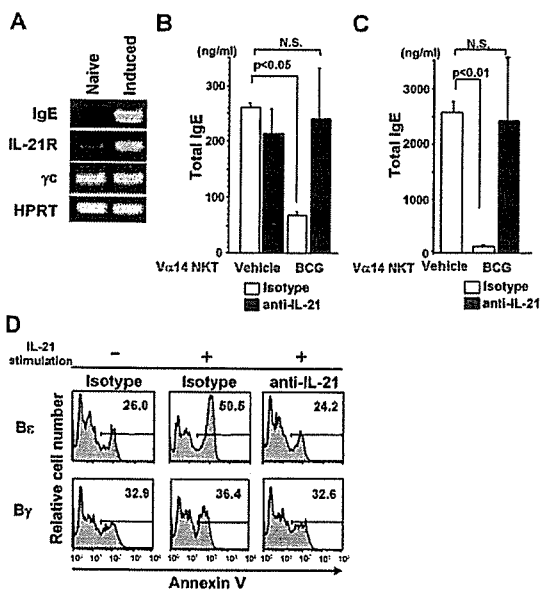

Figure 4. IL-21–mediated Bε cell apoptosis. (A) RT-PCR analysis. Expression of IgE (Cε), IL-21R, and γc was investigated in naive B (left) and Bε (right) cells. (B) Suppression of IgE production in naive B cell cultures. Naive B cells and Vα14 NKT cells (10^5 each) were cocultured in the presence of sCD40L and IL-4. (C) Suppression of IgE production in the Bε cell culture. 10^5 Vα14 NKT cells were added to the Bε cell (10^5) cultures. In B and C, 20 μg/ml anti–IL-21 mAb or isotype control mAb was added at the same time as the Vα14 NKT cells. The concentration of total IgE was measured by ELISA in triplicate. Values are expressed as mean ± SD. N.S., not significant. The experiments were repeated three times with similar results. (D) IL-21–mediated Bε cell apoptosis. 2 × 10^5 Bε and Bγ cells were generated and then further cultured with or without 30 ng/ml IL-21 for 30 h. Annexin V staining was then performed. The numbers represent percentage of the gated cells. Annexin V$^+$ cells among Bε and Bγ cells just before IL-21 treatment was 25.7 and 29.2%, respectively (not depicted). The experiments were repeated three times with similar results.

Requirement for IL-12 and CD1d in IL-21 expression by Vα14 NKT cells

We next analyzed the role of DCs in BCG-induced IL-21 mRNA expression. Co-culture of Vα14 NKT cells with DCs plus IL-12 strongly induced IL-21 mRNA expression, whereas no IL-21 mRNA was induced in the absence of DCs (Fig. 3 C). Furthermore, IL-21 mRNA expression was inhibited by the addition of anti–IL-12, anti-CD1d, or both into the cultures (Fig. 3 D), indicating that both IL-12 and CD1d are required for IL-21 expression by Vα14 NKT cells.

IL-21–mediated IgE suppression

To examine whether BCG-activated Vα14 NKT cells actually suppress IgE production, Bε cells were generated from naive CD19$^+$ splenic B cells using the 3-d culture system described by Snapper et al. (33). The starting population of naive B cells expressed negligible IL-21R and contained no Bε cells as defined by Cε transcripts (Fig. 4 A). However, after 3 d of the culture, the majority of CD19$^+$ B cells became Bε cells and expressed IL-21R (Fig. 4 A). We then investigated the effects of BCG treatment on B cells, before and after IgE class switching. The addition of BCG-treated liver MNCs at the onset of the naive B cell cultures significantly suppressed IgE production (~50%; Fig. 4 B). However, when BCG-activated Vα14 NKT cells were added to the Bε cell culture on day 3 and the cells were further cultivated for 5 d, IgE production was even more strongly inhibited (>90% suppression; Fig. 4 C). These results indicate that, even after B cells have undergone Cε class switching, BCG-activated Vα14 NKT cells can potently suppress IgE production. The inhibition of IgE production was IL-21 dependent, as an anti–IL-21 mAb completely abrogated the inhibitory effects (Fig. 4, B and C). When the B cells in these cultures were assessed for apoptosis by annexin V staining, there was a significant increase

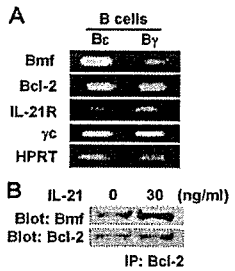

Figure 5. Bmf-mediated Bε cell apoptosis. (A) RT-PCR. RNA from Bε and Bγ cells was analyzed for its expression of the indicated genes by RT-PCR. Note that no significant differences in Bcl-2 and IL-21R expression between Bε and Bγ cells were observed. (B) Western blotting. Bε cells were stimulated with IL-21 at 37°C for 30 min, and their cell lysates (6 × 10^6) were subjected to immunoprecipitation with anti–Bcl-2 mAb and immunoblotting with anti-Bmf antibody (top) or anti–Bcl-2 mAb (bottom). All experiments were repeated three times with similar results.

in apoptotic Bε cells that was not observed in the Bγ cells (Fig. 4 D, middle). Apoptosis of Bε cells was abrogated by the addition of anti–IL-21, a treatment that had no significant effect on Bγ cells (Fig. 4 D, right).

Bmf-induced Bε cell apoptosis

To understand the molecular mechanisms underlying IL-21–induced IgE suppression, we performed DNA microarray analyses to compare gene expression between Bε and Bγ cells. The DNA microarray data were deposited in the Center for Information Biology Gene Expression database (CIBEX; http://cibex.nig.ac.jp/) under accession number CBX15. The proapoptotic Bmf gene (34) was dramatically up-regulated in Bε cells, a finding that was confirmed by RT-PCR (Fig. 5 A). No significant difference in the expression of IL-21R, Bcl-2, or γc was detected (Fig. 5 A), suggesting that elevated Bmf gene expression in Bε, but not in Bγ, cells may account for their differential sensitivity to IL-21–mediated apoptosis.

To investigate whether the Bmf expressed in Bε cells is functional in its proapoptotic activity, Bmf cDNA was isolated from Bε cells and used to prepare several mutants of enhanced GFP–fused Bmf. These mutations included an A69P mutation in the dynein light chain 2 binding motif and an L138A mutation in the BH3 domain. These Bmf mutants were transfected into Baf3 cells. Upon IL-3 deprivation, mock transfectants underwent apoptosis. Transfection with WT Bmf or Bmf-A69P to Baf3 cells also significantly augmented apoptosis (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). However, reduced apoptosis was seen in Baf3 cells transfected with BH3 mutants, such as Bmf-L138A or Bmf-A69P/L138A (Fig. S3), indicating that Bmf in Bε cells is functional and the BH3 domain of the protein is important for mediating its proapoptotic activity.

Based on the understanding of proapoptotic activity of Bmf expressed in Bε cells, we investigated the formation of Bmf–Bcl-2 complexes in Bε cells after activation with IL-21. Bmf in Bε cells faintly binds to Bcl-2 in unstimulated cells (Fig. 5 B, left). However, when Bε cells were stimulated with IL-21, the formation of Bmf–Bcl-2 complexes was significantly augmented (Fig. 5 B, right).

BCG-mediated IL-21 induction in human Vα24 NKT cells

To determine how widespread our findings are, we investigated whether IL-21 and Vα24 NKT cells are required for the BCG-mediated suppression of human IgE responses. When human PBMCs were stimulated with α-GalCer or BCG, a significant up-regulation of IL-21 mRNA was detected by quantitative PCR (Fig. 6 A). The BCG-induced up-regulation of IL-21 mRNA was effectively suppressed by blocking with antibodies against CD1d, IL-12p40/p70, or both (Fig. 6 B), indicating that the CD1d-restricted NKT cell–dependent suppression of IgE responses observed in mice also operates in the human immune system. IL-21 mRNA expression by anti-CD1d and anti–IL-12 treatment was significantly reduced but was not as effective as in the mouse Vα14 NKT cell system (Fig. 3 D), perhaps suggesting a significant contribution of human conventional $CD4^+$ T cells (Fig. S2 B).

To evaluate in vivo responses, we inoculated BCG into healthy volunteers and examined IL-21 mRNA levels in PBMCs 1 wk later. There was a significant up-regulation of IL-21 mRNA levels in five out of six individuals (Fig. 6 C), and, furthermore, IL-21 suppressed IgE production by human Bε cells (Fig. 6 D, left). As expected, the addition of BCG-stimulated, but not control, PBMCs significantly inhibited IgE production (Fig. 6 D, right).

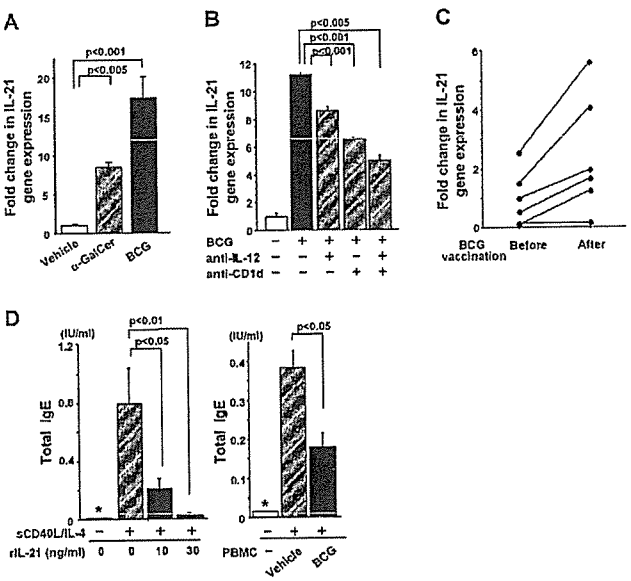

Figure 6. IL-21 mRNA expression and IL-21–induced IgE suppression in humans. (A) IL-21 mRNA expression in PBMCs. 10^6 human PBMCs were stimulated with 100 ng/ml α-GalCer or 50 μg/ml BCG and examined for IL-21 expression by quantitative real-time PCR with Taqman probes. The data are representative of five donors. (B) IL-12 and CD1d are required for IL-21 expression. 10^6 PBMCs were stimulated in vitro with 50 μg/ml BCG in the presence of 10 μg/ml anti-CD1d and/or anti–IL-12p40/p70 mAb. Representative data from five donors are shown. (C) IL-21 mRNA expression in PBMCs. Healthy volunteers were inoculated intradermally with BCG (two drops of 26.7 mg/ml of BCG emulsion per person). In A–C, the data for IL-21 expression were normalized to 18S ribosomal RNA expression, and relative expression levels are shown. Statistical analysis was performed using a matched pairs *t* test in C. (D) Suppression of IgE production. Left, suppression of IgE production by IL-21. 2×10^5 human B cells were cultured with sCD40L and IL-4 in the presence of human IL-21 for 14 d. Right, suppression of IgE production by BCG-activated human PBMCs. 10^5 Bε cells were co-cultured with 10^5 PBMCs, sCD40L, and IL-4 in the presence of 50 μg/ml BCG for 14 d. Total IgE was measured by ELISA. Values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the IgE levels are below the detection limit for total IgE (<0.014 IU/ml). Data shown are representative of three donors. Results were expressed as a fold difference in human IL-21 gene expression relative to a control sample (vehicle) after being normalized with 18S ribosomal RNA expressions in each sample.

DISCUSSION

It is widely accepted that the mechanistic basis of the hygiene hypothesis for suppression of IgE responses is an increase in the Th1/Th2 ratio (12). However, in reality, the Th1 response exacerbates allergic reactions, as human asthma is associated with the production of IFN-γ, a cytokine that appears to contribute to the pathogenesis of the disease (35). Furthermore, the adoptive transfer of allergen-specific Th1 cells causes severe airway inflammation (36). Thus, a shift in the Th1/Th2 ratio alone cannot explain all of the immunological findings observed in allergic diseases (1). Furthermore, there are several studies suggesting that BCG vaccination has little or no effect on the development and prevalence of allergic diseases (37, 38). Therefore, it is necessary to better understand the precise mechanism of IgE suppression in BCG-treated animals or humans.

In this study, neither a Th1/Th2 imbalance nor an involvement of regulatory T cells was observed in response to BCG treatment (Fig. 1). Instead, we demonstrated that IL-21–induced Bε cell apoptosis is the mechanism responsible for BCG-mediated suppression of IgE production (Figs. 1, 3, and 5). Because the human IL-21 responses to BCG vaccination were heterogeneous (Fig. 6 C), it seems likely that the magnitude of the response in each individual could cause different degrees of BCG-induced IgE suppression and might be prognostic.

Previous studies have indicated that IL-21 is preferentially expressed by activated $CD4^+$ T cells (20), the results that are partially in agreement with the present data, as half of peripheral Vα14 NKT cells are $CD4^+$ (39, 40). Interestingly, upon anti-CD3 mAb stimulation, Vα14 NKT cells, but not conventional T cells, preferentially expressed IL-21 (Fig. S2 A), similar to the results with BCG (Fig. 3 B). Therefore, the major IL-21 producers in response to BCG in mice are Vα14 NKT cells.

It has been proposed that, for full activation of Vα14 NKT cells to produce IFN-γ, two signals are required: one CD1d-dependent and the other TLR-mediated IL-12–dependent signals (31). In agreement with this, IL-21 expression by BCG-activated Vα14 NKT cells was significantly inhibited by blocking with antibodies to IL-12 and/or CD1d (Fig. 3 D). Therefore, it is likely that Vα14 NKT cells recognize endogenous antigens presented by CD1d molecules but require IL-12 signals to produce IL-21. Nevertheless, it is still possible that glycolipid BCG components such as phosphatidylinositol mannoside may directly stimulate Vα14 NKT cells to produce IL-21 in a CD1d-dependent manner (41, 42).

In terms of the receptors on DCs that are required for BCG recognition and signal transduction, we showed in this study that BCG-induced IL-12 production is IRAK-4 and MyD88 dependent (Fig. 2 E and Fig. S1). These results in mice are consistent with a recent report indicating that BCG cannot induce IL-12 or IFN-γ production by PBMCs from IRAK-4–deficient patients (43). In addition, it has been reported that BCG enhances NF-κB–dependent gene transcription through the activation of phosphatidylinositol 3 kinase and c-Jun N-terminal kinase cascades (44). The activated NF-κB is then liberated for nuclear translocation and transactivates a variety of immune response genes, including IL-12.

In contrast to a previous report that implicated TLR2 and TLR4 in the recognition of mycobacterial antigens (32), we could not identify any involvement of these receptors in IL-12 production by BCG-stimulated BM-DCs (Fig. 2, C and D). In agreement with our findings, it has recently been reported that TLR2/4 double KO mice infected with live BCG have normal adaptive immune responses and survived as long as WT mice (45). As whole BCG contains multiple components including mycobacterial glycolipids, proteins, and DNA, several receptors that use IRAK-4 and MyD88 as the signal transducer appear to be involved in the complex recognition of BCG.

In IL-21R–deficient mice, the level of circulating IgE is high, whereas that of IgG1 is low (23, 46). Similarly, in human B cells, IL-21 inhibits IgE production and stimulates IgG4 (analogous to mouse IgG1) production (19). These results suggest that IL-21 differentially regulates IgE and IgG1 (IgG4 in humans) class switching. In fact, Suto et al. (18) reported that IL-21 specifically suppresses IgE production by inhibiting germ line Cε transcripts. Our present findings do not exclude this possibility. IL-21 has also been reported to induce apoptosis in resting and activated B cells by reducing the expression levels of apoptosis-related genes (25, 26). However, in this report, we have shown that IL-21 selectively induces apoptosis in Bε, but not Bγ, cells (Fig. 4 D). Thus, our findings that BCG-activated IL-21–expressing Vα14 NKT cells suppressed IgE production even after class switching (Fig. 4 C) suggests that the role of IL-21 on Bε cells is to control cell growth and viability, rather than to regulate the differentiation and maturation of these cells.

We found that expression of a proapoptotic gene, Bmf, was significantly higher in Bε cells than in Bγ cells (Fig. 5 A). Under physiological conditions, Bmf, which is a BH3 domain–only Bcl-2 family member that inhibits Bcl-2 function and accelerates apoptosis, binds to myosin V motors via the dynein light chain 2 domain of Bmf (34). In response to certain cellular damage signals, Bmf is supposed to be released from the myosin V motors and trigger apoptosis (34). Because Bmf from Bε cells induced apoptosis and a mutation in the BH3 domain of Bmf failed to induce apoptosis (Fig. S3), we confirmed that Bmf expressed in Bε cells is functional, and that the BH3 domain is important for the binding to Bcl-2 and is essential for its proapoptotic activity. In fact, the binding of Bmf with Bcl-2 was up-regulated by IL-21R signaling (Fig. 5 C). Therefore, BCG-mediated Bε cell apoptosis is due to the augmented formation of Bmf–Bcl-2 complexes generated by IL-21R signaling in Bε cells.

Finally, we defined the mechanism of BCG-induced IL-21–dependent suppression of IgE production in humans (Fig. 6). In a broader context, these findings may explain the mechanisms underlying the BCG-mediated suppression of allergic diseases and the epidemiological data indicating a reduction in the morbidity of allergic diseases in patients who

have been infected with *Mycobacterium tuberculosis*. Interestingly, IL-21–mediated B cell responses in C57BL/6 mice differ from those in BALB/c mice (26), suggesting that there is a genetic polymorphism with respect to the outcome of IL-21 signaling in B cells. In fact, a recent report indicated that polymorphisms in the IL-21R gene locus differentially affect serum IgE levels in humans (47). In this study, consistent with our data, the levels of IL-21 expression induced by BCG stimulation varied among the individuals examined (Fig. 6 C). These results suggest that the response to BCG in humans is dependent, at least in part, on genetic background. The specific genes responsible for the heterogeneity in BCG-mediated IL-21 production have not been identified. However, this observation may be applied to the development of diagnostic or therapeutic strategies in which the levels of IL-21 expression are used to evaluate the efficacy of BCG treatment, or in determining the potential benefit of therapy using bacterial products such as CpG for allergic diseases.

MATERIALS AND METHODS

Mice. 7–10-wk-old female BALB/c mice were purchased from Japan CREA Inc. Vα14 NKT-deficient (Vα14 NKT KO) mice on a BALB/c background (48), IRAK-4 KO (49), TLR2 KO, TLR4 KO, and MyD88 KO mice (50, 51) have been described. TLR2 and TLR4 double KO mice were generated by breeding. Mice were kept under specific pathogen-free conditions, maintained on an OVA-free diet, and treated in accordance with the guidelines for animal care at RIKEN Research Center for Allergy and Immunology.

Allergic sensitization and BCG. Allergic epicutaneous sensitization was performed as described previously (27). In brief, a 1-cm^2 sterile patch infused with 100 μl of PBS solution with or without 100 μg OVA (grade V; Sigma-Aldrich) was placed on the shaved back of mice and fixed in place with a bio-occlusive dressing and an elastic bandage. Patches were left on for 48 h and removed. The sensitization course was repeated at the same skin site every week for 4 wk. For BCG vaccination, mice were given a weekly i.p. injection of BCG (500 μg/mouse) or PBS at the time of OVA sensitization. The attenuated BCG (strain Tokyo) was purchased from the Japan BCG Laboratory.

Flow cytometry. Cells were stained with antibodies after adding 2.4G2 (BD Biosciences) for Fc blocking. The following antibodies were used: FITC–anti-CD19 (1D3), FITC–anti-IgE (R35-72), APC–anti-IgG1 (X59), FITC–anti-TCRβ (H57-597), APC–anti–IL-12p40/70 (C15.6), and PE–anti-CD11c (HL3; BD Biosciences). PE-conjugated α-GalCer–loaded CD1d tetramer (α-GalCer/CD1d tetramer) was prepared as described previously (52). For intracellular staining, BM-DCs were fixed and permeabilized with BD Cytofix and Cytoperm kits after staining with PE–anti-CD11c. They were then stained with APC–anti–IL-12p40/70. FACS analysis of at least 10,000 cells and cell sorting were performed with a FACSCalibur (BD Biosciences) with FlowJo software (TreeStar) or with a MoFlo cell sorter (DakoCytomation).

Cell preparations and cultures. 2×10^6 BM-DCs obtained by culturing BM for 6 d with 10 ng/ml GM-CSF were further cultured in the presence or absence of BCG, CpG, LPS (Invivogen), PGN from *Escherichia coli* (Invivogen), or 10 μg/ml anti-CD3 mAb (2C11; BD Biosciences) for 48 h at 37°C. For blocking experiments, mAb against CD1d or IL-12p40/p70 (clones 1B1 and C17.8, respectively; BD Biosciences), or an isotype control was added at a concentration of 20 μg/ml after 2.4G2 treatment. $TCR\beta^+$ cells or Vα14 NKT cells with a purity of >98% were obtained from liver MNCs (52) using an Auto MACS (Miltenyi Biotec) after staining with FITC–anti-TCRβ and sorting with anti-FITC magnetic beads (Miltenyi Biotec). Vα14 NKT cells were then isolated from $TCR\beta^+$ cells by MoFlo using PE–α-GalCer/CD1d tetramer. Conventional T or $CD4^+$ T cells were isolated from an α-GalCer/CD1d tetramer$^-$ fraction of $TCR\beta^+$ liver MNCs. Bε and Bγ cells generated from splenic $CD19^+$ cells in the presence of 10 μg/ml sCD40L (ALX-850-075; Qbiogene) and 20 ng/ml of recombinant IL-4 (PeproTech) for 3 d (33) were cultured for 30 h for the apoptosis assay or for an additional 5 d to investigate IgE responses.

ELISA. Cytokines (IL-12p70 and IL-6) and Ig subclasses (IgG1, IgG2a, and IgE) were measured by ELISA using kits or sets of antibodies (BD Biosciences) according to the manufacturer's protocol. Specific antibodies were also measured as described previously (7).

RT-PCR. Total RNA was extracted by RNAeasy (QIAGEN), and cDNA was synthesized with random primers after DNase treatment. The following RT-PCR primer sets were used for mouse genes: IL-21, 5′-CCCTTGTCTGTCTGGTAGTCATC-3′ and 5′-ATCACAGGAAGGGCATTTAGC-3′; IgE (Cε), 5′-AGGAACCCTCAGCTCTACCC-3′ and 5′-GCCAGCTGACAGAGACATCA-3′; mIL-21R, 5′-TGTCAATGTGACGGACCAGT-3′ and 5′-CAGCATAGGGGTCTCTGAGG-3′; γc, 5′-GTCGACAGAGCAAGCACCATGTTGAAACTA-3′ and 5′-GGATCCTGGGATCACAAGATTCTGTAGGTT-3′; Bmf, 5′-CAGACCCTCAGTCCAGCTTC-3′ and 5′-CGTATGAAGCCGATGGAACT-3′; Bcl-2, 5′-GGTGGTGGAGGAACTCTTCA-3′ and 5′-CATGCTGGGGCCATATAGTT-3′; and HPRT, 5′-AGCGTCGTGATTAGCGATG-3′ and 5′-CTTTTATGTCCCCCGTTGAC-3′. The numbers of PCR cycles were as follows: 30 for HPRT; 35 for IgE, γc, and IL-21R; 40 for IL-21 and Bmf; and 45 for Bcl-2. The amounts of cDNA were standardized by quantification of the housekeeping gene HPRT using primers for mouse samples. The human IL-21 mRNA levels were quantified by real-time quantitative PCR on the ABI Prism 7000 sequence detection system (Applied Biosystems) by using TaqMan assay kits and TaqMan Gene Expression Assays (primers and TaqMan probes).

Electrophoretic mobility shift assay. 2×10^6 BM-DCs were stimulated with 50 μg/ml BCG or 1.0 μM CpG-B for the indicated periods. Nuclear extracts were prepared and used for Gel Shift Assay Systems (Promega) as described previously (50).

Bε cell–derived Bmf and its mutants. cDNAs encoding bmf were amplified from Bε cells by PCR using primers 5′-CCGAATTCGGATGGAGCCACCTCAGTGTGT-3′ and 5′-GCGGCCGCCTGCATTCCTGGTGATCCAT-3′ (EcoRI and NotI sites for cloning are underlined). The amplified products were cloned using the pGEM-T Easy Vector System (Promega). Mutant cDNAs were generated by PCR using point-mutated primer pairs.

Immunoprecipitation and Western blotting. Interaction of Bmf with Bcl-2 in Bε cells was detected by immunoprecipitation with anti–Bcl-2 mAb (clone 7; BD Transduction Laboratories) and subsequent immunoblotting with anti-Bmf rabbit antibody (Cell Signaling). The protein levels were visualized by ECL (GE Healthcare) using horseradish peroxidase–conjugated Protein A/G (Pierce Chemical Co.).

Human studies. All human specimens were obtained under informed consent. The protocol for the human research project has been approved by the Ethics Committee of Chiba University and RIKEN, and conformed to the provisions of the Declaration of Helsinki in 1995. 10^8 PBMCs from healthy volunteers were prepared by Ficoll-Paque density gradient centrifugation and used for the cultures. Human recombinant IL-21 was purchased from BIOSOURCE Inc. Human total IgE was measured with a sensitive immune assay (GE Healthcare).

Statistical analysis. Statistical analyses were performed using the Student's *t* test or matched pairs *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 provides data demonstrating that MyD88 signaling in DCs is required for BCG-induced activation. Fig. S2 contains data demonstrating IL-21 mRNA expression by NKT cells, $CD4^+$ T cells, and $CD8^+$ T cells of murine and human origin. Fig. S3 provides the data indicating proapoptotic activity of Be cell–derived Bmf and functional domain analysis using mutant Bmf in Baf3 cells. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062206/DC1.

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Involvement of TNF Receptor-Associated Factor 6 in IL-25 Receptor Signaling[1]

Yuko Maezawa,* Hiroshi Nakajima,[2]* Kotaro Suzuki,* Tomohiro Tamachi,* Kei Ikeda,* Jun-ichiro Inoue,† Yasushi Saito,* and Itsuo Iwamoto*

IL-25 (IL-17E) induces IL-4, IL-5, and IL-13 production from an unidentified non-T/non-B cell population and subsequently induces Th2-type immune responses such as IgE production and eosinophilic airway inflammation. IL-25R is a single transmembrane protein with homology to IL-17R, but the IL-25R signaling pathways have not been fully understood. In this study, we investigated the signaling pathway under IL-25R, especially the possible involvement of TNFR-associated factor (TRAF)6 in this pathway. We found that IL-25R cross-linking induced NF-κB activation as well as ERK, JNK, and p38 activation. We also found that IL-25R-mediated NF-κB activation was inhibited by the expression of dominant negative TRAF6 but not of dominant negative TRAF2. Furthermore, IL-25R-mediated NF-κB activation, but not MAPK activation, was diminished in TRAF6-deficient murine embryonic fibroblast. In addition, coimmunoprecipitation assay revealed that TRAF6, but not TRAF2, associated with IL-25R even in the absence of ligand binding. Finally, we found that IL-25R-mediated gene expression of IL-6, TGF-β, G-CSF, and thymus and activation-regulated chemokine was diminished in TRAF6-deficient murine embryonic fibroblast. Taken together, these results indicate that TRAF6 plays a critical role in IL-25R-mediated NF-κB activation and gene expression. *The Journal of Immunology,* **2006, 176: 1013–1018.**

Interleukin-25 has recently been identified as the fifth member of the IL-17 cytokine family (IL-17E) by database searching (1–4). The IL-17 family now consists of six family members, namely IL-17 (IL-17A), IL-17B, IL-17C, IL-17D, IL-25, and IL-17F (5–7). Among IL-17 family members, IL-25 is less homologic to other IL-17 family members, e.g., 18% homology to IL-17A at amino acid level. Accordingly, in vivo biologic activities of IL-25 are markedly different from those described for IL-17 and other IL-17 family cytokines (2–4, 8–10). Remarkably, it has been shown that the enforced expression of IL-25 induces IL-4, IL-5, and IL-13 production from an unidentified non-T/non-B cell population and subsequently induces Th2-type immune responses such as eosinophilic airway inflammation, mucus production, and airway hyperreactivity (2–4, 8).

IL-25R, which is also called IL-17BR, IL-17Rh1, or Evi27, is a 56-kDa single transmembrane protein with homology to IL-17R (1, 11, 12). IL-25R was first identified as a receptor for IL-17B (11) but IL-25R has subsequently been shown to exhibit a higher affinity for IL-25 than for IL-17B (1). IL-25 has also been demonstrated to activate NF-κB and induce IL-8 production in a human renal carcinoma cell line (1). However, the molecular components consisting of IL-25R signaling pathways and their regulation are still largely unknown.

It has recently been shown that TNFR-associated factor (TRAF)[3] family proteins play a critical role in a number of signaling pathways that activate NF-κB (13–16). TRAF family proteins contain a conserved TRAF-C domain that is essential for the interaction with their cognate receptors or cytoplasmic signaling proteins (13–16). Among TRAF family proteins, TRAF6 exhibits the unique properties in that its TRAF-C domain interacts with a peptide motif distinct from that recognized by other TRAF proteins (17), supporting the findings that TRAF6 exhibits various functions in regulating adaptive and innate immunity, bone metabolism, and cell apoptosis (13–16). The structural analysis of the peptide-TRAF6 interaction has clarified the TRAF6-binding motif as X-X-Pro-X-Glu-X-X-(aromatic/acidic residue) (17). The TRAF6-binding motif is found not only in adaptor proteins such as IL-1R-associated kinase (17) and TIFA (18) but also in membrane-bound proteins such as CD40 and the receptor activator of NF-κB RANK (17). Importantly, the TRAF6-binding motif is present in human and murine IL-25R.

In the present study, we investigated whether TRAF6 is involved in IL-25R signaling. Our results have clearly demonstrated a critical involvement of TRAF6 in IL-25R-mediated NF-κB activation and gene expression.

Materials and Methods

Cell culture

X63 cells were maintained in RPMI 1640 medium with 10% FCS, 50 μM 2-ME, and antibiotics (complete RPMI 1640 medium). Ba/F3 cells were cultured in complete RPMI 1640 medium supplemented with 10% (v/v) of the supernatant of murine IL-3-producing X63 cells (X63-IL-3; a gift from Dr. H. Karasuyama, Tokyo Medical and Dental University, Tokyo, Japan) (19). COS7 cells were cultured in DMEM supplemented with 10% FCS

*Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan; and †Division of Cellular and Molecular Biology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Hiroshi Nakajima, Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba City, Chiba 260-8670, Japan. E-mail address: nakajimh@faculty.chiba-u.jp

[3] Abbreviations used in this paper: TRAF, TNFR-associated factor; TARC, thymus and activation-regulated chemokine; MEF, murine embryonic fibroblast; DN, dominant negative; MKK, MAPK kinase; WT, wild type.

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and antibiotics (complete DMEM). Wild-type (WT) murine embryonic fibroblast (MEF), TRAF6-deficient ($TRAF6^{-/-}$) MEF (20), and Plat-E cells (21) were established and maintained as described elsewhere.

Plasmids

DNA fragment coding the extracellular domain of murine IL-25R, a gift from Dr. J. D. Shaughnessy (University of Arkansas for Medical Sciences, Little Rock, AR) (12) was fused to the fragment coding C-terminal 187 aa of MPL, a receptor for thrombopoietin (22), and cloned into expression vector pCDNA3 (pCDNA3 IL-25R-MPL). Expression vectors for WT TRAF2, dominant negative (DN) TRAF2, Flag-tagged WT TRAF6, and Flag-tagged DN TRAF6 were previously described (23). Expression vectors for Flag-tagged IL-25 (BCMGS Flag-IL-25), Flag-tagged IL-25R (pCMV1 Flag-IL-25R), and *myc*-tagged intracellular region of IL-25R (pCDNA3 *myc*-IL-25R) were constructed by PCR amplification using PFU polymerase (Stratagene). The DNA fragment coding Flag-tagged IL-25R was subsequently subcloned into the retrovirus vector pMX IRES-GFP to generate pMX Flag-IL-25R-IRES-GFP. Alanine substitution of IL-25R on glutaminic acid at aa 338 (IL-25R E338A) was generated by using a PCR-based site-directed mutagenesis kit (Stratagene). The mutation was confirmed by DNA sequencing.

Cytokines

X63 cells were transfected with BCMGS Flag-IL-25 to generate murine IL-25-producing X63 cells (X63-IL-25). The supernatant of X63-IL-25 cells was collected and used as a source of IL-25. The supernatant of murine IL-3-producing X63 cells (X63-IL-3) and the empty vector (BCMGS neo)-transfected X63 cells (X63-control) were also used as controls.

Bioassay for IL-25

IL-3-dependent Ba/F3 cells were transfected with pCDNA3 IL-25R-MPL and Ba/F3 cells that stably expressed IL-25R-MPL were selected by G418 (Ba/F3 IL-25R-MPL cells). The expression of IL-25R-MPL was evaluated not only at mRNA levels by RT-PCR analysis but also at protein levels with the response to the supernatant of X63-IL-25 cells. Subsequently, bioactivity of IL-25 was assessed by the proliferative response of Ba/F3 IL-25R-MPL cells. Briefly, Ba/F3 IL-25R-MPL cells (2×10^3 cells/well) were cultured in triplicate at 37°C in 96-well plates in the complete RPMI 1640 medium in the presence of X63-IL-25 conditioned medium or X63-IL-3 conditioned medium (as a positive control) for 36 h with 0.5 μCi of [^{3}H]thymidine added for the final 12 h. Empty vector (pCDNA3)-transfected Ba/F3 cells were used as a negative control.

Retrovirus-mediated expression of IL-25R in MEF

A transient retrovirus packaging cell line of Plat-E cells (2×10^6) was transfected with 3 μg of pMX Flag-IL-25R-IRES-GFP using FuGENE6 transfection reagents (Roche Diagnostics). At 24 h after the transfection, the medium was once changed and another 24 h later, the supernatant was harvested as virus stocks and stored at −80°C until use. WT MEF or $TRAF6^{-/-}$ MEF (1×10^6) were infected with 2 ml of virus stocks for 4 h in the presence of polybrene (1 μg/ml) and then diluted and maintained in the complete DMEM. Under these conditions, the efficiency of infection was 90% as assessed by GFP^+ cells by FACS.

Luciferase assay

COS7 cells (1×10^5) were transfected with 1.0 μg of pCMV1 Flag-IL-25R and 0.3 μg of NF-κB-responding *Photinus pyralis* luciferase reporter vector pNF-κB-Luc (Stratagene) using FuGENE6. In some experiments, expression vector for DN TRAF6 or DN TRAF2 was cotransfected. Empty vector was added to adjust the total amount of plasmid DNA for transfection. To normalize for transfection efficiency, 10 ng of *Renilla reniformis* luciferase reporter vector pRL-TK was added to each transfection. At 24 h after the transfection, cells were stimulated with X63-IL-25 condition medium or anti-Flag M2 mouse mAb (2 μg/ml; Sigma-Aldrich) at 37°C for 24 h, and the luciferase activity of *Photinus pyralis* and *Renilla reniformis* were determined by the Dual-Luciferase Reporter Assay System (Promega). *Photinus pyralis* luciferase activity of pNF-κB-Luc was normalized by *Renilla reniformis* luciferase activity of pRL-TK. Condition medium of X63-control cells or mouse monoclonal IgG1 (Ancell) was used as controls.

Nuclear accumulation of NF-κB p65

WT MEF or $TRAF6^{-/-}$ MEF were infected with retrovirus of pMX Flag-IL-25R-IRES-GFP as described earlier and Flag-IL-25R-expressing WT MEF or $TRAF6^{-/-}$ MEF were stimulated with X63-IL-25 condition medium or anti-Flag M2 mAb (2 μg/ml) at 37°C for 30 min. Nuclear extracts were prepared as described elsewhere (24), and DNA-binding activity of NF-κB p65 in the nuclear extracts was detected by Transfactor NF-κB chemiluminescent kit (BD Biosciences) according to the manufacturer's instruction. Briefly, nuclear extracts (5 μg) were added to wells coated with NF-κB consensus oligonucleotides and incubated for 1 h at room temperature. After washing, the wells were incubated with anti-NF-κB p65 rabbit polyclonal Ab, followed by anti-rabbit IgG HRP and then chemiluminescent substrate mixture. Chemiluminescent intensities were measured with Arvo 1420 multilabel counter (Wallac). For DNA competition experiments, 0.5 μg of unlabeled competitor oligonucleotide was added to the nuclear extracts.

Immunoblotting

MEF (1×10^5) were starved from FCS for over 12 h and then stimulated with anti-Flag mAb (2 μg/ml), mouse rIL-17 (100 ng/ml; R&D Systems), or mouse rIL-1β (10 ng/ml; PeproTech) for 30 min. The cells were then lysed with cell lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.875% Brij97, 0.125% Nonidet P-40, 8 mM DTT, and 1% protease inhibitor mixture (Sigma-Aldrich)) supplemented with 1 mM Na_3VO_4, 10 mM NaF, and 60 mM β-glycerophosphate. The aliquot of lysates was applied for SDS-PAGE. The following Abs were used for immunoblotting: anti-IκB-α (MBL Japan), anti-p38α/SAP2a, anti-p38 (pT180/pY182), anti-ERK1, anti-ERK1/2 (pT202/pT204), anti-pan JNK/SAPK1, and anti-JNK (pT183/pT185) (BD Transduction).

Coimmunoprecipitation assay

COS7 cells (4×10^5) were transfected with pCDNA3 *myc*-IL-25R (1.0 μg) and/or pME18S Flag-TRAF6 (1.0 μg), pME18S Flag-TRAF2 (1.0 μg), or pME18S Flag-TRAF5 (1.0 μg) using FuGENE6. Twenty-four hours after the transfection, cells were harvested, lysed with cell lysis buffer, and centrifuged to remove cellular debris. After preclearation, the supernatants were immunoprecipitated with either anti-*myc* mAb (9E10; Santa Cruz Biotechnology) or anti-Flag M2 mAb and 100 μl of protein G-Sepharose (Pharmacia). The immunoprecipitates or the aliquot of whole cell lysates were applied for immunoblotting with rabbit polyclonal anti-Flag Ab (Sigma-Aldrich) or biotin-labeled anti-*myc* mAb (9E10; Santa Cruz Biotechnology).

RT-PCR

Total cellular RNA was prepared, and RT-PCR analysis was performed as previously described (24). In brief, Flag-IL-25R-expressing WT MEF or $TRAF6^{-/-}$ MEF were stimulated with anti-Flag mAb (2 μg/ml) at 37°C for 3 h and the total cellular RNA was isolated using Isogen solution (Nippon Gene) according to the manufacturer's instruction. The following primer pairs were used for PCR: IL-6 (ATGAAGTTCCTCTCTGCAA GAG and GTTTGCCGAGTAGATCTCAAAG), G-CSF (GCTGTG GCAAAGTGCACTATG and AAGCCCTGCAGGTACGAAATG), TGF-β (ATTCAGCGCTCACTGCTCTTG and TCAGCTGCACTTG CAGGAGC), and thymus and activation-regulated chemokine (TARC) (TGAGGTCACTTCAGATGCTGC and ACCAATCTGATGGCCT TCTTC). RT-PCR for β-actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t test. Values for $p < 0.05$ were considered significant.

Results

Establishment of a bioassay for IL-25

It has been reported that IL-25 activates NF-κB in a renal carcinoma cell line (1), but the signaling pathway under IL-25R is largely unknown. To examine IL-25 signaling in detail, we first prepared rIL-25 and an assay that verifies the bioactivity of rIL-25. Because IL-25 belongs to the cystine knot family and correct refolding and dimer formation seem to be required for its biological activity (6, 7), we used the mammalian cell-based cytokine expression system (19) rather than the *Escherichia coli*-based expression system. We first established X63 cells that stably produced mouse IL-25 (X63-IL-25 cells) and used the supernatant of