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Critical Role of p38 and GATA3 in Natural Helper Cell Function

Jun-ichi Furusawa,*† Kazuyo Moro,*‡§,S Yasutaka Motomura,*¶,§ Kazuo Okamoto,#,**,†† Masato Kubo,*¶ and Shigeo Koyasu*†

Natural helper (NH) cells, a member of Lin–IL-2R–IL-7R–IL-25R–IL-33R–GATA3* group 2 innate lymphoid cell subset, are characterized by the expression of transcription factors GATA3 and RORγt and production of large amounts of Th2 cytokines such as IL-5, IL-6, and IL-13 upon IL-33 stimulation or a combination of IL-2 and IL-25. We have studied the signal transduction pathways critical for the cytokine expression and development of NH cell. Either stimulation with IL-33 or a combination of IL-2 and IL-25 induced p38 activation and phosphorylation of GATA3 in NH cells, and the phosphorylated form of GATA3 bound to the IL-5 and IL-13 promoters. All these events were blocked by SB203580, a p38 inhibitor. Inhibition of p38 also blocked IL-6 production. The mature NH cells lacking Gata3 were impaired in the proliferation and production of IL-5 and IL-13, but not IL-6, indicating that both p38 and GATA3 are critical for the proliferation and production of IL-5 and IL-13 and that the mechanisms downstream of p38 differ between IL-6 and IL-5/IL-13. In contrast, the NH cells lacking RORγt showed no impairment in the proliferation and cytokine production, indicating that GATA3 but not RORγt plays a pivotal role in the effector functions of mature NH cell. However, deletion of either GATA3 or RORγt in hematopoietic stem cells severely blocked the development into NH cells. Our results demonstrate the important roles of p38 and GATA3 in NH cell functions. The Journal of Immunology, 2013, 191: 1818–1826.

We have previously identified an Id2-dependent novel innate lymphocyte subset named natural helper (NH) cells present in a novel lymphoid tissue, fat-associated lymphoid cluster (FALC), in mouse, rat and human adipose tissues (1). Recent reports showed that NH cells also exist in the lung, small, and large intestines (2–4). NH cells do not express lineage (Lin) markers but express IL-2R, IL-7R, IL-25R, and IL-33R. IL-7 is critical for the differentiation of NH cells as well as NH cell survival. IL-2 induces proliferation of NH cells and IL-33 or a combination of IL-2 and IL-25 (IL-2+25) activates NH cells to produce large amounts of Th2 cytokines IL-5, IL-6, and IL-13. NH cells have important roles in innate immune reactions against helminth infections (1, 4–8). A distinct Id2-dependent innate lymphocyte subset, retinoic acid receptor-related orphan receptor γt (RORγt)* lymphoid tissue inducer (LTi)-related cells present in the gut regulates intestinal homeostasis by producing IL-17 and IL-22 (9–11).

IL-33 is a member of the IL-1 family and is constitutively expressed in the nuclei of a variety of cells including fibroblasts, epithelial cells, endothelial cells, and adipocytes (12, 13). The IL-33 receptor consists of T1/ST2 and IL-1RAcP and receptor binding of IL-33 activates NF-κB transcription factors and the MAPK family, including JNK and p38, through MyD88, IRAK, binding of IL-33 activates NF-κB transcription factors and the MAPK family, including JNK and p38, through MyD88, IRAK, TAK1 (14, 15). Administration of IL-33 in vivo induces Th2 cytokine production and associated physiologic changes in mice including airway hyperresponsiveness, eosinophilia, and goblet cell hyperplasia (16). Previous studies have shown that polymorphisms of IL-33 and T1/ST2 are associated in asthma in human, demonstrating that IL-33 and T1/ST2 have a role in human allergic diseases (17). The levels of IL-33 are increased in smooth muscle cells in the airways of severe asthma patients compared with healthy individuals (18). It is thus likely that NH cells have a major role in those IL-33-mediated responses.

Transcription factors GATA3 and retinoic acid receptor-related orphan receptor α (RORα) but not RORγt are highly expressed in NH cells and have important roles in the differentiation of NH cells (1, 3, 6, 19–21). GATA3 selectively activates the IL-4, IL-5, and IL-13 promoters through chromatin remodeling in Th2 cells (22). Interestingly, GATA3 is required for the continuous pro-
duction of IL-5 and IL-13, but dispensable for maintaining the expression of IL-4 by Th2 cells (23). ROXs is induced in Th17 cells and functions together with RORγt to induce IL-17 expression in Th17 cells (24). Although IL-33 induces Th2 cytokine production by various cells, roles of GATA3 and ROXs in IL-33 signaling have been obscure.

In this study, we demonstrate that a p38 inhibitor strongly suppresses IL-33-induced production of IL-5, IL-6, and IL-13 by NH cells. Inhibition of p38 blocks both GATA3 phosphorylation and GATA3 binding to the IL5 and IL13 promoters. GATA3 deletion in mature NH cells impairs the expression of IL-5 and IL-13 without affecting IL-6 production. Deletion of GATA3 significantly decreases proliferation of NH cells by cytokine stimulation. Contrary to GATA3, the mutation of RORγt showed no effect on the proliferation and Th2 cytokine production of NH cells.

Materials and Methods

Mice

Mice used in this study were on a C57BL/6 background and were maintained in our animal facility under specific pathogen-free conditions. Wild-type (WT) C57BL/6 mice, B6.6F1-Ki67−/− and B6.6F1-Ki67−/− mice were obtained from Japan SLC (Tokyo, Japan). B6.6F1-Rag2−/− mice and γc−/− Rag2−/− mice were obtained from Taconic Farms (Germantown, NY). Cre-ERT2 transgenic mice and Rosa26loxP/cre mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Gata3flox/flox mice were purchased from Calbiochem (Darmstadt, Germany). Corn oil was from Sigma-Aldrich (St. Louis, MO), and ionomycin was purchased from位列科技（Danvers, MA), Abcam (Cambridge, MA), Millipore (Temecula, CA), Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, MA), Millipore (Temecula, CA), Invitrogen (Carlsbad, CA), and GE Healthcare (Waukesha, WI). Recombinant mIL-3 and mIL-7 were purchased from PeproTech (Rocky Hill, NJ). mIL-2 was purchased from Shionogi Pharmaceutical (Osaka, Japan). mIL-25 and mIL-33 were purchased from R&D Systems (Minneapolis, MN), PMA and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma-Aldrich (St. Louis, MO), and ionomycin was purchased from Calbiochem (San Diego, CA). SB203580, SP600125, and BAY11-7082 were purchased from Calbiochem (Darmstadt, Germany). Corn oil was purchased from Ajinomoto (Tokyo, Japan).

Preparation of cells

NH cells were isolated from mesentry as described previously (1), except that we used 10 µg/ml Liberase DH from Roche (Tokyo, Japan) and gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA) for the digestion of mesentery. Bone marrow-derived mast cells (BMMCs) and Th2 cells were prepared as described previously (1,27). To isolate lymphocytes from the small intestinal lamina propria, gut fragments without Peyer’s patches were treated with 1 mM EDTA for 20 min to remove epithelial cells.

Sorted NH cells were seeded at 5 × 10^3 cells/well into 96-well round-bottom tissue culture plates with various stimulants including IL-2 (10 ng/ml), IL-7 (10 ng/ml), IL-25 (10 ng/ml), IL-33 (10 ng/ml), stem cell factor (SCF; 50 ng/ml), and PMA (30 ng/ml) plus ionomycin (500 ng/ml). Supernatants were collected after 3–96 h. Amounts of cytokines in culture supernatants were determined by ELISA using Quantikine Kits (R&D Systems) for IL-5, IL-6, and IL-13. Cell numbers and viability were examined using a Countess Automated Cell Counter (Invitrogen). Cytokine production was calculated and presented as picograms of production per 1 × 10^5 cells.

Flow cytometry

Flow cytometry was performed on a FACSAriaII (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo Software (Tree Star, Ashland, OR). For intracellular cytokine staining, cells were pretreated with Brefeldin A (eBioscience) for 3 h before harvest. Cells were fixed and permeabilized with IntraPrep (Beckman Coulter, Marseille, France) and then stained intracellularly with the indicated Abs.

Immunoblot analysis

After stimulation, cells were washed once with PBS and lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. Lysates were separated by SDSPAGE, followed by transfer to polyvinylidene difluoride membranes (Pall Corporation, Washington, NY). Membranes were probed using the designated Abs and visualized with an ECL Advance western blotting detection kit (GE Healthcare, Buckinghamshire, U.K.).

Quantitative real-time PCR

Total RNA was prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with Ready-To-Go T-Primed First-Strand kit (GE Healthcare). A CFX96 Real-Time PCR system, C1000 Thermal Cycler and SsoFast EvaGreen Supermix (both from Bio-Rad, Hercules, CA) were used to evaluate gene expression. The expression levels of Gata3 were normalized to 18s rRNA expression. PCR cycling was as follows: 95°C for 3 min for 1 cycle, 98°C for 3 s, 60°C for 3 s for 40 cycles, and 98°C for 10 s. Primer pair sequences, specific for Gata3 were (forward, 5’-GAAGACGGGAGGGGCAAGT-3’), reverse, 5’-AGCTGGGAGGATGC-3’), those for Rora were (forward, 5’-TTCCTGTTGAGGCTCTCAAG-3’), reverse, 5’-GGGTGAGTGGGCTTGCT-3’), and those for 18s rRNA were (forward, 5’-GCCGCTGAGGAAAACTTTCT-3’), reverse, 5’-CGAAGCTCCAGCTTCTCTTC-3’).

Bone marrow transplantation

Whole bone marrow cells (BMMCs) were transferred into sublethally irradiated (2 Gy) γc−/− Rag2−/− or B6.6F1-Rag2−/− mice. Cre-mediated recombination was induced on day 1–3. Mesenteric Lin Thy1.2+CD25+ T1/22+Gata3−/− or B6.6F1-Ki67−/− bone marrow (BM) Lin−/−CD25+T1/22IL-7Rα+ NH cells were analyzed by flow cytometry after indicated time periods. In the case of transfer into B6.6F1-Rag2−/− mice, donor-derived cells were gated with anti-CD45.2 mAb.

Cre-mediated recombination

For in vivo assays, 4-hydroxytamoxifen (4-OHT; 1 mg per 25 g mouse body weight) was diluted with a combination of 90% corn oil (Ajinomoto, Tokyo, Japan) and 10% ethyl alcohol (EIOH) and injected i.p. daily for 3 consecutive days. For in vitro assays, recombination was induced by incubation of NH cells cultured in IL-2 (10 ng/ml) with 100 µM 4-OHT or 0.01% EtOH for 48 h.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (26). Primer pair sequences specific for each locus were as follows: Il3 (forward, 5’-TCGCTTTATAGGTGTCCTC-3’), reverse, 5’-GGCTCTCAAGAAAGGAGAG-3’), Cgre (forward, 5’-GTCCTCTTACGACCCCTAC-3’), reverse, 5’-AAAGCTTTGCGGAAAACAC-3’). Results are presented as the ratio of the cycling threshold value of immunoprecipitated DNA to that of input DNA (2^(-CTinput-CTIP)), where CT indicates the cycling threshold and IP is immunoprecipitated chromatin.

Statistical analysis

Data are shown as the mean and SEM. Statistical analysis was performed using the repeated measures one way ANOVA. Bonferroni’s multiple

Contrary to GATA3, the mutation of RORγt suppresses IL-33–induced production of IL-5, IL-6, and IL-13 without affecting IL-6 production. Deletion of GATA3 significantly decreases proliferation of NH cells by cytokine stimulation. Contrary to GATA3, the mutation of RORγt showed no effect on the proliferation and Th2 cytokine production of NH cells.
comparison adjustment for preplanned contrasts was applied to secure overall type I error, whereas a false discovery rate method for multiple comparison adjustment was applied for pairwise comparisons to avoid over-adjustment.

**Results**

**p38-Mediated signaling pathway is critical for the IL-33–induced cytokine production by NH cells**

NH cells in mesentery (Supplemental Fig. 1) produce large amounts of IL-5, IL-6, and IL-13 upon stimulation by IL-33 (1). After stimulation with IL-33 for 96 h, NH cells produced ~1 pg/cell of IL-5 and IL-13, whereas BMMCs produced much lower amounts (Fig. 1A). In contrast, production of IL-6 was more comparable between NH cells and BMMCs (Fig. 1A). Although NH cells express the SCF receptor, c-Kit, c-Kit is dispensable for IL-33–mediated proliferation (Fig. 1B) and cytokine production (Fig. 1C) by NH cells and SCF had little effect on the production of IL-6 by NH cells, whereas SCF and IL-33 had a synergistic effect on IL-6 production by mast cells (Fig. 1D and data not shown) as reported previously (28). Although IL-6 production by BMMCs reached a plateau by 24 h after IL-33 stimulation, cytokine production by NH cells continued to increase over time (Fig. 1E).

We then examined the signal transduction pathways downstream of the IL-33 receptor in NH cells and BMMCs. IL-33 induced phosphorylation of NF-kB p65, JNK, and p38 MAPK in the cytosol of both BMMCs and NH cells (Supplemental Fig. 2A). Intriguingly, NH cells have phosphorylated NF-kB p65, JNK, and p38 MAPK in the nucleus even before stimulation. IL-33 induced biphasic phosphorylation of NF-kB p65, c-Jun, and ATF2 in the nucleus of NH cells (Supplemental Fig. 2B). Phosphorylation of these molecules peaked 30–60 min after stimulation by IL-33, decreased somewhat, and then increased again after 48 h of activation. Stimulation-dependent complete degradation of an adaptor molecule, IRAK1, was not observed in NH cells in contrast to BMMCs (Supplemental Fig. 2C). In addition, 24 h after stimulation by IL-33, expression of Gfi-1, c-Maf, and NFATc1, molecules highly expressed in Th2 cells, was strongly induced in NH cells (Supplemental Fig. 2D).

To determine signaling pathways important for IL-33–induced Th2 cytokine production in NH cells, we examined the effects of various inhibitors. Among them SB203580, a p38 inhibitor, significantly suppressed IL-33–induced production of IL-5, IL-6, and IL-13 in NH cells, whereas JNK and NF-kB inhibitors (SP600125 and BAY11-7082, respectively) had little effect (Fig. 2A, Supplemental Fig. 3A). Inhibition of p38 during early phase of stimulation reduced IL-5, IL-6, and IL-13 production by IL-33, whereas inhibition in late phase showed little effect (Supplemental Fig. 3B). SB203580 and BAY11-7082 suppressed the growth of NH cells without affecting their viability (Fig. 2B) or the expression level of T1/ST2 (Fig. 2C). Intracellular staining confirmed that SB203580 significantly inhibited the amounts of these Th2 cytokine in NH cells (Fig. 2D), indicating that SB203580 suppressed NH cell production of IL-33–induced Th2 cytokines rather than the secretion step. Because IL-2+25 also induces Th2 cytokine production and cell expansion of NH cells (1), we examined the effect of p38 inhibitor upon IL-2+25 stimulation. In this case, too, SB203580 significantly inhibited IL-2+25–induced Th2 cytokine production and cell proliferation of NH cells in a dose-dependent manner without affecting cell viability (Fig. 2E, 2F).

**GATA3 regulates IL-5 and IL-13 expression in NH cells**

Although recent studies have shown that GATA3 is critical for cytokine production by NH cells (6, 19, 20), mechanisms how GATA3 is involved in the cytokine expression of NH cells have

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**FIGURE 1.** NH cells produce IL-5, IL-6, and IL-13 upon stimulation by IL-33 in a different manner from BMMCs. (A) Five thousand mesenteric NH cells or BMMCs were stimulated with IL-33 (10 ng/ml) for 96 h. The amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production per 1 × 10^3 cells. Error bars show SEM (n = 2–3). *p < 0.05. (B and C) Five thousand mesenteric NH cells from Kit^w/w^ or Kit^w/Wv^ mice were stimulated with IL-33 (10 ng/ml) for 96 h. (B) Cell numbers and viability at the end of culture are shown. (C) Amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production per 1 × 10^3 cells. Error bars show SEM (n = 3). (D) Five thousand mesenteric NH cells or 100,000 BMMCs were stimulated with SCF (50 ng/ml), IL-33 (10 ng/ml), or SCF plus IL-33 for 96 h. The amounts of IL-6 in the supernatants were detected by ELISA. Error bars show SEM (n = 3). *p < 0.05. (E) Mesenteric NH cells or BMMCs were stimulated by IL-33 (10 ng/ml) and supernatants harvested at the indicated times. The amounts of IL-5, IL-6, and IL-13 were detected by ELISA. Error bars show SEM (n = 3). *p < 0.05. All results are representatives of two or three independent experiments with similar results.
been obscure. As reported previously (1), mesenteric naive NH cell express high amounts of GATA3 in the nucleus whose levels were similar to those in differentiated Th2 cells (Fig. 3A). IL-33 stimulation induced the phosphorylation of GATA3 and enhanced GATA3 expression (Fig. 3B). SB203580 inhibited IL-33–induced phosphorylation of GATA3 in the nuclei of NH cells (Fig. 3C), whereas JNK and NF-κB inhibitors showed little effect (Fig. 3D). These data collectively indicate that IL-33 activates the p38-mediated phosphorylation of GATA3 in NH cells. IL-25 is also known to activate TRAF6, TAK1, and p38 (29). In fact, stimulation of NH cells by IL-2+25 induced the activation of p38 and phosphorylation of GATA3 (Fig. 3E). Furthermore, although phosphorylation of p38 and GATA3 in the nucleus of NH cell was observed even 96 h after IL2+25 or IL-33 stimulation, phosphorylation of p38 and GATA3 peaked at 30 min after stimulation (Fig. 3E), suggesting that the early phase of signal transduction is important for Th2 cytokine production in NH cells. Phosphorylation of GATA3 was induced in the order of IL-33, IL2+25, and IL-2, all of which were significantly inhibited by SB203580 (Fig. 3F).

We next determined the role of GATA3 in IL-33–induced Th2 cytokine production using NH cells from Cre-Ert2:Gata3flx/flx

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**FIGURE 2.** p38 MAPK is important for cytokine production in NH cells. (A) Five thousand mesenteric NH cells were pretreated with DMSO (0.1%), SB203580 (10 μM), SP600125 (3 μM), or BAY11-7082 (100 nM) for 1 h prior to IL-33 (10 ng/ml) stimulation for 96 h. Amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production per 1 × 10^5 cells. (B) Cell numbers and viability at the end of culture are also shown. Error bars show SEM (n = 3). *p < 0.05. (C) Mesenteric NH cells were pretreated as in (A) and stimulated IL-33 (10 ng/ml) for 24 h. Cells were incubated with Brefeldin A for the last 3 h of IL-33 treatment. IL-5, IL-6, and IL-13 expression levels were detected by intracellular cytokine staining. Numbers indicate the percentage of each population within the gate. (D) Five thousand mesenteric NH cells were pretreated with DMSO (0.1%) or SB203580 (10 μM) for 1 h before IL-2+25 (10 ng/ml each) or IL-33 (10 ng/ml) stimulation for 96 h. Amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production per 1 × 10^5 cells. (E) Cell numbers and viability at the end of culture are also shown. Error bars show SEM (n = 3). *p < 0.05. All results are representatives of two or three independent experiments with similar results.

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**FIGURE 3.** IL-33–induced GATA3 phosphorylation by p38 MAPK in NH cells. (A) Total RNA was extracted from the indicated cells, and Gata3 mRNA levels were detected by real-time PCR (upper panel). Error bars show SEM (n = 3). *p < 0.05. Nuclear fractions were prepared from IL-33–stimulated BMDCs and mesenteric NH cells, as well as Th2 cells and GATA3 levels examined by Western blotting (lower panel). (B) Purified mesenteric NH cells and BMDCs were stimulated with IL-33 for the indicated periods. Phosphorylation and expression levels of GATA3 in nuclear extracts were detected by immunoblot analysis. (C and D) NH cells were pretreated with DMSO (0.1%) or SB203580 (10 μM) (C), or DMSO (0.1%), SB203580 (10 μM), or BAY11-7082 (100 nM) (D) prior to IL-33 (10 ng/ml) stimulation for 1 h. Phosphorylation and expression levels of GATA3 in cytoplasmic and nuclear extracts were detected by immunoblot analysis. (E) NH cells were stimulated with IL-2+25 (10 ng/ml each) or IL-33 (10 ng/ml) for the indicated periods. Phosphorylation and expression levels of p38 and GATA3 in nuclear extracts were detected by immunoblot analysis. We used anti-pGATA3 (phosphor-S308) Ab purchased from Abcam (Cat No. ab61052). (F) NH cells were pretreated with DMSO (0.1%) or SB203580 (10 μM) prior to IL-2 (10 ng/ml), IL-25 (10 ng/ml), IL-2+25 (10 ng/ml each), or IL-33 (10 ng/ml) stimulation for 30 min. Phosphorylation and expression levels of GATA3 in nuclear extracts were detected by immunoblot analysis. All results are representative of two or three independent experiments with similar results.
mice. NH cells derived from WT and control Cre-Ert2:Gata3+/+ mice with or without 4-OHT treatment produced large amounts of IL-5, IL-6, and IL-13 in response to IL-33 (Fig. 4A). In contrast, upon 4-OHT treatment, NH cells derived from Cre-Ert2:Gata3+Cre-Ert2:Gata3 mice produced significantly less IL-5 and IL-13 upon IL-33 induction, whereas IL-6 production was unaffected (Fig. 4A). Production of IL-5 and IL-13 in response to a combination of either IL-2 and IL-25 or PMA and ionomycin was also impaired upon deletion of GATA3 (Fig. 4B). Intriguingly, at the same time, deletion of IL-25 or PMA and ionomycin was also impaired upon deletion of GATA3 (Fig. 4B). These results demonstrate that in IL-33–stimulated NH cells, GATA3 activates Il5 and Cgre-containing loci by inducing histone H3 acetylation. In addition, GATA3 binds to the Il5 promoter and the Cgre site of the IL-13 promoter in a manner dependent on p38.

GATA3 and RORαs is critical for the differentiation of NH cells

It was recently reported that GATA3 is critical for the differentiation of NH cells by deleting Gata3 in Id2+ cell lineage (6). NH cells are derived from lymphoid progenitor cells within the hematopoietic stem cell (HSC) population (32). To investigate the roles of GATA3 in mesenteric NH cell differentiation from HSCs, we examined the effect of GATA3 deletion using 4-OHT treat-

FIGURE 4. IL-33–induced p38-mediated GATA3 phosphorylation is critical for IL-5 and IL-13 but not IL-6 production in NH cells. (A) Mesenteric NH cells (5 × 10^3 cells) isolated from WT, Cre-Ert2:Gata3+/+, or Cre-Ert2:Gata3+Cre-Ert2:Gata3 mice were cultured in media containing IL-2 (10 ng/ml) with EtOH (0.01%) or 4-OHT (100 nM) for 48 h. After deleting Gata3, cells were stimulated with IL-33 (10 ng/ml) for 96 h. Amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production by 1 × 10^3 cells. Error bars show SEM (n = 3). *p < 0.05. (B and C) After deleting Gata3 as in (A), cells were cultured with IL-7 (10 ng/ml), IL-2 (10 ng/ml), IL-2 and IL-25 (10 ng/ml each), IL-33 (10 ng/ml), or PMA (30 ng/ml) plus ionomycin (500 ng/ml) for 96 h. (B) Amounts of IL-5, IL-6, and IL-13 in the supernatants were detected by ELISA and are presented as picograms of production by 1 × 10^3 cells. (C) Cell numbers and viability at the end of culture are shown. Error bars show SEM (n = 2–3). *p < 0.05. (D and E) WT mesenteric NH cells were pretreated with DMSO (0.1%) or SB203580 (10 μM) for 1 h prior to IL-33 (10 ng/ml) stimulation for the indicated times. ChIP analysis for the acetylation of histone H3 at Lys9 and Lys14 (H3K9ac-H3K14ac) (D) or GATA3 (E) binding to the Il5 and Il13 loci were detected by quantitative real-time PCR. Results are presented as relative enrichment compared with input DNA prepared from untreated cells. Error bars show SEM (n = 3–4). All results are representative of two or three independent experiments with similar results. *p < 0.05.
ment of Cre-Ert2:Gata3\textsuperscript{flox/flox} and Cre-Ert2:Gata3\textsuperscript{3/4} mice. Although Cre recombinase expression sometimes results in cellular toxicity (33–35), no significant differences between lymphocyte populations of Cre-Ert2:Gata3\textsuperscript{flox/flox} and Cre-Ert2:Gata3\textsuperscript{3/4} mice were observed after administration of 40 \( \mu \)g/g body weight 4-OHT (data not shown). BMcs from Cre-Ert2:Gata3\textsuperscript{flox/flox} or Cre-Ert2:Gata3\textsuperscript{3/4} mice were transplanted into \( \gamma_c^-\) Rag2\textsuperscript{2/2} mice lacking NH cells. We then injected 4-OHT and analyzed mesenteric as well as BMcs 1 month after 4-OHT treatment. Lin\textsuperscript{+}Thy1.2\textsuperscript{high}T1/ST2\textsuperscript{+}CD25\textsuperscript{+} NH cells were readily observed in mesentery of \( \gamma_c^-\) Rag2\textsuperscript{2/2} mice transplanted with BMcs from Cre-Ert2:Gata3\textsuperscript{flox/flox} mice, but were absent in 4-OHT-treated \( \gamma_c^-\) Rag2\textsuperscript{2/2} mice transplanted with BMcs from Cre-Ert2:Gata3\textsuperscript{flox/flox} mice (Fig. 5A). NH cells were present in 4-OHT-treated \( \gamma_c^-\) Rag2\textsuperscript{2/2} mice transplanted with BMcs from Cre-Ert2:Gata3\textsuperscript{3/4} mice. NH cells are also present in the BM (3, 6, 36), and the differentiation of NH cells in the BM was also impaired in the absence of GATA3 (Fig. 5A). Essentially the same results were obtained by transplantation of hematopoietic stem cell fraction (data not shown). Similar results were also obtained when we transplanted BMcs into Rag2\textsuperscript{2/2} mice expressing a congenic marker (Fig. 5B), indicating that the defect of NH cell differentiation is cell intrinsic and the effect of other \( \gamma_c^-\)-dependent cells is minimal. Deletion of GATA3 (Fig. 5C) or administration of 4-OHT into Cre-Ert2:Gata3\textsuperscript{flox/flox} mice (data not shown) had no effect on ROR\gamma\textsuperscript{+} LTi-related populations in the gut.

In contrast to the deletion of GATA3, deletion of E4bp4 encoding E4BP4 or NF-IL3, a transcription factor critical for NK cell differentiation (37, 38), or Cgre encoding a GATA3-binding site within the Il13 promoter (25), did not affect NH cell differentiation (Supplemental Fig. 4B). NH cells express a high level of ROR\alpha (1, 3, 21), which is important for the differentiation of NH cells in the lung and small and large intestines (3, 21). We examined the role of ROR\alpha on mesenteric NH cell differentiation using Rora\textsuperscript{sg/sg} (staggerer, sg) mice carrying a mutation in the Rora locus. We transplanted BMcs of Rora\textsuperscript{sg/sg} mice into \( \gamma_c^-\) Rag2\textsuperscript{2/2} and Rag2\textsuperscript{2/2} mice. The differentiation of NH cells in both the mesentery and BM was impaired in \( \gamma_c^-\) Rag2\textsuperscript{2/2} and Rag2\textsuperscript{2/2} mice (Fig. 6A, 6B) without affecting the differentiation of ROR\gamma\textsuperscript{+} LTi-related populations in the gut (Fig. 6C).

RORA is dispensable for Th2 cytokine production in NH cells

We next examined the effect of GATA3 deletion in the steady state to clarify the role of GATA3 in the maintenance of NH cells. When we administered 4-OHT into Cre-Ert2:Gata3\textsuperscript{flox/flox} mice, the number of NH cells in the mesentery or BM were significantly decreased and the expression levels of T1/ST2, and IL-2R\alpha (CD25) on NH cells were greatly reduced (Fig. 7A). Deletion of GATA3 in purified NH cells by treatment with 4-OHT also downregulated T1/ST2 and CD25, but the expression level of ROR\alpha and IL-7R\alpha (CD127) was unaffected (Fig. 7A–C, data not shown). These results collectively indicate that GATA3 positively regulates the expression of IL-33 and IL-2 receptors in NH cells and is important in the maintenance of NH cells in vivo.

Although RORA is important for the differentiation of NH cells (Fig. 6 and Refs. 3, 21), Rora\textsuperscript{sg/sg} mice have a significant number of NH cells albeit in reduced numbers (Fig. 7D). Mesenteric NH cells isolated from Rora\textsuperscript{sg/sg} mice expressed T1/ST2 and GATA3 at the same levels to control WT-derived NH cells (Fig. 7E). Furthermore, we observed that isolated Rora\textsuperscript{sg/sg} NH cells normally responded to IL-33 to proliferate and to produce Th2 cytokines as WT NH cells (Fig. 7F, 7G), indicating that GATA3 and ROR\alpha independently function in NH cells and that ROR\alpha is dispensable for the function of mature NH cells.

**Discussion**

Lin\textsuperscript{+}IL-2R\alpha\textsuperscript{IL-7R}IL-3R\textsuperscript{IL-25R}GATA3\textsuperscript{+} NH cells were originally discovered in lymphoid cluster located along the blood vessels in the mouse and human adipose tissues, such as mes-
entry named “fat-associated lymphoid clusters” or FALCs (1).

Upon stimulation with IL-33, NH cells produce large amounts of IL-5 and IL-13, and the amounts produced by 5000 cells for 5 d reach microgram quantities (1). NH cells were also found in the lung and gut (3, 4). In human, CRTH2+ NH cells expressing high levels of GATA3 were found in nasal polyps (39). These cells are classified as a member of group 2 innate lymphoid cells (40).

NH cells express high levels of GATA3 and RORγ (1, 3, 6, 19–21), and the importance of those transcription factors for the differentiation of NH cells has been reported (3, 6, 19–21). We confirmed that both GATA3 and RORγ have critical roles in the differentiation of NH cells from HSCs in vivo using transplantation of BMCs (Figs. 5, 6). Contrary to NH cells, development of intestinal RORγ+ LTi-related population, a member of group 3 innate lymphoid cells (40), was unaffected by the deletion of GATA3 or RORγ. Id2 is an essential transcription factor for the differentiation of all innate lymphoid cells (40, 41). It has been reported that the expression of both Id2 and GATA3 is controlled by E4BP4 in NK cells (37) and that NK cell differentiation is dependent on E4bp4 (37, 38). As demonstrated in this study, the differentiation of NH cells was intact in the absence of E4bp4, suggesting differences in the regulatory mechanisms of Id2 and GATA3 expression between NK cells and NH cells.

NH cells are well known to produce large amounts of Th2 cytokine, such as IL-5, IL-6, and IL-13 upon IL-33 or IL-25 stimulation, and they have important roles in anti-helminth immunity (1, 5). Helminth infection resulted in the production of IL-33 in the body fluid that activates NH cells to produce IL-5 and IL-13, which leads to eosinophilia and goblet cell hyperplasia that are critical for anti-helminth immunity (1, 5, 42). NH cells are thus an important population governing the innate immune response against helminth. In addition to anti-helminth immunity, NH cells are involved in allergic inflammation associated with eosinophilia and goblet cell hyperplasia that are induced by IL-5 and IL-13 produced by NH cells, respectively (2, 4, 39–41, 43).

The mechanisms of signal transduction for the cytokine expression have been elusive. Both IL-33 and IL-25 activate NF-κB and MAPK pathways through MyD88, IRAK, TRAF6, and TAK1 (13, 14, 29). We demonstrated that IL-33 stimulation induced strong and sustained activation of NF-κB and MAPK pathways in NH cells compared with BMMCs by IL-33. IRAK1 induces a negative feedback loop downstream of TLRs (44, 45), and complete degradation of this adapter molecular was not observed in NH cells. In addition, IL-33 strongly enhanced the expression of Th2-related molecules such as GATA3, Gfi-1, c-Maf, and NFATc1. Sustained expression of these molecules could be the reasons for the strong and sustained activation of cytokine production in NH cells. In T cells, TCR-stimulated phosphorylation of GATA3 by p38 is critical for the nuclear translocation of GATA3 and enhanced Th2 cytokine production (46, 47). Interestingly, NH cells expressed GATA3 protein in the nucleus without stimulation, and IL-33 strongly increased GATA3 binding to the Il5 promoter and the Cgre site of the Il13 promoter in a manner dependent on phosphorylation by p38. Although the production of IL-5, IL-6, and IL-13 was accelerated 48 h after IL-33 stimulation, our data indicate that the early phase rather than late phase of IL-33–mediated p38 signal transduction is important for Th2 cytokine production, because the inhibition of p38 at later
time points was less effective. Consistent with these data, the phosphorylation of GATA3 peaked at 30 min to 6 h after IL-33 stimulation. Our data also indicate that IL-2+25-induced Th2 cytokine production was also regulated by GATA3 in a p38-dependent manner. It should be determined in future experiments whether phosphorylation of GATA3 by p38 directly regulates the binding of GATA3 to the promoters of IL5 and IL13 and the expression of IL-5 and IL-13 in NH cells. It should be noted that GATA3 deletion in mature NH cells impaired the production of IL-5 and IL-13 upon IL2+25, IL-33, or PMA plus ionomycin induction, whereas IL-6 production was unaffected, indicating that IL-6 expression is dependent on p38 but independent of GATA3. It has been shown that deletion of GATA3 in IL-13 expressing cells in worm-infected mice impaired Th2 cytokine expression in innate helper type 2 cells (19). Our results indicate the importance of GATA3 in regulating the expression of IL-5 and IL-13 in naive NH cells.

Surprisingly, deletion of GATA3 in cultured NH cells resulted in growth retardation of NH cells in response to IL-33 and IL-2+IL-25 without affecting cellular viability, although responses to IL-2 and IL-7 were not significantly affected. Because IL-6 production in response to IL-33 or IL-2+IL-25 was not affected by GATA3 deletion, impaired production of IL-5 and IL-13 in the absence of GATA3 is unlikely due to the growth retardation. GATA3 deletion in mature NH cells in vivo also significantly decreased NH cells in the mesentery and BM, which is consistent with a recent report regarding lamina propria NH cells (6), collectively indicating that GATA3 is important in the maintenance of NH cells in vivo. GATA3 deletion caused downregulation of T1/ST2 and CD25 on mature NH cells (Fig. 7 and Ref. 6) and overexpression of GATA3 induced T1/ST2 expression in human NH cells (20). Because NH cell differentiation is not impaired in IL-2−/−, IL-2Rb−/−, IL-25−/−, IL-33−/−, or T1ST2−/− mice (Ref. 1 and data not shown), the reduction of NH cells after deletion of GATA3 is unlikely due to the reduction of T1ST2 or IL-2R expression. On the other hand, IL-7 is critical for the differentiation and maintenance of NH cells (1), but the expression level of IL-7 receptor is unaffected by the deletion of GATA3. It is unknown how GATA3 regulates the binding of GATA3 to the promoters of RORa and RORc receptors as T1/ST2 and CD25 expression levels of NH cells from GATA3−/− mice were equal to those of WT mice. In addition, isolated NH cells from Rora−/− mice were not affected in terms of proliferation and Th2 cytokine production, suggesting that RORa is dispensable for the function of mature NH cells.

The present results demonstrate the critical role of p38 and GATA3 in IL-5 and IL-13 expression in NH cells. GATA3 is also critical for the differentiation, maintenance, and proliferation of NH cells. The lack of RORa impairs the differentiation but not cytokine production and proliferation of NH cells, suggesting that RORa mainly plays a role in the differentiation process of NH cells but is dispensable for the function of mature NH cells. It is important in future studies to clarify how GATA3 and RORa regulate differentiation or maintenance of NH cells. GATA3 has been known to associate with allergic diseases such as asthma (48–50). As NH cell-derived IL-5 and IL-13 mediate eosinophilia and goblet cell hyperplasia, respectively, which are important for pathophysiology of asthma, GATA3 in NH cells could be a good target for new strategy to treatment asthmatic disease.

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Disclosures
S.K. is a consultant for Medical and Biological Laboratories, Co. Ltd. The other authors have no financial conflicts of interest.

References


Corrections


In Fig. 3D, there was a splicing between “Cytosol” and “Nuclear” samples by removing one lane of the same Western blot, but we did not add a line to indicate the splicing. The corrected Fig. 3D is shown below. The figure legend was correct as published.

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Supplemental Figure 1. The gating strategy of NH cells. Mesenteric (upper) and BM (lower) NH cells were identified as Lin\(^{−}\)Thy1.2\(^{+}\)T1/ST2\(^{+}\)CD25\(^{+}\) lymphocytes. Mesenteric NH cells express cKit at a higher level and IL-7R\(\alpha\) at a lower level than BM NH cells.
Supplemental Figure 2. Signal transduction downstream of IL-33 receptor in NH cells.

(A) Phosphorylation of NF-kB p65, JNK and p38 in cytoplasmic and nuclear extracts of mesenteric NH cells or BMMC was examined by western blotting. (B) Phosphorylation of NF-kB p65, c-Jun and ATF2 in nuclear extracts was examined by western blotting. (C) Mesenteric NH cells and BMMC were stimulated with IL-33 (10 ng/ml) for the indicated time periods. Expression levels of MyD88, IRAK1, TRAF6 and TAK1 in the cytoplasmic fraction were examined by western blotting. (D) Mesenteric NH cells were stimulated with IL-33 (10 ng/ml) for the indicated time periods. Nuclear extracts were immunoblotted with anti-Gfi1, anti-c-Maf, anti-Ets1, anti-NFAT or Lamin B antibodies. All results are representatives of three independent experiments with similar results.
Supplemental Figure 3. Early phase of p38 signal is important for IL-33 induced Th2 cytokine production in NH cells.

(A) Five thousand mesenteric NH cells were pretreated with DMSO (0.1%), SB203580 (3-30 μM), SP600125 (1-10 μM) or BAY11-7082 (10-1000 nM) for 1 hr prior to IL-2-25 (10 ng/ml each) or IL-33 (10 ng/ml) stimulation for 96 hrs. (B) Five thousand NH cells were treated with DMSO (0.1%) or SB203580 (10 μM) at the indicated time point during 96 hrs of IL-33 (10 ng/ml) stimulation. (A, B) The amounts of IL-5, IL-6 and IL-13 in the supernatants were detected by ELISA and are presented as pg production per 1 x 10^3 cells. Error bars show s.e.m. (n = 3). *, P < 0.05. All results are representatives of two independent experiments with similar results.
Supplemental Figure 4. Role of E4BP4 and CGRE for cytokine production and differentiation of NH cells.

(A) Mesenteric NH cells from WT, E4bp4−/− or Cgre−/− mice were stimulated by IL-33 (10 ng/ml) for 6 days. IL-5 and IL-13 production in the culture supernatants were detected by ELISA. Error bars show s.e.m. (n = 3). *, P < 0.05. (B) Mesenteric NH cells in WT, E4bp4−/− or Cgre−/− mice were analyzed by flow cytometry. Numbers in dot plots are the percentage of each gated cell population. Graphs indicate the numbers of NH cells per 1 x 10⁵ lymphocytes.