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J Immunol 2013; 191:1818-1826; Prepublished online 12 July 2013;
doi: 10.4049/jimmunol.1300379
http://www.jimmunol.org/content/191/4/1818

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/07/12/jimmunol.1300379.DC1

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Critical Role of p38 and GATA3 in Natural Helper Cell Function

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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 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 activates NH cells and have important roles in 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, TRAF6, and 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). ROXes 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 ROXes in IL-33 signaling have been obscure.

In this study, we demonstrated 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 ROXes 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, WBB6F1-Ki–/– and WBB6F1-Ki+/– mice were obtained from Japan SLC (Tokyo, Japan). B6.SJL-Rag2–/– and Thy1.2–/– mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Cre-Ert2:Gata3flox/flox mice were generated previously (23) and were crossed with Cre-Ert2 transgenic mice and Rosa26R mice were obtained from The Jackson Laboratory. Gata3flox/flox mice were previously (23) and were crossed with Cre-Ert2 transgenic mice to generate Cre-Ert2:Gata3flox/flox mice. Conserved GATA3 response element (Cpgr)–/– mice (25) and E4bp4–/– mice (26) were reported previously. All experiments were approved by the Animal Care and Use Committee of Keio University and RIKEN and were performed in accordance with the institutional guidelines.

Abs and reagents

Abs were purchased from BD Pharmingen (San Diego, CA), ebioscience (La Jolla, CA), MD Biosciences (St. Paul, MN), BioLegend (San Diego, 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), hIL-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 Sigma-Aldrich (St. Louis, MO) containing 10 ng/ml hIL-2, 10% FCS, 50 M M2-ME (Life Technologies), 100 U/ml penicillin, and 100 M gentamicin (Life Technologies), 50 M gentamicin (Nacalai, Kyoto, Japan), 1 nonessential amino acids (Sigma-Aldrich), 10 M HEPES (Sigma-Aldrich), and 1 mM sodium pyruvate (Life Technologies).

Measurement of cytokines

Sorted NH cells were seeded at 5 × 103 cells/well into 96-well round-bottom tissue culture plates with various stimulators 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 between 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 × 106 cells.

Flow cytometry

Flow cytometry was performed on a FACSariaII (BD Bioscience, 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 SDS-PAGE, 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 5 s, 60°C for 5 s for 40 cycles, and 98°C for 10 s. Primer pair sequences, specific for Gata3 were (forward, 5′-AGAACCGGCCCCTTATGAA-3′, reverse, 5′-AGTTCGGAGGATGTCC-3′), those for Rora were (forward, 5′-TTACGTGTTGAAGGCTGCAAG-3′, reverse, 5′-GGAATGTGCTGGTCTGCT-3′), and those for 18s rRNA were (forward, 5′-GCCGCTAGAGGAAATTTCT-3′, reverse, 5′-CGAAAGTCGCTACTTGTCT-3′).

Bone marrow transplantation

Whole bone marrow cells (BMCs) were transferred into sublethally irradiated (2 Gy) γ−/−, Rag2–/– or B6.SJL-Rag2–/– mice. Cre-mediated recombination was induced on day 1–3. Mesenteric Lin Thy1.2+/+CD25+ T1/ST2+c-Ki– or bone marrow (BM) Lin−/CD25+T1/ST2+IL-7Rα+ NH cells were analyzed by flow cytometry after indicated time periods. In the case of transfer into B6.SJL-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 nM 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: Il5 (forward, 5′-TCCGCTTTATAGGTGTCCTC-3′, reverse, 5′-GGGCTTCAGCAAAAGAAGAG-3′), Cgfr (forward, 5′-GTCCTCTTTAGCACCCCTTCT-3′, reverse, 5′-AAGGGTGGGAGGAAACAC-3′). Results are presented as the ratio of the cycling threshold value of immunoprecipitated DNA to that of input DNA (2CTinput−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
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-κB p65, JNK, and p38 MAPK in the cytosol of both BMMCs and NH cells (Supplemental Fig. 2A). Intriguingly, NH cells have phosphorylated NF-κB p65, JNK, and p38 MAPK in the nucleus even before stimulation. IL-33 induced biphasic phosphorylation of NF-κB 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-κB 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 is expressed in TH2 cells, IL-2 production by NH cells was measured. PHA-stimulated proliferation (Fig. 1B) and cytokine production (Fig. 1C) of IL-33–stimulated NH cells were significantly suppressed by SB203580 (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...
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 IL-2+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:Gata3flox/flox mice.

**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 cultured with IL-2 (10 ng/ml) or IL-33 (10 ng/ml) for the indicated time periods and examined the expression levels of T1/ST2. (D) 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. (E) Five thousand mesenteric NH cells were pretreated with DMSO (0.1%) or SB203580 (3–30 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. (F) 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.

**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 BMMCs and mesenteric NH cells, as well as Th2 cells and GATA3 levels examined by Western blotting (lower panel). (B) Purified mesenteric NH cells and BMMCs were stimulated with IL-33 (10 ng/ml) 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), SP600125 (3 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:Gata3flox/flox 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). We next investigated whether GATA3 directly binds to these loci. IL-33 strongly increased GATA3 binding to the promoter of the Il5 locus and the Cgre site of the Il13 locus, and this GATA3 binding was strongly suppressed by SB203580 (Fig. 4E). 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 × 10^5 cells) isolated from WT, Cre-Ert2:Gata3+/+, or Cre-Ert2:Gata3flox/flox 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{+/+} 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{+/+} 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{+/+} mice were transplanted into \(\gamma_c^-\text{Rag2}^-\) mice lacking NH cells. We then injected 4-OHT and analyzed mesenteric as well as BMCs 1 month after 4-OHT treatment. Lin\^-Thy1.2\textsuperscript{+/+}T1/ST2\^-CD25\^+ NH cells were readily observed in mesentry of \(\gamma_c^-\text{Rag2}^-\) mice transplanted with BMCs from Cre-Ert2:Gata3\textsuperscript{flox/flox} mice, but were absent in 4-OHT-treated \(\gamma_c^-\text{Rag2}^-\) mice transplanted with BMCs from Cre-Ert2:Gata3\textsuperscript{+/+} mice (Fig. 5A). NH cells were present in 4-OHT-treated \(\gamma_c^-\text{Rag2}^-\) mice transplanted with BMCs from Cre-Ert2:Gata3\textsuperscript{+/+} 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 \(\text{Rag2}^-\) 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{+/+} mice (data not shown) had no effect on ROR\^\gamma-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\^\gamma (Fig. 6A–C, data not shown) and ROR\^\gamma 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{+/+} mice, the number of NH cells in the mesentery or BM were significantly decreased and the expression levels of T1/ST2, and IL-2R\^-a\^-a\^- (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\^\gamma and IL-7R\^-a\^- (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 ROR\^\gamma is important for the differentiation of NH cells (Fig. 6 and Refs. 3, 21), Rora\^-a\^- mice have a significant number of NH cells albeit in reduced numbers (Fig. 7D). Mesenteric NH cells isolated from Rora\^-a\^- mice expressed T1/ST2 and GATA3 at the same levels to control WT-derived NH cells (Fig. 7E). Furthermore, we observed that isolated Rora\^-a\^- 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\^\gamma independently function in NH cells and that ROR\^\gamma is dispensable for the function of mature NH cells.

**Discussion**

Lin\^-IL-2\^-IL-7\^-IL-25\^-IL-33\^-GATA3\^+ NH cells were originally discovered in lymphoid cluster located along the blood vessels in the mouse and human adipose tissues, such as mesenchyme.
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 RORγLTi-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 IL-2+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-2Rβ−/−, IL-25−/−, IL-33−/−, or T1/ST2−/− mice (Ref. 1 and data not shown), the reduction of NH cells after deletion of GATA3 is unlikely due to the reduction of T1/ST2 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 controls the maintenance or survival of NH cells. Contrary to GATA3, RORα is not involved in the expression of IL-2 or IL-33 receptors as T1/ST2 and CD25 expression levels of NH cells from Rora−/− mice were equal to those of WT NH cells. In addition, isolated NH cells from Rora−/− mice were not affected in terms of proliferation and Th2 cytokine production, suggesting that RORα 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 RORα impairs the differentiation but not cytokine production and proliferation of NH cells, suggesting that RORα 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 RORα 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.
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.