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Enforced Expression of Gata3 in T Cells and Group 2 Innate Lymphoid Cells Increases Susceptibility to Allergic Airway Inflammation in Mice

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Airway inflammation in allergic asthma reflects a threshold response of the innate immune system, including group 2 innate lymphoid cells (ILC2), followed by an adaptive Th2 cell-mediated response. Transcription factor Gata3 is essential for differentiation of both Th2 cells and ILC2. We investigated the effects of enforced Gata3 expression in T cells and ILC2 on the susceptibility of mice to allergic airway inflammation (AAI). We used CD2-Gata3 transgenic (Tg) mice with enforced Gata3 expression driven by the CD2 promoter, which is active both in T cells and during ILC2 development. CD2-Gata3 Tg mice and wild-type (WT) littermates were analyzed in mild models of AAI without adjuvants. Whereas OVA allergen exposure did not induce inflammation in WT controls, CD2-Gata3 Tg mice showed clear AAI and enhanced levels of IL-5 and IL-13 in bronchoalveolar lavage. Likewise, in house dust mite-driven asthma, CD2-Gata3 Tg mice were significantly more susceptible to AAI than WT littermates, whereby both ILC2 and Th2 cells were important cellular sources of IL-5 and IL-13 in bronchoalveolar lavage and lung tissue. Compared with WT littermates, CD2-Gata3 Tg mice contained increased numbers of ILC2, which expressed high levels of IL-33R and contributed significantly to early production of IL-4, IL-5, and IL-13. CD2-Gata3 Tg mice also had a unique population of IL-33-responsive non-B/non-T lymphoid cells expressing IFN- γ . Enforced Gata3 expression is therefore sufficient to enhance Th2 and ILC2 activity, and leads to increased susceptibility to AAI after mild exposure to inhaled harmless Ags that otherwise induce Ag tolerance. *The Journal of Immunology*, 2014, 192: 000–000.

Asthma is characterized by reversible airway obstruction, pulmonary inflammation, and bronchial hyperreactivity (1). Patients manifest symptoms of asthma in response to inhalation of airborne allergens, such as house dust mite (HDM), molds, or animal dander. Many cell types participate in allergic airway inflammation (AAI), including eosinophils, dendritic cells (DCs), B and T lymphocytes, mast cells, macrophages, epithelial cells, smooth muscle cells, and fibroblasts. Nevertheless, CD4⁺ Th2 cells, which produce a unique profile of IL-4, IL-5, IL-9, and IL-13 proinflammatory cytokines, are thought to be central in the orchestration and amplification of allergic inflammatory events. Th2 cytokines explain many hallmarks of allergic asthma: IgE synthesis (IL-4), airway eosinophilia (IL-5), mast cell accumulation (IL-9),

goblet cell hyperplasia (IL-4, IL-13), and bronchial hyperreactivity acting on bronchial smooth muscle cells (IL-13) (1).

Although adaptive Th2 cells have been identified as important sources of IL-4, IL-5, and IL-13, many studies emphasize the importance of innate cells such as mast cells, eosinophils, and basophils, in cytokine production. Most importantly, a population of non-B/non-T innate lymphoid cells (ILCs) that have the capacity to produce large amounts of IL-5 and IL-13 upon stimulation by IL-33 or IL-25 was recently identified in the intestine and the lung (2, 3). Because of their Th2 cytokine profile, they have been named group 2 ILCs (ILC2) and can be identified as lineage-negative cells expressing receptors for IL-2, IL-7, and IL-33 (4). The involvement of ILC2 in asthma was recently demonstrated in various mouse models, including HDM-driven allergic AAI (5–8). ILC2 have also been identified in human lung and are associated with chronic rhinosinusitis (9–11).

Differentiation of Th2 cells is induced by the key NF Gata3, which acts as a transcription regulator of the Th2 cytokines IL-4, IL-5, and IL-13, and concomitantly suppresses Th1 development (12–14). A suggested mechanism for this activity is that Gata3 causes chromatin remodeling in the Th2 cytokine locus, in conjunction with various other transcriptional regulators including c-maf, Irf4, SatB1, and Ctcf (12, 15, 16). Apart from its function in Th cells, Gata3 is also essential for early stages of T cell development in the thymus (12, 17). Conditional deletion of the *Gata3* gene in established Th2 cells demonstrated that Gata3 is critical for IL-5 and IL-13, but not IL-4 production (18). Deletion of the *Gata3* gene in murine ILC2 also abolished their IL-13 production (19). Moreover, studies of *Gata3*-deficient hematopoietic chimeras and conditional targeting experiments showed that

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Abbreviations used in this article: AAI, allergic airway inflammation; BAL, bronchoalveolar lavage; CLP, common lymphoid progenitor; DC, dendritic cell; HDM, house dust mite; ILC, innate lymphoid cell; i.t., intratracheal; WT, wild-type.

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the *Gata3* gene is required for ILC2 development, as well as maintenance of mature ILC2 in mice (20, 21).

To date, >100 genes have been associated with asthma genome-wide, including the Th2 locus, thymic stromal lymphopoietin, IL-33, and IL-33R/T1ST2, but typically the impact of each of these genes is mild (22–24). Interestingly, haplotype analysis of the GATA3 locus showed associations with asthma and atopy-related phenotypes (25). Nakamura et al. (26) showed that GATA3 expression is increased in lung mucosa and bronchoalveolar lavage (BAL) cells of asthmatic patients, compared with healthy control subjects. Moreover, Gata3 expression was increased after local allergen provocation, in the absence of inflammatory cell recruitment (27).

Mouse studies indicated that blocking Gata3 expression by knockdown strategies can reduce allergic inflammation in mouse model (13), suggesting that Gata3 is an important target for therapeutic strategies (28). Conversely, we have previously shown that enforced expression of Gata3 in T cells under the control of the CD2 promoter in transgenic (Tg) mice resulted in enhanced formation of a IL-33R/T1ST2-expressing Th2-committed T cell compartment in vivo (29), consistent with direct regulation of *ST2* gene expression by Gata3 (30). These CD2-Gata3 Tg mice lacked Th1-mediated hypersensitivity responses and showed reduced differentiation or activity of the Th17 cell subset, and protection from autoimmune encephalomyelitis and rheumatoid arthritis (29, 31, 32). Overexpression of Gata3 in T cells has been associated with enhanced Th2 responses and eosinophilic airway inflammation in vivo (33–36). However, effects of Gata3 overexpression on the ILC2 population in AAI remain unknown.

We recently found that CD2-Gata3 Tg mice contained increased numbers of ILC2, which might be explained by increased Gata3 levels in ILC2 precursors (21). Importantly, we found increased Gata3 expression in CD2-Gata3 Tg common lymphoid progenitor (CLP) cells, which showed an increased capacity to develop into ILC2 in vitro (21). Together with the finding that in mice with one targeted Gata3 allele, the numbers of ILC2 were reduced, we concluded that Gata3 is essential for the development of ILC2 from CLP cells in a dose-dependent fashion (21). However, it remains unknown whether also the function of Gata3 in mature Th2 cells or ILC2 is dose dependent.

In this study, we investigated the effects of Gata3 overexpression on Th2 cytokine production by T cells and ILC2, and the effects on the susceptibility of mice to develop AAI. In particular, we found that enforced Gata3 expression enhanced eosinophilia in acute models of AAI, and increased both Th2 and ILC2 involvement.

Materials and Methods

Mouse and genotyping

CD2-Gata3 Tg mice (29) mice were backcrossed on the C57BL/6 background for >10 generations and genotyped by PCR (5'-CAG CTC TGG ACT CTT CCC AC-3' and 5'- GTT CAC ACA CTC CCT GCC TT-3'). Animals were kept under specific pathogen-free conditions, provided with water and food ad libitum, and were used at the age of 6–11 wk. All experiments were approved by the Erasmus MC Animal Ethics Committee.

OVA and HDM AAI model

To induce airway inflammation, we anesthetized mice with isoflurane and treated them intratracheally (i.t.) with 80 μ l OVA (Worthington) solution (10 mg/ml in PBS; Life Technologies) or PBS as a control on day 0. Ten days later, all mice were exposed to OVA (Sigma OVA type III) aerosols 10 mg/ml during 30 min for 3 consecutive days. In some experiments, HDM (Greer Laboratories) extract was used as an allergen: on day 0, isoflurane-anesthetized mice were sensitized i.t. using 10 μ g HDM extract in 80 μ l PBS or using PBS only. Ten days later, anesthetized mice were challenged with 10 μ g HDM in 50 μ l PBS intranasally for 3 consecutive days. Twenty-four hours after the last challenge, mice were sacrificed and BAL was performed by flushing the lungs three times with 1 ml PBS containing

EDTA (Sigma-Aldrich). Lungs were collected and digested using collagenase I or IV (Life Technologies) containing DNaseI (Life Technologies) for FACS analysis and partly snap-frozen or inflated with PBS/OCT (TissueTek; Sakura Finetek Europe, Zoeterwoude, the Netherlands), snap-frozen in liquid nitrogen, and stored at -80°C until further processing for histological analysis.

In some experiments, DCs were transferred i.t. to induce asthma. In brief, myeloid DCs were grown for 8 d from C57BL/6 bone marrow in the presence of rGM-CSF 20 ng/ml (kindly provided by Dr. K. Thielemans, Belgium) and subsequently pulsed with OVA or PBS. A total of 10^6 DCs was injected i.t. and subsequently challenged three times using OVA aerosols as described earlier.

IL-33 administration

For IL-33-induced airway inflammation, anesthetized mice received 0.5 μ g recombinant mouse IL-33 (BioLegend) in PBS on days 1, 3, and 4 intranasally. Twenty-four hours after the last administration, airway inflammation was investigated (7).

Flow cytometric analysis

BAL, lung, and mediastinal lymph node cells were collected for cellular differentiation by flow cytometry as previously described (37). In some experiments, a fraction of the isolated cells were stimulated with ionomycin (Sigma), PMA (Sigma), and GolgiPlug (BD Biosciences) at 37°C for 4 h. Next, cells were stained for CD3, CD4, and intracellularly for IL-4, IL-5, IL-10, IL-13, IL-17, and IFN- γ , after fixation with 2% PFA and permeabilization using a saponin-containing buffer (7, 21). Fixable Aqua Dead for 405 nm (Invitrogen, Molecular Probes) was used to distinguish live and dead cells.

For Gata3 protein detection in ILC2 and T cells, cells were stained for CD3, CD4, T1ST2, Sca1, CD25, and lineage-positive cells (PE-conjugated Abs against CD8 α , CD11b, CD11c, CD19, CD45R [B220] Ter-119, Gr-1, CD161 [NK1.1], and Fc ϵ R1 α), fixed, and permeabilized by using the Foxp3 staining kit (eBioscience) according to the manufacturer's protocol. Alexa Fluor 647-conjugated Abs against Gata3 and PeCy7-conjugated Abs against T-Bet were purchased from BD Biosciences or eBioscience. Fixable Aqua Life Dead for 405 nm (Invitrogen, Molecular Probes) was used to distinguish live and dead cells. Cells were analyzed using a LSRII Flowcytometer (BD Biosciences) and with FlowJo software (Tree Star).

Immunohistochemistry

Immunohistochemical stainings were performed in a half-automatic stainer (Sequenza) as previously described (38). Acetone-fixed slides were washed with PBS, and incubated and blocked in diluted normal goat serum (CLB, Amsterdam, the Netherlands). Sections were stained with PE-conjugated anti-Siglec-F (E50-2440; BD Pharmingen). The primary Ab was detected using an appropriate alkaline phosphatase-conjugated secondary Ab. After rinsing, slides were incubated with New Fuchsin substrate. Finally, the sections were counterstained with Gills triple strength hematoxylin and mounted in VectaMount (Vector).

Measurements of cytokine concentrations

Cytokine levels in lung homogenates and BAL fluid were determined using commercial ELISA kit for IFN- γ (BD), IL-4 and IL-5 (both eBioscience), IL-13, IL-17, and IL-33 (all R&D) according to manufacturers' protocol.

Statistical analysis

Reported values are shown as mean $\pm$ SEM. Statistical analyses were performed with SPSS (SPSS, Chicago, IL) using a Mann-Whitney *U* test. Resulting *p* values <0.05 (*), <0.01 (**), and <0.001 (***) are indicated and considered significant. Tests that did not reach significance (*p* > 0.05) are not indicated.

Results

Enforced Gata3 expression does not enhance signs of AAI in a severe asthma model

First, we compared asthma susceptibility of CD2-Gata3 Tg mice and wild-type (WT) littermates in an OVA-driven murine AAI model. Sensitization to inhaled OVA was induced by i.t. injection of bone marrow-derived DCs, using PBS-pulsed DCs as a control (39). Upon three OVA aerosol challenges at days 10–12 after sensitization, mice were analyzed at day 13 (Supplemental Fig. 1A). Whereas only a few inflammatory cells were observed in the

BAL fluid of mice that received unpulsed PBS-DC, significant eosinophilic airway inflammation developed upon OVA aerosol challenge of mice that had received OVA-pulsed DCs (Supplemental Fig. 1B). However, we observed no significant differences between CD2-Gata3 Tg and WT mice in the numbers of the individual immune cell populations present in the BAL, determined by flow cytometry (Supplemental Fig. 1B). Likewise, using intracellular FACS staining, we found no differences in the numbers of Th1, Th2, or Th17 cells, typified by IFN- γ , IL-4, or IL-17 production, respectively (Supplemental Fig. 1C). Therefore, we conclude that enforced Gata3 expression in CD2-Gata3 Tg mice

did not enhance AAI in a severe asthma model based on Th2 sensitization by OVA-pulsed DCs.

Enforced Gata3 expression induces susceptibility to AAI upon i.t. OVA sensitization

Next, we determined the susceptibility of CD2-Gata3 Tg mice to AAI in a very mild asthma model based on i.t. sensitization with OVA followed by OVA aerosol challenges (Fig. 1A). When we compared OVA-sensitized and PBS-sensitized WT mice 1 d after the last OVA aerosol challenge, we did not find evidence for eosinophilic inflammation by quantification of BAL cells by flow cytometry, or by immunohistochemical staining of the lungs for

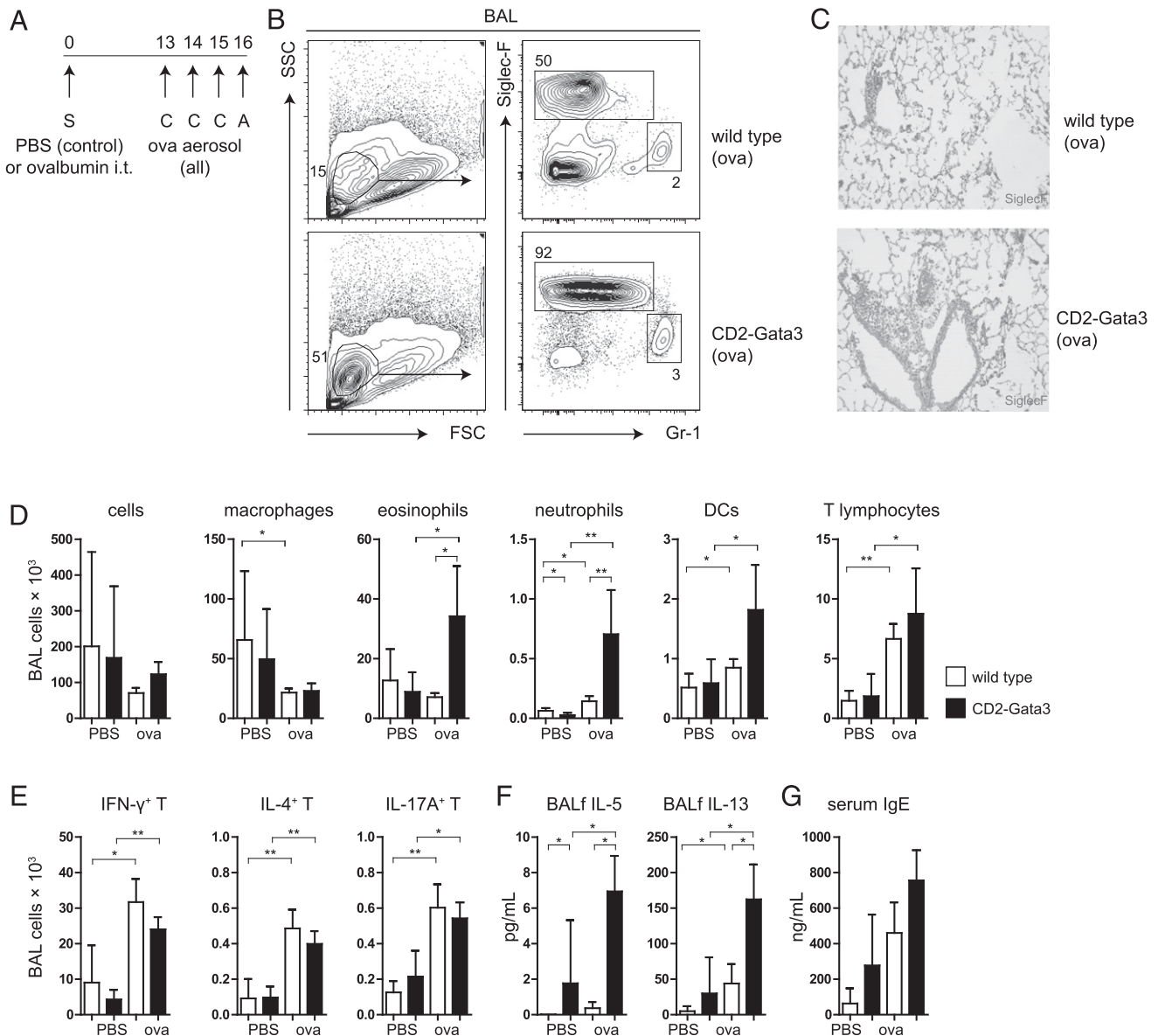


FIGURE 1. Enforced Gata3 expression induces susceptibility to AAI upon i.t. OVA sensitization. **(A)** CD2-Gata3 Tg mice and WT littermates were i.t. sensitized (s) with OVA or PBS as control sensitization followed by OVA aerosol challenges (c). One day after the last challenge, mice were sacrificed and analyzed (a). **(B)** Flow cytometric analysis of BAL showing forward and side scatter profile (FSC/SSC) and cells with eosinophil FSC/SSC characteristics were gated and analyzed for Siglec-F and Gr-1. Proportions of cells in the indicated quadrants are given (in %). **(C)** Bronchial mucosal sections were stained for murine Siglec-F, a specific marker for eosinophils. Murine eosinophils have a ring-shaped or a bilobular nucleus. WT OVA/OVA mice lack eosinophilic infiltrates (*upper panel*), and CD2-Gata3 Tg OVA/OVA mice show some peribronchial and perivascular eosinophilic infiltrates (*lower panel*). Original magnification $\times 100$. **(D)** Quantification of flow cytometric analysis of the indicated populations of leukocytes in BAL of WT (white bars) and CD2-Gata3 Tg mice (black bars). Results are expressed as means $\pm$ SEM. **(E)** Quantification of flow cytometric analysis of CD4⁺ T cells positive for the indicated cytokines upon 4 h of PMA/ionomycin stimulation. Results are expressed as means $\pm$ SEM. **(F)** IL-5 and IL-13 concentrations in BAL fluid measured by ELISA. **(G)** Total IgE in serum by ELISA. Results represent one of two independent experiments with three to six animals per group. * $p < 0.05$, ** $p < 0.01$.

Siglec-F, which is specifically expressed by eosinophils (shown for OVA-sensitized mice in Fig. 1B, 1C; PBS-sensitized mice showed similar FACS plots and immunohistochemical staining). In contrast, we identified substantial peribronchial and perivascular eosinophilic infiltrates in sections of lungs from OVA-sensitized/OVA-challenged CD2-Gata3 Tg mice (Fig. 1C). Numbers of eosinophils in BAL from OVA-sensitized CD2-Gata3 Tg mice were significantly higher than in BAL samples from OVA-sensitized WT littermates ($p = 0.009$; Fig. 1B, 1D). Moreover, quantification of BAL cell populations showed that, in contrast with our findings in WT littermates, CD2-Gata3 Tg mice manifested significantly higher numbers of eosinophils ($p = 0.03$), neutrophils, DCs, and T lymphocytes after OVA sensitization than after PBS sensitization.

We therefore conclude that enforced Gata3 expression induces susceptibility to eosinophilic AAI upon i.t. sensitization in an adjuvant-free OVA model that is associated with immune tolerance in WT control mice (40).

Enforced Gata3 expression results in increased IL-5 and IL-13 in BAL fluid during AAI upon i.t. OVA sensitization

Although we did not find evidence for eosinophilic inflammation 1 d after the last OVA aerosol challenge, we did observe an increase in the number of T cells in BAL when we compared OVA-sensitized and PBS-sensitized WT mice (Fig. 1D). Subsequently, we determined cytokine expression profiles by intracellular flow cytometry upon 4 h of stimulation by PMA/ionomycin. These analyses showed that the observed T cell increase was accompanied by an increase in the numbers of IL-4, IFN- γ , as well as IL-17A⁺ T cells, but no differences were observed between CD2-Gata3 Tg mice and WT littermates (Fig. 1E). In contrast, quantifications in BAL fluid showed significantly increased levels of IL-5 and IL-13 in CD2-Gata3 Tg OVA-sensitized mice, compared with WT OVA-sensitized mice or PBS-sensitized mice (Fig. 1F). Finally, serum levels of total IgE tended to be higher in CD2-Gata3 Tg mice than in WT littermates (Fig. 1G) upon OVA challenge in OVA-sensitized mice, but in these comparisons, no statistical significances were reached.

Taken together, these data in an adjuvant-free OVA-sensitization model demonstrate that the high susceptibility of CD2-Gata3 Tg mice to eosinophilic airway inflammation is associated with increased IL-5 and IL-13 levels in BAL fluid.

Characterization of lung T cells and ILC2 in naive CD2-GATA3 Tg mice

The increased levels of IL-5 and IL-13 in BAL fluid of CD2-Gata3 Tg mice, compared with WT littermates in the i.t. OVA sensitization model, may point to cytokine production by T cells. This would be in agreement with our previously reported findings that the presence of the CD2-Gata3 transgene enhances Th2 cytokine production, both in ex vivo analyzed T cells and in vitro activated T cells (29, 31, 32). However, because the CD2-Gata3 transgene is also expressed during ILC2 development (21), we also investigated ILC2. We quantified Gata3 protein expression by flow cytometry in CD3⁺CD4⁺ T cells, in IL-33R/T1ST2⁺ CD3⁺CD4⁺ Th2 memory cells, as well as in ILC2. The two T cell populations showed higher Gata3 levels in CD2-Gata3 Tg mice, when compared with WT littermates or C57BL/6 controls (Fig. 2A). ILC2 expressed very high Gata3 levels, whereby no differences between CD2-Gata3 Tg mice and WT littermates were detectable (Fig. 2A).

Analysis of BAL fluid cells revealed no differences (absolute numbers, or proportions of T and B cells, neutrophils, eosinophils, or macrophages) between naive CD2-Gata3 Tg mice and WT littermates (Fig. 2B and data not shown). The two groups of mice

did not differ in their numbers of total lung cells upon collagenase digestion. However, in CD2-Gata3 Tg mice, both CD4⁺ and CD8⁺ T cell numbers were significantly reduced (Fig. 2C) and the CD4⁺ T cell population contained increased proportions of IL-33R/T1ST2-expressing cells (Supplemental Fig. 2), in agreement with similar previously reported findings in spleen and lymph nodes (29). Quantification of ILC2 showed an ~2-fold increase in the proportions of ILC2 in CD2-Gata3 Tg mice, compared with WT control littermates. Also, their surface expression of IL-33R/T1/ST2 was slightly but reproducibly enhanced, whereas expression of CD25, CD127, or Sca-1 was similar in CD2-Gata3 Tg and WT ILC2 (Fig. 2D).

Consistent with previous analyses in lymph node and spleen cells (16, 29, 31), lung T cells in naive CD2-Gata3 Tg mice showed increased proportions of IL-4-, IL-5-, and IL-13-expressing cells, compared with WT littermates. Despite our inability to detect increased expression of Gata3 protein in CD2-Gata3 Tg ILC2 by intracellular flow cytometry (Fig. 2A), these cells contained increased proportions of IL-5- and IL-13-expressing cells when compared with WT ILC2 (Fig. 2E). Remarkably, naive CD2-Gata3 Tg ILC2 also contained significant numbers of cells that were positive for IL-4 and IFN- γ , cytokines that are generally not expressed by ILC2 (Fig. 2E).

In summary, these findings show that CD2-Gata3 Tg mice manifest no spontaneous eosinophilic airway inflammation, but the T cell and ILC2 populations in the lungs of these mice contain higher proportions of Th2 cytokine-positive cells than WT mice do.

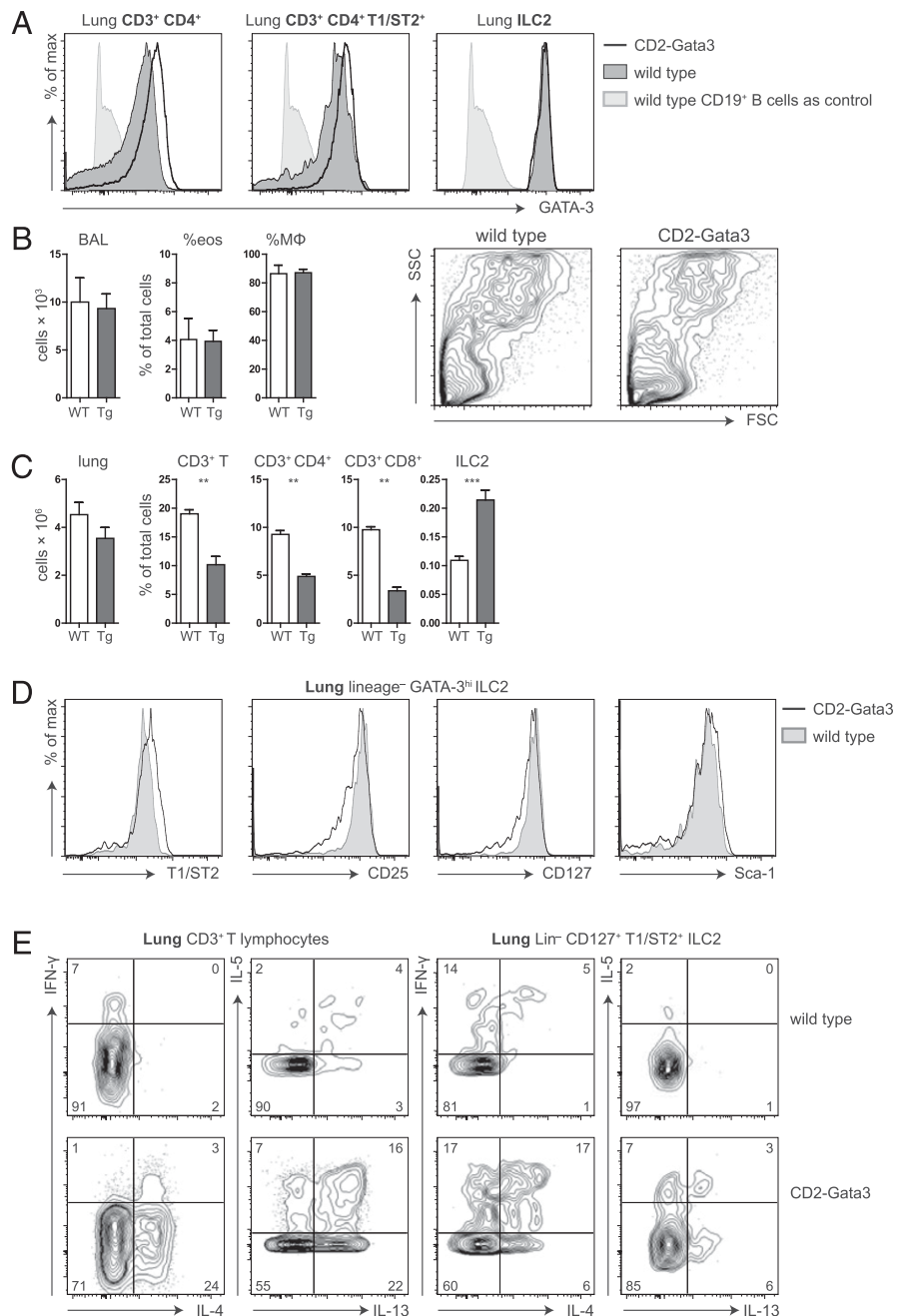
Enforced Gata3 expression enhances AAI in a mild HDM model

Next, we investigated the effects of Gata3 overexpression in an HDM-driven allergic inflammation model, whereby TLR4 on epithelial cells helps drive the development of allergic reactions (41). As shown in Fig. 3A, we performed PBS or HDM sensitizations, followed by three HDM challenges at days 13–15. To focus on the early phase of inflammation, we analyzed CD2-Gata3 Tg mice and WT littermates 1 d after the last challenge. Histological analyses of lung tissue showed more peribronchial and perivascular eosinophilic infiltrates in CD2-Gata3 Tg HDM-sensitized and challenged (HDM/HDM) mice than in WT HDM/HDM or CD2-Gata3 Tg PBS/HDM mice.

Quantification of BAL fluid cells showed that HDM sensitization and challenge did not induce any signs of eosinophilic AAI in WT mice (Fig. 3B). In contrast, CD2-Gata3 Tg mice in the HDM/HDM group showed an ~5-fold increase in eosinophils in BAL fluid, when compared with WT HDM/HDM mice (Fig. 3B). In the HDM/HDM CD2-Gata3 Tg mice, this eosinophilia was accompanied by a more generalized inflammation of innate cells, evidenced by increased numbers of macrophages, neutrophils, and DCs (Fig. 3B). We observed a similar trend in the lungs: the highest numbers of eosinophils were present in the HDM/HDM CD2-Gata3 Tg mice (Fig. 3C).

Both in CD2-Gata3 Tg mice and WT littermates, we observed a significant increase of the total numbers of T lymphocytes in the HDM/HDM group compared with the PBS/HDM group (Fig. 3D). In BAL fluid of CD2-Gata3 Tg mice, we observed a substantial influx of ILC2 after HDM exposure, irrespective of PBS or HDM sensitization (Fig. 3D). In the lungs, the numbers of ILC2 (for gating strategy, see Supplemental Fig. 2B) upon HDM challenge was ~2-fold higher in CD2-Gata3 Tg mice than in WT mice (Fig. 3D).

Collectively, these findings show that enforced CD2-driven Gata3 expression enhances HDM-driven AAI. In this model, the BAL fluid of CD2-Gata3 Tg mice contains increased numbers of leukocytes, in particular, eosinophils and ILC2.



Enforced Gata3 expression enhances cytokine production by Th2 cells and ILC2

Next, we used intracellular FACS analyses to investigate cytokine profiles of Th2 and ILC2 in BAL fluid and lungs in HDM-driven AAI. Fig. 4A shows an example of the cytokine analysis of gated CD3⁺ T cells and ILC2 from the lungs WT and CD2-Gata3 Tg mice.

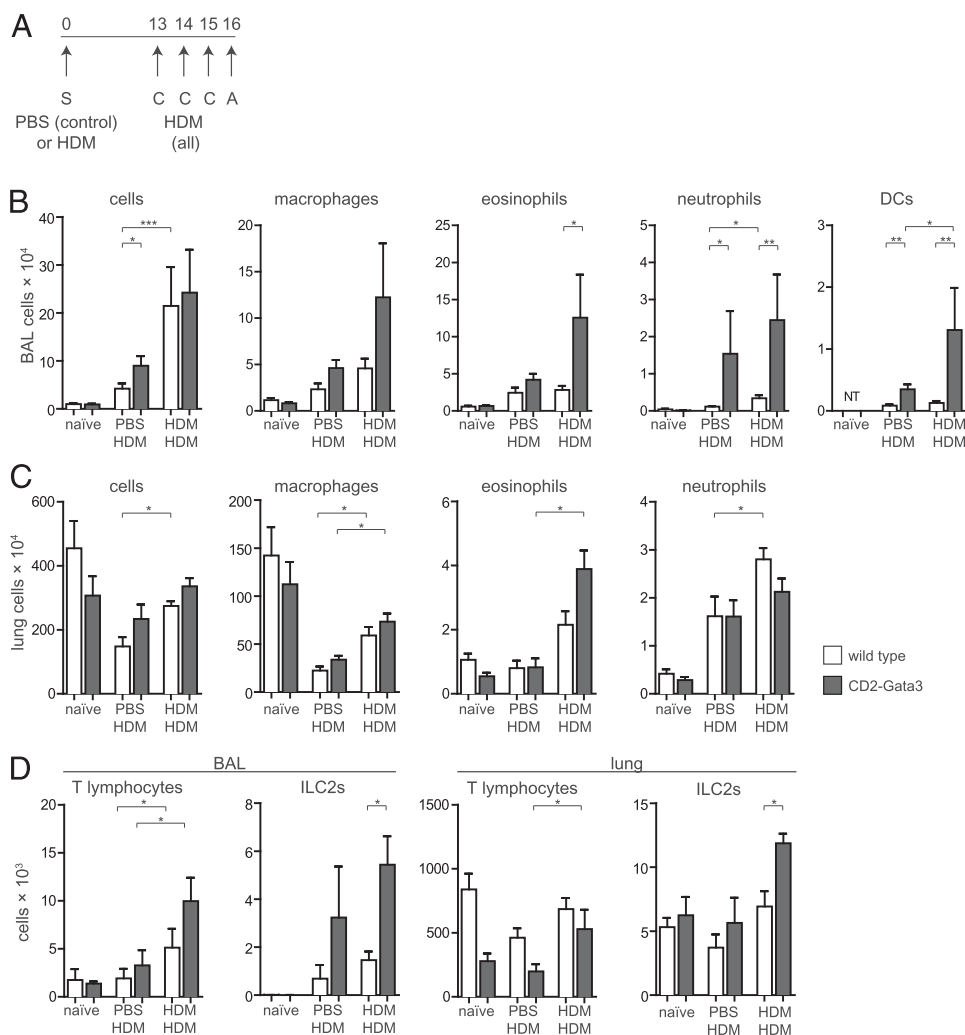
When we quantified cytokine-expressing cells in BAL fluid, we observed that enforced expression of Gata3 increased the numbers of T cells expressing the Th2 cytokines IL-4, IL-5, and IL-13, whereby differences between CD2-Gata3 Tg and WT littermates reached significance in the HDM-sensitized group (Fig. 4B). In contrast, the presence of the CD2-Gata3 transgene did not affect the numbers of T cells positive for the key Th1 cytokine IFN- γ . Remarkably, enforced expression of Gata3 did not only increase the numbers of ILC2 expressing IL-5 and IL-13, both of which are typically produced by ILC2, but also resulted in the appearance of

IL-4⁺ and IFN- γ ⁺ cells within the gate of the IL-33R⁺T1/ST2⁺ CD25⁺CD127⁺Sca-1⁺ population of ILC2 (Fig. 4B).

Whereas CD2-Gata3 Tg and WT littermates had similar numbers of IFN- γ T cells in the lung, CD2-Gata3 Tg mice showed an increase in the numbers of IL-4⁺, IL-5⁺, or IL-13⁺-expressing T cells. Importantly, the average number of Th2-expressing T cells was not different between the groups of naive, PBS/HDM-treated and HDM/HDM-treated CD2-Gata3 Tg mice (Fig. 4C). Parallel to our findings in the BAL fluid, we observed significantly increased numbers of IL-4⁺, IL-5⁺, and IL-13⁺-expressing ILC2 in HDM-sensitized/HDM-challenged CD2-Gata3 Tg mice, compared with WT littermates. We also detected the induction of IFN- γ ⁺ ILC2-like cells in CD2-Gata3 Tg mice (Fig. 4B).

Next, we measured cytokine levels in BAL fluid by ELISA. We found a moderate increase for IFN- γ , IL-4, and IL-5 in CD2-Gata3 Tg mice compared with WT littermates, whereby observed differences were significant for IFN- γ and IL-5 (Fig. 4D). Finally, we

FIGURE 3. Enforced Gata3 expression enhances AAI in a mild HDM model. **(A)** Mild experimental HDM asthma design showing i.t. sensitization (s) of 10 μ g HDM or PBS as control and challenge (c) with 10 μ g HDM intranasal. **(B)** Quantification of flow cytometric analyses of the indicated populations of leukocytes in BAL of WT (white bars) and CD2-Gata3 Tg mice (gray bars). Results are expressed as means $\pm$ SEM. **(C)** Quantification of flow cytometric analyses of the indicated populations of leukocytes in lungs of WT (white bars) and CD2-Gata3 Tg mice (gray bars). Results are expressed as means $\pm$ SEM. **(D)** Numbers of T cells and ILC2 in BAL fluid (left) and collagen-digested lungs (right). Data are shown as median and SEM. Results represent pooled data of two independent experiments with three to six animals per group. Statistical evaluations were performed only between different treatments of groups of the same genotype, or between the two genotypes within one treatment group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



found significantly increased levels of serum IgE in HDM-treated CD2-Gata3 Tg mice, compared with HDM-treated WT control littermates (Fig. 4E).

Taken together, these findings in our HDM-driven AAI show that the presence of the CD2-Gata3 transgene is associated with enhanced Th2 cytokine production by both T cells and ILC2 in BAL and lung. Enforced expression of Gata3 *in vivo* does not affect the numbers of IFN- γ -expressing T cells, but remarkably results in an accumulation of IFN- γ -producing ILC2-like cells in BAL and lung.

Enforced Gata3 expression is associated with an IL-33-inducible population of T1ST2⁺ T-bet⁺Gata3⁺Lin⁻ cells

Because ILC2 normally do not express IFN- γ , we further explored the phenomenon of IFN- γ production by CD2-Gata3 Tg ILC2-like cells. Because Gata3 can directly regulate the expression of IL-33R/T1ST2 in T cells and ILC2 (21, 29, 42), and because the CD2-Gata3 transgene is already expressed in CLP cells, it is conceivable that ectopic Gata3 expression in CLP-derived non-ILC2 cells may switch on IL-33R/T1ST2 expression. In particular, ILC1 cells that express the key transcription factor T-bet and the signature cytokine IFN- γ (4) may become surface IL-33R/T1ST2⁺ and therefore hard to distinguish from ILC2. Because HDM challenge induces IL-33 (1, 3, 43), such IL-33R/T1ST2⁺ ILC1 may become activated in our HDM airway inflammation model and respond to IL-33 by producing IFN- γ .

To address this hypothesis experimentally, we administered IL-33 intranasally to CD2-Gata3 Tg mice and WT littermates,

resulting in eosinophilia in the BAL fluid of both mouse groups (Fig. 5A). Analysis of BAL fluid revealed that the influx of ILC2 and T cells was significantly higher in IL-33-treated CD2-Gata3 Tg mice than in WT littermates (Fig. 5A). Flow cytometric analyses of the lung did not reveal differences in total ILC2 numbers, but demonstrated the presence of a clear and distinct population of IFN- γ ⁺ IL5^{low} ILC2-like cells exclusively in CD2-Gata3 Tg mice (Fig. 5B, Supplemental Fig. 2C for gating strategy). When we gated for total Sca1⁺Lin⁻ cells in the lung, we identified significant populations of IFN- γ ⁺ cells only in CD2-Gata3 Tg mice, but not in WT littermate controls; these IFN- γ ⁺ Lin⁻Sca1⁺ cells were either T1ST2⁻ or T1ST2⁺ and were CD25^{low} (Fig. 5C). Finally, gating for T1ST2⁺Lin⁻Gata3⁺ cells showed that these ILC2-like cells contained a unique population of T-bet⁺ cells that were only observed in the lungs of IL-33-treated CD2-Gata3 Tg mice and not in WT mice (Fig. 5D, Supplemental Fig. 2D for gating strategy). Thus, the presence of CD2-Gata3 gene is associated with an IL-33-inducible population of T1ST2⁺T-bet⁺Gata3⁺ Sca1⁺Lin⁻ cells, consistent with ILC1 cells that have gained IL-33R/T1ST2⁺ expression.

Discussion

The transcription factor Gata3 is highly expressed in Th2 cells and ILC2, is required for the differentiation of these cells, and acts as a key regulator of Th2 cytokine expression. In this study, we found that increased expression of Gata3 in the T cell and ILC2 lineage in mice enhanced their susceptibility to AAI.

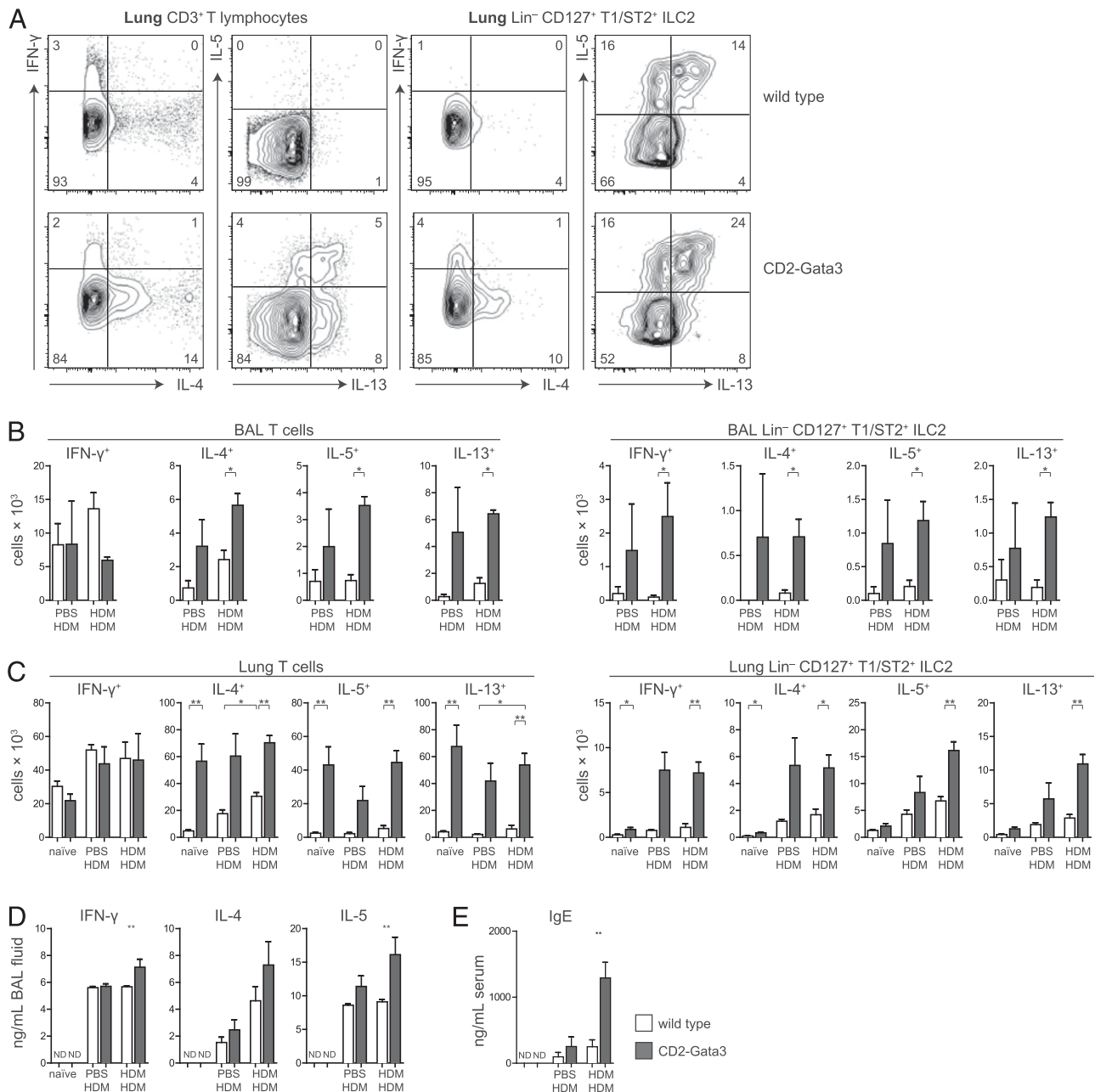


FIGURE 4. Enforced Gata3 expression enhances cytokine production by airway Th2 cells and ILC2. **(A)** Cytokine expression profiles of gated lung T cells (*left*) and ILC2 (*right*) of naive WT or CD2-Gata3 Tg mice upon 4 h of PMA/ionomycin stimulation. Data are shown as contour plots; proportions of cells in the indicated quadrants are given (in %). **(B)** Quantification of flow cytometric analysis of T cells (*left*) and ILC2 (*right*) positive for the indicated cytokines upon 4 h of PMA/ionomycin stimulation from BAL WT (white bars) and CD2-Gata3 Tg mice (gray bars). Results are expressed as means $\pm$ SEM. **(C)** Quantification of flow cytometric analysis of T cells (*left*) and ILC2 (*right*) positive for the indicated cytokines upon 4 h of PMA/ionomycin stimulation from collagenase-treated lung WT (white bars) and CD2-Gata3 Tg mice (gray bars). Results are expressed as means $\pm$ SEM. **(D and E)** Quantification of levels of INF- γ , IL-4, and IL-5 in BAL fluid (**D**) and IgE in serum (**E**) from WT (white bars) and CD2-Gata3 Tg mice (gray bars) as measured by ELISA. Data are shown as average values $\pm$ SEM. Results represent one of two independent experiments with three to six animals per group. * $p < 0.05$, ** $p < 0.01$.

In the presence of the CD2-Gata3 transgene eosinophilic airway, inflammation was observed in mild models (involving i.t. sensitizations with OVA or HDM both without adjuvant), which fail to induce AAI in WT mice. Remarkably, the presence of the CD2-Gata3 Tg transgene resulted in a significant increase of the expression of IL-4, IL-5, and IL-13 (but not INF- γ) in pulmonary T cells already in naive mice (Fig. 2E). Furthermore, the increased numbers of Th2 cytokine-expressing T cells did not change upon HDM challenge in PBS-sensitized or HDM-sensitized mice (Fig. 4C). Together with the findings that the T cell population in CD2-

Gata3 Tg mice contains increased proportions of T cells with a memory phenotype and increased expression of IL-33R/T1ST2 (29), our results suggest that Gata3 supports the formation of a Th2-committed IL-33R/T1ST2⁺ memory T cell compartment in vivo in the lung. ILC2 in the lungs of naive CD2-Gata3 Tg mice manifested slightly increased cytokine expression, compared with WT mice (Fig. 2E), and upon HDM challenge their cytokine expression is induced to levels that are significantly higher than those of WT ILC2 (Fig. 4C). Consistent with the innate characteristics of ILC2, that is, the absence of an Ag receptor, their

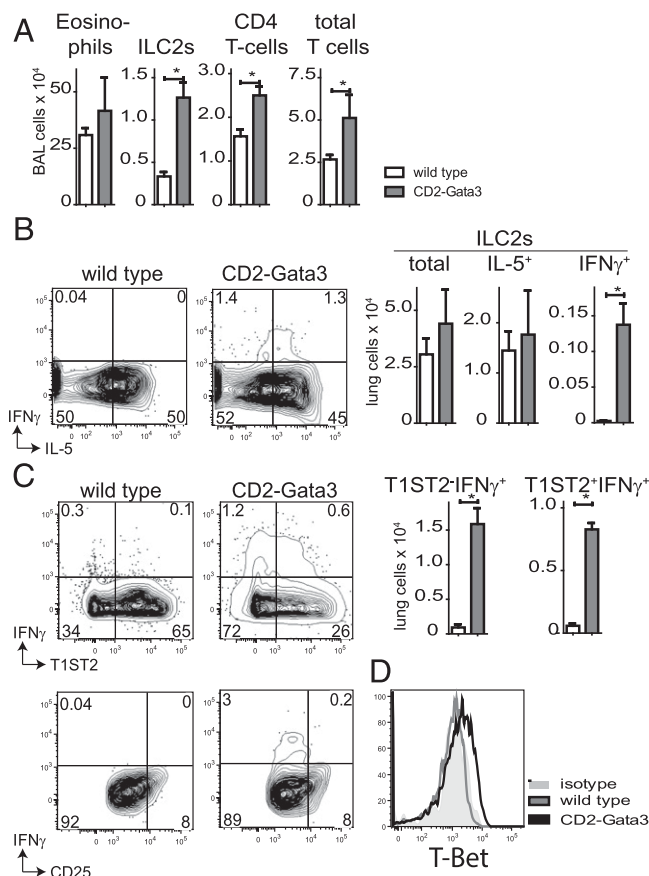


FIGURE 5. The presence of CD2-Gata3 gene is associated with an IL-33-inducible population of T1ST2⁺ T-bet⁺Gata3⁺ Lin⁻ cells. **(A)** Quantification of flow cytometric analyses of the indicated populations in BAL of IL-33-treated WT (white bars) and CD2-Gata3 Tg mice (gray bars). **(B)** Flow cytometric analysis of cells within the ILC2 gate (of CD25⁺T1ST2⁺Sca1⁺Lin⁻ cells) for intracellular expression of IFN- γ and IL-5 in collagen-digested lungs from the indicated mice. Quantification of flow cytometric analyses of the indicated ILC2 populations (of CD25⁺T1ST2⁺Sca1⁺Lin⁻ cells) in lungs of WT (white bars) and CD2-Gata3 Tg mice (gray bars). **(C)** Flow cytometric analysis of Sca1⁺Lin⁻ cells for IFN- γ , together with T1ST2 (top) or CD25 (bottom), and quantification of flow cytometric analyses of the indicated of IFN- γ ⁺ Sca1⁺Lin⁻ populations in lungs of WT (white bars) and CD2-Gata3 Tg mice (gray bars). **(D)** T-Bet protein expression by intracellular flow cytometric analysis in Gata3⁺ T1ST2⁺CD25⁺Sca1⁺ lineage-negative cells in lungs of CD2-Gata3 Tg mice (black line) and WT (C57bl/6) mice (gray line). Histogram of FMO (isotype; bright gray) is shown as a control. Experiments were performed with four mice per group. Data in bar graphs are shown as median and SEM. Statistical evaluations were performed with Mann-Whitney U test; * $p < 0.05$.

cytokine expression profile is not different between mice from the PBS/HDM- or HDM/HDM-challenged group.

The increased cytokine production by ILC2 may be explained by a direct effect of Gata3 overexpression on the Th2 cytokine locus in mature ILC2. However, we were unable to detect higher levels of Gata3 protein in pulmonary or intestinal ILC2 cells by our intracellular flow cytometric assay (Fig. 2A) (21). Alternatively, increased Gata3 levels in developing ILC2 might affect epigenetic modifications at the Th2 locus (12), which may enhance cytokine production in ILC2 upon activation during HDM challenge. Nevertheless, we found that CD2-Gata3 Tg ILC2 expressed higher levels of the IL-33R/T1ST2, thereby increasing their sensitivity to respond to IL-33 (Fig. 5), which is a potent ILC2 activator induced during HDM challenge (1, 3, 43). It is not likely that the presence of the CD2-Gata3 transgene affected IL-33 induction, because we

did not detect differences in IL-33 expression between CD2-Gata3 Tg and WT littermates in lung homogenates and observed a similar increase upon HDM challenge in both mouse groups (data not shown). An important role of Gata3 in enhancing IL-33R-mediated ILC2 activation would be consistent with the finding that in our asthma model based on i.t. instillation of OVA-primed DCs (thus bypassing the role of IL-33-expressing epithelial cells or activated macrophages), we did not detect differences between CD2-Gata3 Tg and WT littermates (Supplemental Fig. 1). The differences between the DC installation and OVA/HDM sensitization models would point to an important role of IL-33 as a trigger in AAI in susceptible individuals. In this context, it was shown that IL-33 is a steroid-resistant cytokine, and because levels are increased in steroid-resistant asthma patients (44), the IL-33-IL-33R/T1ST2 axis could be a potential therapeutic target to inhibit Th2 cytokine induction in susceptible individuals (44, 45). Finally, we found that already in the absence of sensitization, HDM-challenged CD2-Gata3 Tg mice manifested significant recruitment of DCs, next to some eosinophilic inflammation (Fig. 3B). It is therefore conceivable that ILC2 may act in parallel with epithelial cells that are induced by HDM to produce CCL20 and GM-CSF to support recruitment and activation of DCs (46). Further experiments should identify chemokines and cytokines that are produced by activated ILC2. Just like IL-5 and IL-13, such factors may be regulated by Gata3.

Several other groups have generated Tg mice with overexpression of Gata3 and have consistently reported enhanced eosinophilic airway inflammation (33–36). In chronic models that involve repeated allergen exposure, enforced Gata3 expression was associated with increased subepithelial fibrosis and airway smooth muscle hyperplasia (35). Although in most of these published models Gata3 expression was also driven by the human CD2 promoter, the effects of Gata3 overexpression on cytokine profiles appeared variable: limited effects on IL-4 but increased IL-5/IL-13 expression (33), increased IL-4 but similar IL-5 levels (35), and increased production of IL-4, IL-5, and IL-13 (36) have all been described. Because we observed the largest effects of Gata3 in naive or nonsensitized mice, it is conceivable that acute and more chronic models for allergic inflammation may show diverging effects of Gata3. Furthermore, Gata3 overexpression may differentially affect Th2 cells and ILC2. In this context, a paradoxical finding was that enforced Gata3 expression, which does not affect IFN- γ production by circulating (29, 31, 32) or pulmonary T cells (Fig. 4A, 4C), resulted in increased expression of IFN- γ by cells that had characteristics of pulmonary ILC2 (Fig. 4A, 4C). Our finding of a population of IL-33-inducible T1ST2⁺ T-bet⁺Gata3⁺ Lin⁻ cells exclusively in CD2-Gata3 Tg mice would support our hypothesis that ectopic expression of Gata3 in T-bet⁺ ILC1 may induce T1ST2⁺ expression in these cells. Stimulation of these cells by IL-33, induced by HDM challenge (1, 3, 43), would then signal for production of IFN- γ in these Gata3⁺T-bet⁺ ILC1. Our findings also imply that ectopic expression of Gata3 is not sufficient to downregulate T-bet and IFN- γ expression in ILC1. Nevertheless, we cannot formally exclude the alternative explanation for the finding of IFN- γ expression in cells with ILC2 characteristics, that is, ILC2 plasticity. It remains possible that ILC2 show plasticity in cytokine expression, because this has also been demonstrated for ILC3 (47–51). This plasticity is also reflected by the apparently variable capacity of ILC2 to produce IL-4 (3, 9, 43) (Fig. 4B, 4C).

In summary, we have shown that enforced expression of Gata3 is sufficient to enhance Th2 and ILC2 activity. As a result, because of joint efforts between Th2 cells and ILC2, production of the proinflammatory cytokines IL-4, IL-5, and IL-13 is enhanced and mice become remarkably susceptible to eosinophilic inflammation,

even after mild exposure to inhaled harmless Ags that usually leads to inhalational tolerance. Interestingly, because IL-33R/T1ST2 expression is directly controlled by Gata3 (42), our findings suggest that the observed association in human GWAS studies of asthma with the IL-33R/T1ST2 gene might be based on effects in the T cell, as well as the ILC2 lineage.

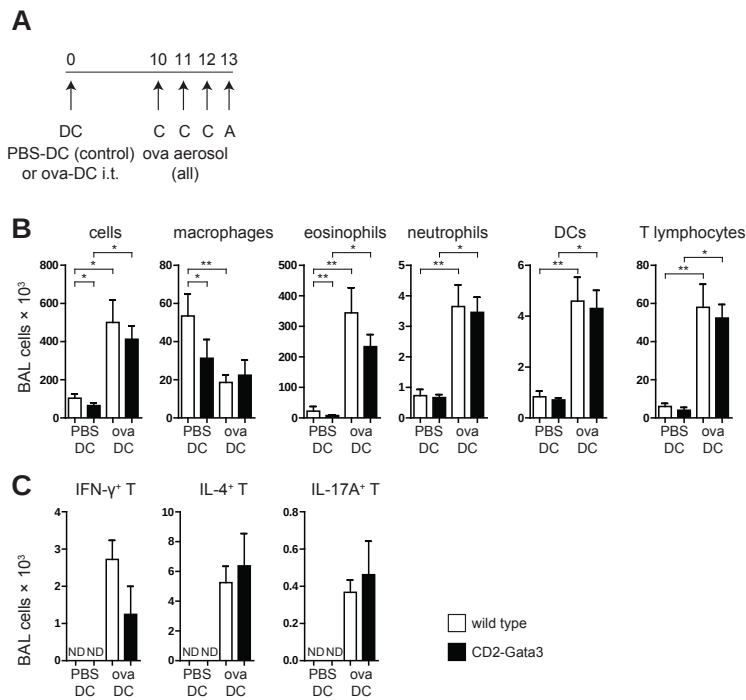
Disclosures

The authors have no financial conflicts of interest.

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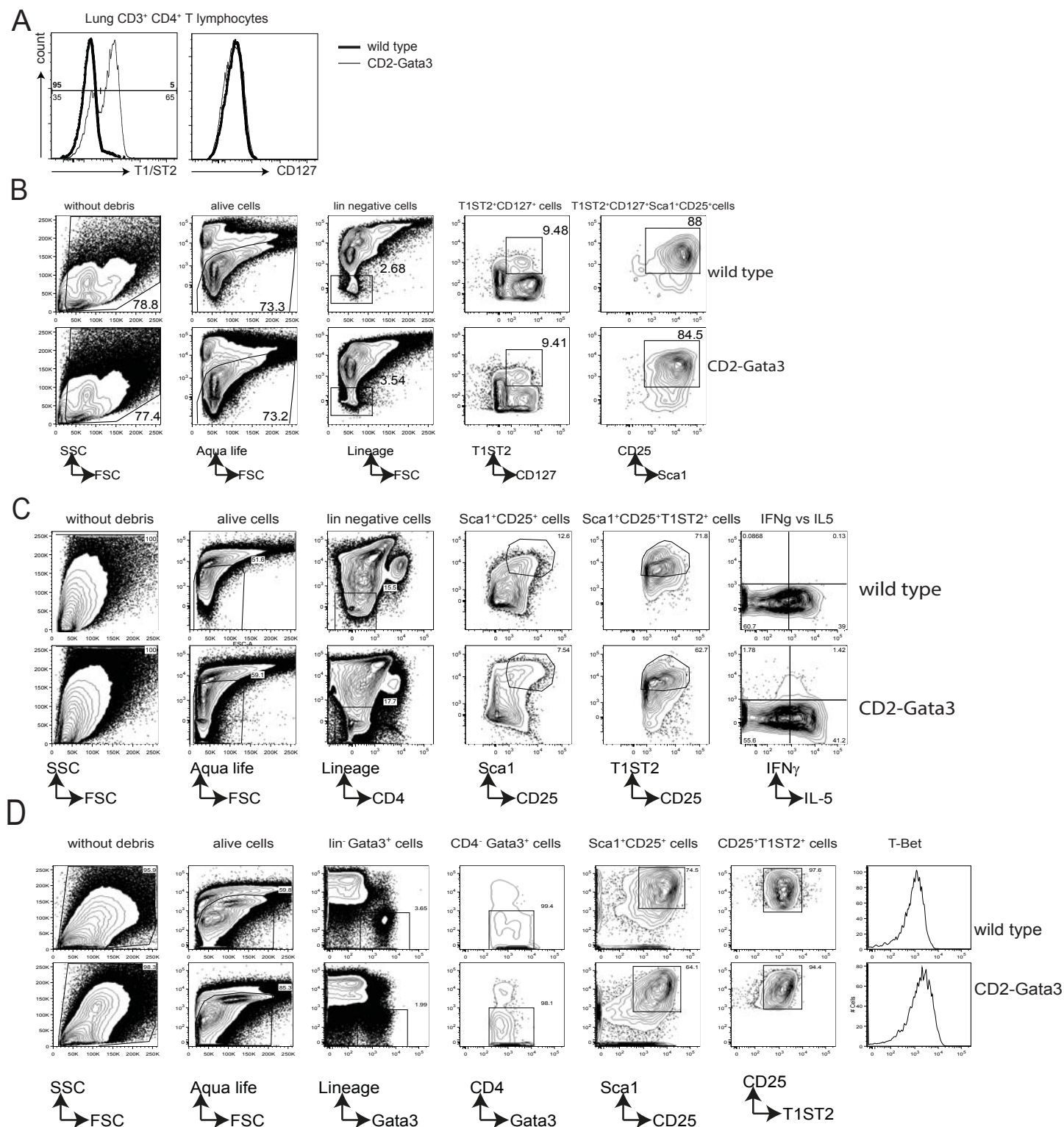
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Supplemental Figure 1

Enforced Gata3 expression does not enhance symptoms in a severe asthma model

(A) Mice were intratracheally (i.t.) sensitized with PBS or OVA-pulsed dendritic cells (ova-DC) One day after the last OVA aerosol challenge (c), allergic airway inflammation was investigated in wildtype (WT) (white bars) and CD2-Gata3 Tg (black bars) mice. (B) Quantification of flow cytometric analysis of the indicated populations of leukocytes in bronchoalveolar lavage (BAL) of WT (white bars) and CD2-Gata3 Tg mice (gray bars). The results shown are expressed as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$. (C) Quantification of flow cytometric analysis of T-cells positive for the indicated cytokines in bronchoalveolar (BAL) fluid upon 4hrs of PMA/ionomycin stimulation. The results shown are expressed as means $\pm$ SEM. The results shown represent one out of two independent experiments with 3-6 animals per group.



Supplemental Figure 2

Increased expression of IL-33R/T1ST2 on CD2-Gata3 Tg CD4⁺ T cells and gating strategy for identification of ILC2s. (A) Flow cytometric analysis of surface expression of IL-33R/T1ST2 and IL-7R/CD127 on collagenasedigested lung CD3⁺CD4⁺ T cells from CD2-Gata3 Tg mice (thin black line) and WT (C57bl/6) mice (bold line) mice, shown as histogram overlays. (B) Gating strategy for identification of ILC2s in Lungs of HDM sensitized and challenged WT and CD2-Gata3 Tg mice. (C) Gating strategy for identification of ILC2s in Lungs of IL-33 challenged WT and CD2-Gata3 Tg mice. ILC2 cells gated on lineage negative and positive for Sca1, CD25 and T1ST2 are for approximatele for 40% positive for IL-5. A substantial proportion of the ILC2s was positive for IFN γ which was only observed in CD2-Gata3 tg mice. (D) Gating strategy for identification of Gata3 positive ILC2 expressing T-Bet in Lungs of IL-33 challenged lungs of WT and CD2-Gata3 Tg mice. T-Bet and Gata3 expression was analyzed ex vivo without restimulation. Gata3 + cells were characterized as lineage negative cells that express Sca1, CD25 and T1ST2 being ILC2s. These cells express T-Bet.