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*J Immunol* published online 13 September 2013
http://www.jimmunol.org/content/early/2013/09/13/jimmunol.1301228

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/09/13/jimmunol.1301228
8.DC1

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TCF-1 Controls ILC2 and NKp46+RORγt+ Innate Lymphocyte Differentiation and Protection in Intestinal Inflammation

Lisa A. Mielke,* Joanna R. Groom,* Lucille C. Rankin,* Cyril Seillet,* Frederick Masson,* Tracy Putoczki, † and Gabrielle T. Belz*‡

Innate lymphocyte populations play a central role in conferring protective immunity at the mucosal frontier. In this study, we demonstrate that T cell factor 1 (TCF-1; encoded by Tcf7), a transcription factor also important for NK and T cell differentiation, is expressed by multiple innate lymphoid cell (ILC) subsets, including GATA3+ nuocytes (ILC2) and NKp46+ ILCs (ILC3), which confer protection against lung and intestinal inflammation. TCF-1 was intrinsically required for the differentiation of both ILC2 and NKp46+ ILC3. Loss of TCF-1 expression impaired the capacity of these ILC subsets to produce IL-5, IL-13, and IL-22 and resulted in crippled responses to intestinal infection with Citrobacter rodentium. Furthermore, a reduction in T-bet expression required for Notch–2-dependent development of NKp46+ ILC3 showed a dose-dependent reduction in TCF-1 expression. Collectively, our findings demonstrate an essential requirement for TCF-1 in ILC2 differentiation and reveal a link among Tcf7, Notch, and Tbx21 in NKp46+ ILC3 development. The Journal of Immunology, 2013, 191: 000–000.

Innate lymphoid cells (ILCs) are a developmentally related family of cells that have emerged more recently as critical effectors of innate immunity, particularly at mucosal surfaces, such as the intestine and the lung. ILCs can be activated by inflammatory signals, independent of Ag presentation, to rapidly produce cytokines, suggesting that ILCs form part of the front line of defense to infection and provide important cues for the development of the adaptive immune response (1).

Although the classical members of the innate lymphocyte family—NK cells and lymphoid tissue inducer (LTI) cells—have been known for >2 decades, newly described family members, such as the nuocyte (2), natural helper cell associated with fat clusters (3), and NKp46+ innate lymphocytes (NKp46+ ILC, also known as natural cytotoxicity receptor [NCR]+ ILC) have greatly expanded the family membership (4). Molecular analysis of these ILC subsets demonstrated that there is considerable similarity in the types of transcription factors (including Id2, Rorgt, and Tbx21) and cytokines (such as IL-5, IL-13, IL-17, and IL-22) produced by ILCs and Th cells to control cell fate and function, suggesting that a common program of differentiation is used by the innate and adaptive immune systems (5).

Inhibitor of DNA binding 2 (Id2) is a helix–loop–helix transcription factor that lacks a DNA binding domain and heterodimerizes with E-box proteins to prevent gene transcription (6). It is a key regulatory protein required for a wide range of developmental and cellular processes and is essential for the development of all ILC subsets (7, 8). Downstream of Id2, specific transcription factors and the cytokines that they produce appear to define individual ILC lineages. Group 1 ILCs (ILC1) are composed of classical NK cells, which are distinct from other ILC subsets because they do not express CD127 and depend on IL-15, and an innate lymphocyte subset, both of which express IFN-γ (9, 10). Group 2 ILCs (ILC2s) require GATA3 (11, 12) and RORα (13) for their development. They produce IL-5 and IL-13 and have been implicated in the development of airway hypersensitivity responses (14). In addition, ILC2 are critical for defense against intestinal parasites (2) and have both pro- and anti-inflammatory properties in the skin (15). Group 3 ILCs (ILC3) are found predominantly in the GALT and require the transcription factor RORγt for their development (16, 17). This last subset can produce IL-17 or IL-22, and both cytokines play a fundamental role in intestinal immune responses and tissue repair (18). IL-22 is thought to act exclusively on epithelial cells to promote proliferation and barrier function in the intestine and, therefore, plays a protective role in inflammatory bowel disease and infections against attaching and effacing bacterial pathogens (19, 20). ILC3 are made up of a number of subsets, including the classical LTi cells, which are required for initiation of lymphoid tissue development during embryogenesis (21–25), and a second subset that expresses the NCR NKp46 [or NKp44 in humans (26)] and is known as NKp46+ or NCR+ ILC3. NKp46+ ILC3 produce IL-22, which is further enhanced by stimulation with IL-23. In mice, these cells do not produce IL-17 in response to IL-23, and this distinguishes them from NCR- or LTi ILC3, which can produce both IL-17 and IL-22 (26).
The molecular pathways that diversify these cell types into functionally distinct subsets are not well characterized. NKp46+ ILC3 are intriguing because they appear to represent a terminally differentiated effector subset that is greatly expanded in response to inflammation. Recently, we (27) and other investigators (28, 29) showed that T-bet (encoded by Tbx21) is essential for the development of NKp46+ ILC but not the NCR+ ILC3 or ILC2 populations. T-bet is required to allow the transition of NCR− ILC3 into NKp46+ ILC3 through Notch2-dependent signaling (27). Following activation, the intracellular domain of Notch dissociates and interacts with the transcription factor RBP-Jκ; mice lacking this factor have fewer NKp46+ ILC3 (30). Differentiation of NKp46+ ILC3 progenitors in an in vitro culture system required the expression of Notch ligands (31), which also appear to be important for the in vitro differentiation of ILC2 (13, 32).

To further define the molecular pathways and transcription factors that give rise to functional NKp46+ ILC3, we investigated Notch target genes for their potential role in the differentiation of NKp46+ ILC3. T cell factor 1 (TCF-1; encoded by Tcf7) was identified as a direct target gene of Notch in T cells to promote T cell development in the thymus (33, 34). In line with this, we identified TCF-1 as a novel transcription factor required for the development of NKp46+ ILC3 in addition to ILC2 (32). TCF-1 was required for IL-22 production and protection against the intestinal pathogen Citrobacter rodentium. Furthermore, expression of a single allele of T-bet resulted in a significant reduction in TCF-1 expression in NKp46+ ILC3 but not other ILC3 subsets. These findings suggest that TCF-1 acts downstream of T-bet and Notch to facilitate NKp46+ ILC3 development.

### Materials and Methods

**Mice**

Id2GFP mice (35), RorcγtGFP mice (16), Prdm16GFP mice (36), Tbx21<−/−> mice (37), and Tcf7<−/−> mice (38) were described previously. Ly5.1+ × Ly5.2+ mice were bred and maintained in-house. Mice were used at 8–12 wk of age, unless otherwise stated. All procedures involving animals were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research.

**Infection with C. rodentium**

Mice were inoculated with 2 × 10⁵ CFU of C. rodentium by oral gavage. Mice were analyzed 8 d postinfection, and bacterial dissemination was determined by culture of livers and spleens from infected mice on agar plates containing nalidixic acid, as previously described (27).

**Papain-induced lung inflammation**

Mice were treated intranasally with 20 μL papain (5 mg/ml diluted in PBS) or PBS alone on three consecutive days. Twenty-four hours following the last treatment, lung cells were isolated and stained with Abs for CD45, CD11c, and Siglec F to identify infiltrating eosinophils and then analyzed by flow cytometry.

**Mixed fetal liver chimeras**

Ly5.1 mice were lethally irradiated and reconstituted with a 1:1 mixture of wild-type (Ly5.2) and Tcf7<−/−> (Ly5.2<Ly5.1+) fetal liver cells. Mice were allowed to reconstitute their hematopoietic system for 8 wk before use.

**Isolation of lymphocytes and flow cytometry**

Intestinal lamina propria lymphocytes were isolated as previously described by Rankin et al. (27). Mononuclear lung cells were isolated from the lung by generating single-cell suspensions following digestion with Collagenase II (145-2C110), CD4 (GK1.5), CD8 (53-6.7), ICOS (C398.4A), NKp46 (29A1.4), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A34), Sca1 (E13-161.7), and ST2 (DI8). Intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience) and Abs to GFP (AFKJS-9), IL-13 (eBio13A), IL-5 (TRFK5), and IL-22 (IL22JOP). Intracellular cytokine staining for IL-22 was performed following stimulation for 4 h with IL-23 (10 ng/ml) and IL-1β (10 ng/ml) and intracellular cytokine staining for IL-13 and IL-5 was performed following stimulation for 4 h with PMA (50 ng/ml) and ionomycin (100 ng/ml), in the presence of brefeldin A (1 μg). Cells were analyzed using a FACSanto II (BD Biosciences), and FlowJo software was used for analysis. Flow cytometric sorting was performed with a FACSaria (BD Biosciences).

**Histology**

Tissues were fixed in 10% neutral buffered formalin and embedded in wax, by standard protocols, prior to sectioning and staining with H&E. Images were captured with an Aperio ImageScope. Colitis severity was assessed by a combined score of colon cellular inflammation and tissue disruption. The histological scoring was performed in a blinded fashion.

**Quantitative RT-PCR**

Total RNA was prepared from purified lymphocyte populations with an RNeasy Mini kit (Qiagen), and cDNA was synthesized with oligo(dT) and Thermoscript reverse transcriptase (Invitrogen). Real-time PCR was done with the SensiMix SYBR no-Rox kit (Bioline). Primer sequences were as follows: TCF7L1: 5′-CCGTGCTGATAACAAACGCCC-3′ and 5′-CCACCTCACAGTATTGGG-3′; Left: 5′-TGTATTATCCCATACCGGTGGG-3′; Right: 5′-CAGTGTTGTCGCTGCTGACAG-3′; Gafla: 5′-CCTGCGACCATTGCCATAGGAA-3′ and 5′-GGATACCTTCCAGGTACGAC-3′; and Rora: 5′-GACATCAGCCGAAAATGGAACA-3′ and 5′-GTOGAGAAATGACATCAGGAA-3′.

**Results**

Tcf7 is highly expressed in ILCs and controls development of Id2+ innate immune cells

ILCs exhibit a shared requirement for the transcriptional repressor Id2, which acts to inhibit E protein transcription factor activity and antagonize the development of B and T cell fates during ILC development (9, 23, 39). Therefore, Id2 expression marks hematopoietic precursors that give rise to the different ILC subsets (27). Using our Id2-GFP reporter mouse, in which endogenous expression of Id2 is tagged using GFP, we analyzed the development of ILC populations in the presence or absence of Tcf7. This showed that the overall expression of Id2-GFP+ lymphocytes was substantially reduced in the absence of TCF-1 in the lung, lamina propria of the small intestine and colon, and the mesenteric lymph node (MLN) (Fig. 1A). Although Id2-GFP was reduced in T cell lineages (TCRβ+), as expected, this loss of expression was also found in CD8+ TCRβ− cells, suggesting the involvement of TCF-1 in the regulation of innate lymphocyte populations (Fig. 1B). To investigate which ILC subsets expressed Tcf7, quantitative RT-PCR analysis was performed on ILC subsets isolated from the small intestinal lamina propria (Fig. 1C). This revealed that Tcf7 transcripts were highly expressed in all ILC subsets, including ILC1 (NK cells), ILC2 (nuocytes), and NCR− and NKp46+ ILC3 (Fig. 1C). Lef1, a closely related HMG transcription factor that functions redundantly with Tcf7 in promoting thymocyte maturation, was not expressed in lamina propria ILC subsets, but it was highly expressed in CD8+ T cells, as expected (data not shown). These results suggest that TCF-1 may play an important role in the development of Id2+ ILCs. Consistent with previous reports (40), we did not observe a developmental defect in NK cells (data not shown); thus, we proposed that TCF-1 may influence the development of ILC2 and/or ILC3 innate subsets.

**TCF-1 is required for development of ILC2 and ILC3 subsets**

To delineate which ILC subsets were influenced by TCF-1, the expression of lineage-specific markers (i.e., GATA3, Sca1, and ICOS for ILC2 and RORγt and NKp46 for ILC3) was analyzed in...
Tcf7\(^{+/+}\) and Tcf7\(^{-/-}\) mice. Strikingly, both the frequency and total number of lin\(^{-}\)GATA3\(^{+}\)Sca1\(^{+}\) ILC2 (Fig. 2A, 2B) were markedly reduced compared with wild-type control animals. Of the remaining GATA3\(^{+}\) ILC2 in TCF-1–deficient mice, expression of the IL-33R subunit, ST2, was consistently reduced, whereas other cell surface molecules commonly expressed on ILC2 remained unchanged, including KLRG1 and ICOS (Supplemental Fig. 1A) and IL-17RB (IL-25R; Supplemental Fig. 1B). Consistent with the decreased expression of ST2, ILC2s from Tcf7\(^{-/-}\) mice did not proliferate in response to IL-33, and we could not detect the proliferation of either wild-type or Tcf7\(^{-/-}\) ILC2 in response to IL-25 (Supplemental Fig. 1C). These data are in line with recent reports suggesting that IL-33 is more potent than IL-25 in promoting ILC2 (41). These results highlight the importance of TCF-1 in the development of ILC2 and suggest that TCF-1 may regulate the expression of some, but not all, cell surface markers on ILC2. Analysis of the small intestine revealed that TCF-1 is required for normal Peyer’s patch development: Peyer’s patches develop but the size of each is reduced compared with wild-type mice (data not shown). Interestingly, we observed that TCF-1 was required for the development of ROR\(^{yt}\)NKp46\(^{+}\) ILC3 but not NCR\(^{-}\) ILC3, including the CD4\(^{+}\) or CD4\(^{-}\) subset (Fig. 2C, 2D). A significant proportion of Ror\(^{yt}\)NKp46\(^{+}\) ILC3 expressed KLRG-1, and the frequency of these cells was reduced 10-fold in the absence of TCF-1 (Fig. 2C, right panels). Previously, we showed that NKp46\(^{+}\) ILC3, but not NCR\(^{-}\) ILC3, express the transcriptional repressor Blimp-1 (encoded by Prdm1) (27). NKp46\(^{+}\) ILC3 do not require Blimp-1 for their differentiation (27); therefore, we were able to use Blimp-1 reporter mice to provide a means by which to determine whether TCF-1 might also regulate NKp46 expression. Blimp1-GFP expression in ILC3 populations remained unchanged in the presence or absence of TCF-1, indicating that loss of the NKp46\(^{+}\) ILC3 subset was a TCF-1–dependent developmental defect rather than the result of modulation of cell surface receptor expression (Supplemental Fig. 1D). The development of NCR\(^{-}\) ILC3 were not dependent on TCF-1, suggesting that TCF-1 plays a specific role in the generation of ILC3 cells by controlling the differentiation of NKp46\(^{+}\) ILC3.
ILC2 and ILC3 subsets are regulated by an intrinsic TCF-1 pathway.

Tcf7−/− mice lack expression of TCF-1 in both hematopoietic and nonhematopoietic (stromal) compartments, and TCF-1 has multiple roles in the development of the hematopoietic cells and tissues (33, 38, 40, 42), both of which could influence the development of ILC populations. To determine whether loss of ILC2 and ILC3 cells was a TCF-1–driven cell-intrinsic effect, we generated mixed fetal liver chimeras by combining congenically marked Tcf7−/− (Ly5.1+ Ly5.2+) and Tcf7+/+ (Ly5.2+/+) fetal liver cells at a 1:1 ratio and adoptively transferred them into lethally irradiated Ly5.1+/+ recipient mice. This approach allowed us to trace the hematopoietic origin of ILC2 and ILC3 subsets. Eight weeks after transplantation, reconstitution of ILC2 and ILC3 populations in the lung and small intestine was analyzed. Ly5.2+/+ wild-type cells reconstituted ILC2 compartments normally in the lung and intestine (Fig. 3A, 3B) and ILC3 compartments in the intestine (Fig. 3C, 3D). In contrast, Ly5.1+Ly5.2+ Tcf7−/− fetal liver cells completely failed to repopulate ILC2 (Fig. 3A, 3B). The frequency and total number of ILC3 were reduced (Fig. 3C, 3D). Analysis of the ILC3 subsets revealed that Tcf7−/− fetal liver cells did not fully repopulate NKp46+ ILC3, whereas in NCR2 ILC3 repopulations, CD4− and CD4+ were not affected (Fig. 3C, 3D). Thus, the loss of ILC2 and NKp46+ ILC3 populations in both intact mice and fetal liver chimeras showed that TCF-1 is required in a cell-intrinsic manner.

**FIGURE 2.** TCF-1 is essential for development of ILC2 and NKp46+ ILC3. (A) Analysis of GATA3- and Sca1-expressing ILC2 in CD3−CD19− lymphocytes isolated from the lung, lamina propria of small intestine or colon, and MLN of wild-type (Tcf7+/+) or TCF-1–deficient (Tcf7−−/−) mice (left panels) stained for intracellular expression of RORγt. Expression of NKp46, CD4, and KLRG1 in RORγt+ ILC3 isolated from the small intestine of wild-type (Tcf7+/+) or TCF-1–deficient (Tcf7−−/−) mice (middle and right panels). Results show one representative experiment of three (n = 6 mice analyzed/genotype). (B) Total cell number of ILC3 (CD45+CD3−CD19− RORγt+) subsets isolated from the small intestine of wild-type (Tcf7+/+) or TCF-1–deficient (Tcf7−−/−) mice. Data are mean ± SE pooled from three independent experiments (n = 6). *p < 0.05, **p < 0.01.
development of allergic responses (11, 13, 14), whereas ILC3 can produce IL-17 and IL-22 (1). To determine whether loss of TCF-1 resulted in impaired cytokine production, ILC from various tissues (lung, small intestine, colon, and MLNs) were stimulated in vitro to induce IL-5 and IL-13. We observed large numbers of IL-5- and IL-13–producing cells in the lungs of wild-type mice (Fig. 4A, Supplemental Fig. 2A), and these cells were almost completely absent in Tcf7-/- mice (Fig. 4A). We also observed decreased IL-5 and IL-13 production from the small intestine, colon, and MLN (Fig. 4A, Supplemental Fig. 2A). To determine whether loss of Tcf7 influences ILC2-mediated lung inflammation in vivo, we used a model in which mice are treated intranasally with papain; this induces ILC2 to produce IL-5 and IL-13 to enhance lung eosinophil infiltration. Tcf7-/- deficient mice showed reduced eosinophil infiltration. Tcf7-/- mice showed reduced eosinophil infiltration to the lung following papain treatment (Supplemental Fig. 2B). These results confirm the importance of TCF-1 in innate immune-mediated inflammatory responses. To determine whether loss of TCF-1 also impaired the production of cytokines characteristic for ILC3, cells were stimulated ex vivo to induce production of IL-17 and IL-22. Analysis of IL-22 production by ILC3 from the small intestine revealed that the frequency and total number of IL-22–producing NKp46+ ILC3 were significantly reduced in Tcf7-/- mice (Fig. 4B, Supplemental Fig. 2C), although IL-22 production by NCR2 ILC3 was not influenced by the loss of TCF-1 (Fig. 4B). Similar to previous reports showing that TCF-1 acts to suppress the Il17 promoter (43–45), we observed a significant increase in IL-17 production by NCR2 ILC3 from Tcf7-/- mice. Although IL-17 production by NKp46+ IL3 was slightly increased in the absence of Tcf7, the total number of NKp46+ IL3 producing IL-17 was extremely small, and the differences were not statistically significant (Fig. 4B, Supplemental Fig. 2C). Loss of functional NKp46+ IL3 and increased IL-17 production indicated that TCF-1 may play a critical role in maintaining intestinal homeostasis.

FIGURE 3. TCF-1 controls ILC2 and ILC3 development by a cell-intrinsic mechanism. Reconstitution of innate cells in Ly5.1+/+ mice reconstituted with a 1:1 mixture of Tcf7+/+ (Ly5.1+) and Tcf7-/- (Ly5.2-) fetal liver cells. Frequency (A) and total cell number (B) of ILC2 in the lung and small intestinal lamina propria expressing GATA3 and ICOS. Frequency (C) and total cell number (D) of ILC3, CD4+ NKp46+ ILC3, CD4+ ILC3, or NKp46+ ILC3 expressing RORyt and CD4 in CD3-CD19- lymphocytes isolated from the small intestinal lamina propria. All cells were gated on CD45+CD3-CD19- lymphocytes. Data show one of two similar independent experiments (n = 6 mice/group). **p < 0.01.
of TCF-1 in regulating ILC populations, Tcf7\(^{+/−}\) ILC2 and T-bet\(^{+/−}\) NKp46\(^{+}\) ILC3 were analyzed by quantitative RT-PCR for their expression of Gata3 and Tcf7, respectively, in the different subsets. In addition, Tcf7, Lef1, and Rora were analyzed in ILC2; as expected, Tcf7 expression decreased in Tcf7\(^{+/−}\) ILC2, whereas Lef1 expression was increased in Tcf7 heterozygous mice, consistent with previous reports that Lef1 is regulated by Tcf7 and can partially compensate for the loss of Tcf7 (Fig. 4C) (44). Both Gata3 and Rora expression were decreased in Tcf7\(^{+/−}\) ILC2, suggesting that TCF-1 is a critical transcription factor required for expression of GATA3 and ROR\(_{α}\), two transcription factors known to be required for the differentiation and function of ILC2 (Fig. 2C, 2D); however, Tcf7 expression was higher in NCR\(^{−}\) ILC3 cells compared with NKp46\(^{+}\) ILC3 (Fig. 1C), suggesting that Tcf7 might be important in the transition to NKp46\(^{+}\) ILC3, similar to that of T-bet (27). Therefore, we analyzed ILC3 isolated from Tbx21\(^{+/−}\)Rorc\(_{γ}^{+/−}\)gfp\(_{δ}\) heterozygous mice and found that the expression of Tcf7 in the NKp46\(^{+}\) ILC3 subset was reduced by approximately half (Fig. 4D). These data, combined with our previous findings that T-bet is required to induce Notch2 in NCR\(^{−}\) ILC3 for the differentiation of NKp46\(^{+}\) ILC3, imply that Tcf7 plays an important role in optimal signaling through the T-bet/Notch pathway.

**Discussion**

The mucosal immune system constantly balances the response to environmental Ags, such as virus, bacteria, and nutrients. This sentinel role is orchestrated by the interplay of a network of innate lymphocytes, such as NK cells (ILC1), nuocytes (ILC2), and NKp46\(^{+}\) ILC3. Our previous findings that T-bet is required to induce Notch2 in ILC3, similar to that of Tcf7 (27), suggest that TCF-1 plays a crucial role in the innate immune response to infection with *C. rodentium*.

Previous reports suggest that NKp46\(^{+}\) ILC3 play a crucial role in protection against *C. rodentium* infection (27). In this model, NKp46\(^{+}\) ILC3 produce IL-22 to enhance epithelial cell repair and prevent dissemination of *C. rodentium* (19). To assess innate immune responses, we analyzed infected mice on day 8 following infection before they start to clear the bacteria. Intestinal inflammation caused by *C. rodentium* infection induces colon shortening, which was exacerbated in the absence of TCF-1 (Fig. 5A). Histological analysis revealed increased mucosal hyperplasia and crypt damage in Tcf7\(^{+/−}\) mice (Fig. 5B, 5C). Furthermore, infected Tcf7\(^{+/−}\) mice displayed increased bacterial dissemination to the liver and spleen compared with wild-type controls (Fig. 5D). We observed that ILC3 make up >95% of IL-22–producing cells at this time point, and the overall IL-22 production was decreased in the absence of TCF-1 (data not shown, Supplemental Fig. 3). Enhanced susceptibility to *C. rodentium* in Tcf7\(^{+/−}\) mice correlated with reduced IL-22 production by total ILC3 (Fig. 5E) and loss of IL-22–producing NKp46\(^{+}\) ILC3 (Fig. 5F, 5G). Therefore, TCF-1 plays a crucial role in the innate immune response to infection with *C. rodentium*.

**TCF-1 controls NKp46\(^{+}\) ILC3 via the T-bet/Notch–signaling pathway**

GATA3 is essential for ILC2 development and controls IL-5 and IL-13 production (11), whereas T-bet influences the differentiation of NKp46\(^{+}\) ILC3 by inducing Notch (27). To further explore the role of TCF-1 in regulating ILC populations, Tcf7\(^{+/−}\) ILC2 and T-bet\(^{+/−}\) NKp46\(^{+}\) ILC3 were analyzed by quantitative RT-PCR for their expression of Gata3 and Tcf7, respectively, in the different subsets. In addition, Tcf7, Lef1, and Rora were analyzed in ILC2; as expected, Tcf7 expression decreased in Tcf7\(^{+/−}\) ILC2, whereas Lef1 expression was increased in Tcf7 heterozygous mice, consistent with previous reports that Lef1 is regulated by Tcf7 and can partially compensate for the loss of Tcf7 (Fig. 4C) (44). Both Gata3 and Rora expression were decreased in Tcf7\(^{+/−}\) ILC2, suggesting that TCF-1 is a critical transcription factor required for expression of GATA3 and ROR\(_{α}\), two transcription factors known to be required for the differentiation and function of ILC2 (Fig. 2C, 2D); however, Tcf7 expression was higher in NCR\(^{−}\) ILC3 cells compared with NKp46\(^{+}\) ILC3 (Fig. 1C), suggesting that Tcf7 might be important in the transition to NKp46\(^{+}\) ILC3, similar to that of T-bet (27). Therefore, we analyzed ILC3 isolated from Tbx21\(^{+/−}\)Rorc\(_{γ}^{+/−}\)gfp\(_{δ}\) heterozygous mice and found that the expression of Tcf7 in the NKp46\(^{+}\) ILC3 subset was reduced by approximately half (Fig. 4D). These data, combined with our previous findings that T-bet is required to induce Notch2 in NCR\(^{−}\) ILC3 for the differentiation of NKp46\(^{+}\) ILC3, imply that Tcf7 plays an important role in optimal signaling through the T-bet/Notch pathway.

**Discussion**

The mucosal immune system constantly balances the response to environmental Ags, such as virus, bacteria, and nutrients. This sentinel role is orchestrated by the interplay of a network of innate lymphocytes, such as NK cells (ILC1), nuocytes (ILC2), and NKp46\(^{+}\) and NCR\(^{−}\) innate lymphocytes (ILC3), and their environment. In this study, we identify an essential requirement for TCF-1 in the generation of the ILC2 and NKp46\(^{+}\) ILC3 subsets.

TCF-1 (encoded by Tcf7) was identified as a critical regulator of T cell specification and is highly expressed in early thymic progenitors (34); however, its expression pattern and role in other lymphocyte populations are less clear. We found that TCF-1 was expressed by all ILC subsets, but it was essential for the development of ILC2 cells and also was required for the development of NKp46\(^{+}\) ILC3 cells. Loss of TCF-1 did not impair the development of NK cells in the steady-state or NCR\(^{−}\) ILC3.
Despite this, Tcf7 expression in NCR\(^2\) cells was high, suggesting that induction of expression might play an important role in the transition of NCR\(^2\) cells into NKp46\(^+\) ILC3. Recently, we (27) and other investigators (28, 29) showed that the transcription factor T-bet was critical for the development of NKp46\(^+\) ILC3 and that this depended on T-bet activation of Notch2. We found in our analyses of T-bet heterozygous ILC that loss of a single allele of T-bet diminished Tcf7 expression in a dose-specific manner in NKp46\(^+\) ILC3, but not other ILC3, subsets. We similarly found a loss of Notch expression in the absence of T-bet (27). This loss was associated with a significant reduction in IL-22 production and impaired protection during \(C.\) rodentium infection. In thymocytes, Notch signals induce TCF-1, which, in turn, imprints T cell fate by switching on the expression of T cell essential genes (34). Collectively, these findings suggest that a key action of Notch is to induce TCF-1 generally in lymphocytes; in addition, it acts to integrate the signals between T-bet and TCF-1 in the transcriptional network to determine the fate outcome of different ILC subsets (Fig. 5H).
Although the major focus of TCF-1 has been in fate decisions of thymocytes and in the development of memory CD8+ T cells, a more significant role for TCF-1 was recently implicated in the development of innate lymphocytes (32, 43). Yang et al. (32) observed a significant reduction in the production of IL-5 and IL-13 in the lungs of Tcf7−/− mice, impairing their capacity to mount ILC2-mediated innate type 2 immune responses. This was attributable to the requirement for TCF-1 in promoting ILC2 generation, which appeared to act through both GATA3-dependent and -independent pathways. TCF-1 also appeared to be an important factor for the expression of IL-33R by ILC2. IL-33 plays a critical role in inducing ILC2 effector functions; however, because we did not find TCF-1 consensus binding sites in the promoter or enhancer regions of ST2, we hypothesize that it may indirectly regulate IL-33R/ST2 expression. The molecular regulation of ST2 has not been studied in depth. However, Tcf7 was recently reported to regulate the expression of IL-7R and GATA3 (32), whereas PU.1, a known target gene of Tcf7 (46), was shown to induce the ST2 promoter (47). Investigation of these pathways will be important in future studies to better understand the regulation of ILC2 function and development. We found that ILC2 intrinsically depended on TCF-1 for their development; however, in addition, we discovered that Nkp46+ ILC3, but not NCR+ ILC3, also depended on TCF-1 in a cell-intrinsic manner. Consistent with previous reports that show TCF-1 acts to suppress IL-17 production (43–45), TCF-1–deficient NCR+ ILC3 displayed elevated IL-17 expression; however, we observed a decrease in total IL-22–producing ILC3 in the intestine, which may be attributed to the loss of Nkp46+ ILC3 in the gut. We also noted that, in TCF–deficient mice, Peyer’s patches were considerably smaller, but not absent, compared with wild-type control mice, in contrast to a previous report (43). This suggests that the development of fetal LTi cells occurs, allowing early colonization of the anlage, but downstream signals that result in the full recruitment of lymphocytes to the Peyer’s patch are impaired. Further investigation will be required to fully delineate how loss of TCF-1 interrupts this sequence of events. Nkp46+ ILC3 play a pivotal role in controlling early susceptibility to infection with C. rodentium by increasing IL-22 production and tissue repair in the intestine (19). In line with this, failure to develop Nkp46+ ILC3 in the absence of TCF-1 resulted in diminished protection to infection, indicating that other ILC subsets do not compensate for the loss of Nkp46+ ILC3 and that these cells are an essential component of intestinal immune responses.

Strong parallels have been drawn between T cells, particularly CD4+ Th cell subsets in the adaptive arm of the immune system, and ILC subsets in the innate arm (48). Broad similarities in the signature cytokines produced and transcription factors led to the notion that very highly conserved transcriptional networks underpin the development of these two types of immune cells. Our data highlight these similarities, demonstrating that both TCF-1 and Notch are coordinately regulated in the thymus during early T cell development (34) and now are also required for ILC differentiation. However, prominent differences in exactly how this circuitry is put together suggest that the signaling networks in different lymphoid cells can be distinct. This is underlined by the requirement for TCF-1 in both ILC2 and Nkp46+ ILC3. Although activity of TCF-1 in ILC2 cells is akin to early development of T cells in the thymus and Th2 cells in the periphery, TCF-1 appears to modulate the terminal differentiation of Nkp46+ ILC3, which is more similar to the differentiation pathway used by CD8+ T cells (49, 50). However, in contrast to CD8+ T cells that express both T-bet and its paralog, eomesodermin (encoded by Eomes), Th1 cells that express T-bet, and Th2 cells that express T-bet, Eomes, and Gata3, neither ILC2 nor ILC3 express Eomes which, at least in Th2 cells, controls IL-5 production through Gata3, a signature cytokine of ILC2 (51). Thus, it appears that unique, but overlapping, combinations of factors enable differentiation and cytokine production. Our work emphasizes the need to further delineate differences between the molecular circuitry that guides the development and cytokine expression of innate lymphocyte subsets from other lymphocytes, such as T cells.

Acknowledgments

We thank Rebecca Cole and Jayne Vella for maintaining and caring for the mice and the Walter and Eliza Hall Institute of Medical Research flow cytometry core facility for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


