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T-bet is essential for NKp46⁺ innate lymphocyte development through the Notch pathway

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Abstract

Innate lymphoid cells (ILCs) including lymphoid tissue-inducer (LTi) cells, IL-22-producing NKp46⁺ innate cells and IL-13-producing nuocytes play important roles in regulating intestinal microbiota, defence against pathogens and formation of lymphoid tissue^{1–4}. Their development is dependent on Id2 and Ror γ t or Ror α ^{5–7}. Lineage tracing experiments have shown that the common lymphoid precursor gives rise to nuocytes, LTi cells and NKp46⁺ ILCs^{6,8,9}, but these studies have not deciphered the discrete steps and transcription factors that specify ILC subset development, activation and maintenance. Whether NKp46⁺ ILCs arise directly from LTi cells, or rather represent a separate lineage that diverges earlier in development, remains controversial^{10–12}. We investigated the requirement for the transcriptional master regulator T-bet (encoded by *Tbx21*) which is critical for the development of both T cells and NK cells^{13,14}, in driving differentiation of ILC populations. Here we report that T-bet played an essential role for the development of NKp46⁺ ILCs, but was dispensable for LTi cells or nuocytes. *Tbx21*^{+/+} LTi cells adopted an NKp46⁺ phenotype *in vitro* and *in vivo* but not in the absence of *Tbx21*. Decrease of T-bet expression coordinately reduced Notch1 and Notch2 and we show Notch signaling is necessary for the transition of LTi cells into NKp46⁺ ILCs. In addition, *Tbx21*^{-/-} mice have an accumulation in CD4⁻ LTi cells and differentiation into NKp46⁺ ILCs came solely from this population. Our results pinpoint T-bet as the critical regulator of NKp46⁺ ILC differentiation by regulation of Notch2 signaling. NKp46⁺ cells are an important element of the protective intestinal mucosal cellular arsenal, and here, we uncover the distinct molecular pathways that guide the development of NKp46⁺ ILCs.

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Keywords

Lymphoid tissue inducer cells; innate lymphoid cells; nuocytes; NKp46; GFP reporter mouse

An emerging theme linking the innate and adaptive arms of the immune system is the striking conservation of genetic regulatory programs driving cell differentiation and fate decisions¹⁵. ILC populations, namely LTi cells ($\text{Lin}^- \text{NKp46}^- \text{NK1.1}^- \text{c-Kit}^{\text{high}}$), NKp46⁺ ILCs ($\text{Lin}^- \text{NKp46}^+ \text{NK1.1}^{\text{low/-}}$) and NK cells ($\text{Lin}^- \text{NKp46}^+ \text{NK1.1}^+$) require the transcription factor Id2 for their development and Ror γ t is also essential for LTi cells and NKp46⁺ ILCs^{5,7}. The transcription factor T-bet (encoded by *Tbx21*) has been implicated in the development of both CD8⁺ T cells and NK cells^{13,16,17}, but has not been investigated in the lineage specification of these newly defined innate lymphocyte populations. To determine whether T-bet plays a role in ILC development, we took advantage of the *Rorc*(γ)^{gfp/+7} reporter mice and surface receptor staining to track each of these populations (Supplementary Fig. 1). We investigated ILC populations in naive *Rorc*(γ)^{gfp/gfp}, *Rorc*(γ)^{gfp/+}, *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice (Fig. 1). Strikingly, T-bet deficient mice completely lacked NKp46⁺ ILCs, while LTi cells could readily be detected (Fig. 1a). Immunohistochemistry on frozen sections confirmed that NKp46⁺Ror γ t-GFP⁺ cells could not be detected in the tissues of the small intestine in *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice despite the presence of cryptopatches (Fig. 1b and Supplementary Fig. 2), an important site of localization of NKp46⁺ ILCs in normal gut tissues¹⁸. Enumeration of the prevalence of ILC populations in lymphoid and gastrointestinal tissues confirmed a lack of NKp46⁺ ILCs and showed that NK cells were reduced in liver and spleen in *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice, consistent with previous reports^{13,19} (Supplementary Fig. 2).

Analysis of T-bet expression in innate lymphocyte populations by intracellular staining and mRNA levels revealed high expression of T-bet in both NKp46⁺ ILCs and NK ($\text{lin}^- \text{Ror}\gamma\text{t}^- \text{NKp46}^+ \text{NK1.1}^+$) cells isolated from the intestine, while T-bet was not expressed in LTi cells (Fig. 1c). The related T-box factor Eomes, that performs similar functions to T-bet in CD8⁺ T cells and NK cells, was not expressed in NKp46⁺ ILCs or LTi cells (Supplementary Fig. 3a). Thus, T-bet appears to play a crucial role in the development of NKp46⁺ ILCs. Similar to T-bet, Blimp1 (encoded by *Prdm1*) was strongly differentially expressed between LTi and NKp46⁺ ILC populations (Supplementary Fig. 3b). However, mice homozygously deficient for Blimp1 (*Blimp1*^{gfp/gfp}) did not show any apparent defect in the development of ILC subsets (Supplementary Fig. 3c). Thus, Blimp1 is dispensable for ILC differentiation while T-bet is essential.

T-bet is a critical regulator of many aspects of lymphocyte differentiation and may affect the expression of key cell surface receptors such as NKp46 used to identify ILCs. As *Tbx21*^{-/-} NK cells retained normal expression of NKp46, failure to detect NKp46⁺ ILCs in *Tbx21*^{-/-} mice was unlikely to be due to dysregulation of the NKp46 receptor itself. To further discount an effect of *Tbx21* on NKp46 expression in ILCs, we examined the expression of *Blimp1*. *Blimp1*^{gfp/+}*Tbx21*^{-/-} LTi cells did not express altered levels of Blimp1-GFP or other markers that would be characteristic of a contaminating NKp46⁺ ILC population

(Supplementary Fig. 4a,b). Thus, NKp46⁺ ILCs require T-bet for their development and failure to detect this population is not attributable to loss of receptor expression.

To uncover where the defect in generation of NKp46⁺ ILCs arises, we investigated ILC precursor cells found in foetal liver²⁰. Analysis of E14.5 embryonic liver cells showed no significant differences between the *Rorc*(γ)^{gfp/+} and *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} strains (Supplementary Fig. 5). Additionally, in adult mice the size and number of Peyer's patch did not differ between the two strains (Supplementary Fig. 6; data not shown) and tissues showed normal architecture with no evidence of inflammation (Supplementary Fig. 6) indicating that the loss of *Tbx21* did not obviously disrupt secondary lymphoid tissue formation. As no adverse affects were noted in gross histology, we investigated the importance of NKp46⁺ ILCs following *Citrobacter rodentium* infection. In this model, IL-22 production has a crucial role in the protection against *C. rodentium* dissemination, and NKp46⁺ ILCs are a key producer of this cytokine^{21,22}. Eight days following infection, *Tbx21*^{-/-} mice exhibited shorter colon lengths and increased bacterial dissemination into the liver and spleen (Supplementary Fig. 7a). The increased susceptibility to *C. rodentium* infection correlated with the loss of NKp46⁺ ILCs and fewer *Rorc*(γ)⁺ IL-22 producing ILCs (Supplementary Fig. 7c).

Loss of T-bet within the haematopoietic compartment results in dysfunction of various immune cells in the intestine. For example, T-bet deficient dendritic cells produce markedly elevated levels of tumour necrosis factor (TNF)- α that could influence the developing ILCs²³. Therefore, we investigated whether the absence of T-bet affected NKp46⁺ ILC development *via* a cell intrinsic mechanism. Mixed foetal liver chimeras were generated in which congenically marked wild-type *Ror* γ ^{gfp/+} (Ly5.1⁺Ly5.2⁺) and *Ror* γ ^{gfp/+}*Tbx21*^{-/-} (Ly5.2) foetal liver cells were mixed in a 1:1 ratio and transplanted into irradiated Ly5.1^{+/+} recipients allowing us to trace the hemopoietic origin of LTi, NKp46⁺ ILCs and NK cells. Eight weeks following reconstitution, T-bet-sufficient foetal liver progenitors reconstituted the NK, LTi and NKp46⁺ ILC compartments. In contrast, *Tbx21*^{-/-} foetal liver progenitors reconstituted LTi normally, NK cells poorly, and completely failed to reconstitute NKp46⁺ ILCs (Fig. 1d). Thus, the similar and specific loss of NKp46⁺ ILCs in both intact and foetal liver chimeric mice shows that this defect is attributable to a cell-intrinsic mechanism. To determine whether T-bet influences the capacity of mature LTi cells to differentiate into NKp46⁺ ILC *in vivo*, purified innate lymphocyte subsets from *Rorc*(γ)^{gfp/+} and *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice were adoptively transferred into *Rag2*^{-/-} γ ^c^{-/-} recipient mice that lack all ILCs¹⁵. After 14 days, transferred LTi cells and NKp46⁺ ILCs (*Ror* γ ⁺ intermediate and high) were recovered from the small intestinal lamina propria. While *Ror* γ ^{gfp/+} LTi cells were able to differentiate into NKp46⁺ ILCs, *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} cells failed to generate NKp46⁺ ILCs (Fig. 2a). Notably, NKp46⁺ ILCs generated from T-bet sufficient LTi cells did not express NK1.1 or the cytolytic molecule perforin distinguishing them from classical NK cells (data not shown). This was also the case when recipient mice were infected with *C. rodentium* to induce an inflammatory state (data not shown). Thus, mature LTi cells can give rise to NKp46⁺ ILCs *in vivo* in line with previous observations²⁴ but are unable to do this in the absence of T-bet.

The cues responsible for driving transition between LT α cells and NKp46 $^{+}$ ILCs are largely unknown, although Notch signaling has previously been implicated^{25,26}. To further explore the relationship between T-bet and Notch in NKp46 $^{+}$ ILC differentiation, we cultured purified ILC populations from *Rorc*(γ)^{gfp/+} and *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice on OP9 or Notch ligand expressing OP9-DL1 stromal cells in the presence of IL-7, SCF and Flt3L and analysed their differentiation (Fig. 2b). After 9 days, wild-type LT α cells generated NKp46 $^{+}$ ILCs only when exposed to a Notch ligand while *Tbx21*^{-/-} LT α cells failed to generate this subset. *Rorc*(γ)^{gfp/+} NKp46 $^{+}$ ILCs generated *in vitro* also upregulated their expression of T-bet solely in the presence of Notch ligands (Fig. 2c). However, in the absence of T-bet or when a Notch blocking antibody was added to wild-type cultures, induction of the NKp46 $^{+}$ ILC population was respectively blocked or markedly reduced (Fig. 4b,c, and data not shown). Combined, this data demonstrates an unappreciated link between T-bet and Notch signaling for the differentiation of NKp46 $^{+}$ ILCs.

NKp46 $^{+}$ ILCs express two distinct levels of Ror γ t-GFP, however the similar expression and requirement for T-bet in both populations suggests that these subsets are highly related, although the exact relationship between them remains unclear. *In vitro* exposure to Notch ligands showed that LT α cells generated predominantly Ror γ t-GFP^{int} cells (Fig. 2b). In contrast, adoptive transfer experiments showed the Ror γ t-GFP^{high} phenotype to be relatively stable and the Ror γ t-GFP^{int} NKp46 $^{+}$ subset produced a large proportion of Ror γ t-GFP^{high} cells (Fig. 2a), which suggests interconversion between these populations.

ILCs arise as progeny of LT α cells²⁴ (Fig. 2), but the contribution of CD4 $^{+}$ and CD4 $^{-}$ LT α cells to the differentiation of NKp46 $^{+}$ ILCs is unclear. Following the enumeration of all ILCs in *Rorc*(γ)^{gfp/+} and *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} mice, we found an increase in LT α cells in the gastrointestinal tract (Supplementary Fig.2). Interestingly, this increase was only observed within the CD4 $^{-}$ LT α subset, suggesting that loss of T-bet may prevent the developmental progression specifically from CD4 $^{-}$ LT α cells into NKp46 $^{+}$ ILCs (Fig. 3a,b). Indeed, using our OP9-DL1 Notch ligand culture system, NKp46 $^{+}$ ILCs could only be differentiated from sorted CD4 $^{-}$ and not from CD4 $^{+}$ LT α cells (Fig. 3c).

We further examined whether T-bet expression was also essential for other related ILC populations, such as nuocytes, in addition to its requirement for the development of NKp46 $^{+}$ ILCs (Supplementary Fig. 8)⁶. Similar to LT α and NKp46 $^{+}$ ILCs, nuocytes also expressed high levels of Id2-GFP (Supplementary Fig. 8a). However, in contrast to NKp46 $^{+}$ ILCs, *Tbx21*^{-/-} mice showed normal nuocyte numbers, frequency and Id2-GFP expression (Supplementary Fig. 8b-d). In addition, their capacity to produce the cytokine IL-13 and mediate eosinophil recruitment was unaltered (Supplementary Fig. 8e-g). Thus, T-bet appears to be specifically involved in the regulation of NKp46 $^{+}$ ILCs.

To determine whether a direct interaction between T-bet and Notch was required for differentiation of NKp46 $^{+}$ ILCs, we first analyzed ILC populations from the small intestine of *Tbx21*^{+/+} wild-type and haploinsufficient *Tbx21*^{+/-} mice for their expression of *Notch1* and *Notch2* (Fig. 4a). Both Notch family members exhibited a reduction in expression in NKp46 $^{+}$ ILCs isolated from *Tbx21*^{+/-} mice (Fig. 4a). To search for putative T-bet binding sites in the Notch genes we examined published ChIPseq analysis for T-bet in Th1 cells²⁷.

This revealed a T-bet binding site to intron 2 of *Notch1* and in the promoter of *Notch2*. To examine whether T-bet also bound in the Notch genes in ILCs we performed ChIP on *in vitro* cultivated NK cells, an ILC lineage that could be obtained in sufficient numbers for ChIP analysis. T-bet occupancy was only observed in *Notch2* and not *Notch1* (Fig. 4b.). While these data strongly suggest that T-bet directly regulates *Notch2* in NKp46⁺ ILC differentiation, the mechanism by which T-bet controls *Notch1* remains to be determined.

To further understand the relationship between T-bet and Notch-signaling in NKp46⁺ ILC differentiation, we expressed T-bet or the intracellular form of Notch (ICN) by lentiviral transduction into *Tbx21*^{-/-} LTi cells and cultured them on OP9 or OP9-DL1 cells for 5 days (Fig. 5c). Enforced expression of T-bet was able to rescue the formation of NKp46⁺ ILCs from *Tbx21*^{-/-} LTi cells in a Notch-signaling dependent manner. Most importantly, expression of a constitutively activated form of Notch (ICN) was sufficient to mediate differentiation of NKp46⁺ ILCs. The T-bet regulation of *Notch2* (and potentially *Notch1*) identifies a novel and distinct pathway that drives the differentiation of NKp46⁺ ILCs from LTi cells to promote the lineage diversity observed within the innate lymphocyte family.

Our finding that Notch is downstream of T-bet suggests a model whereby the induction of T-bet in mature LTi cells is the critical step in defining the NKp46⁺ ILC lineage by enabling a response to Notch-ligand mediated cues on adjacent cells. Interestingly, Notch expression in ILCs has also been proposed to partially depend on another environmental ligand inducible transcription factor, the aryl hydrocarbon receptor²⁶. Collectively, this implies a complex but highly regulated interplay between the gut microenvironment and innate lymphocytes in order to control gastrointestinal homeostasis.

Our data demonstrates CD4⁻ LTi cells are the direct precursor to NKp46 ILCs and identifies T-bet as the essential transcriptional regulator of NKp46⁺ ILCs, a key innate cell type required for intestinal immune protection. The ability of NKp46⁺ ILCs to rapidly expand from LTi cells in response to T-bet/Notch-signaling shows them to be a highly specialized innate cell type and suggests NKp46⁺ ILC expansion may be driven by integration of signals delivered by gut microbial communities through the T-bet pathway. Our studies have implications for how modulation of T-bet expression promotes tolerance or uncontrolled immune reactions leading to intestinal disease and pathology.

METHODS SUMMARY

All mice used were bred and maintained in specific pathogen-free facilities of WEHI. C57BL/6, Ly5.1, C57BL/6 × Ly5.1 (F1), B6.129S7-*Rag1*^{tm1Mom/J} (*Rag1*)²⁸, *Rag2*^{tm1.1Flv} × *Il2rg*^{tm1.1Flv} (*Rag2*^{γc}), *Id2*^{gfp} ²⁹, *Rorc*(*γ*)^{gfp} (*Rorγ*^{gfp}) ⁷, *Tbx21*^{-/-} ³⁰, *Tbx21*^{-/-} *Rorc*(*γ*)^{gfp} and *Blimp1*^{gfp} ³¹ were used. Flow cytometric analyses was performed on antibody stained lymphocyte populations and analysed using a CantoII flow cytometer (BD Biosciences) and FlowJo analysis software. Intracellular staining for cytokines was performed using the Cytofix/Cytoperm kit (BD Biosciences). Mice were lethally irradiated (2 × 0.55 Gy), reconstituted with a mixture of *Tbx21*^{-/-} (Ly5.2⁺) and *Tbx21*^{+/+} (Ly5.1⁺/5.2⁺) foetal liver cells at a ratio of 1:1 and mice were allowed to reconstitute for 8 weeks. Intestinal lymphoid cells were isolated from intestine by incubations in 5mM EDTA Ca²⁺ Mg²⁺ free Hanks

medium (2 × 20 min) followed by digestion with Collagenase III (2 × 30 min, 37 °C, 1 mg/ml; Worthington)/DNase I (200 µg/ml; both from Roche) and Dispase (4U/mL) then Mononuclear cells were isolated with 40%/80% Percol gradient. Purified lymphoid progenitors and ILC populations were cultured on OP9 or OP9-DL1 cells in complete IMDM supplemented with Flt3L (20 ng/ml), 2% supernatant of an IL-7-producing cell line and SCF (10 ng/mL)²⁴. Cells were cultured for 9 days prior to analysis. Total RNA was prepared from purified ILC populations for quantitative RT-PCR using an RNeasy mini kit (Qiagen). cDNA was synthesized from total RNA with OligodT and thermoscript reverse transcriptase (Invitrogen). Real-time PCR was performed using the SensiMix SYBR no-Rox kit (Bioline). Tissues for histological analyses were fixed in 10% neutral buffered formalin and processed routinely. Immunohistological tissues were fixed in 4% paraformaldehyde, sucrose infiltrated and embedded in OCT freezing medium prior to staining for CD3, B220, CD326, NKp46. Images were acquired on a LSM780 confocal microscope (Carl Zeiss Mircoimaging) and quantified using IMARIS image analysis software (Bitplane). Statistical analysis was performed using unpaired student's *t*-test, two tailed.

Full Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NKp46⁺ ILCs fail to form in the absence of the transcription factor T-bet

Innate lymphocytes isolated from the small intestinal lamina propria were analysed for T-bet expression. **a**, Lamina propria cells were isolated from the small intestine (upper panels) and colon (lower panels) of *Rorc*(γ)^{gfp/+}, *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-}, and *Rorc*(γ)^{gfp/gfp} mice. It should be noted that in the colon, only a single population of NKp46⁺Ror γ ⁺ cells exists in *Tbx21*^{+/+} mice and this population was also lacking in *Tbx21*^{-/-} mice. Numbers indicate the percentage of live CD45⁺lin⁻ (CD11c⁻Gr-1⁻CD19⁻CD3ε⁻CD45⁺) cells for gated type of cell. Plots are representative of

at least 3 independent experiments with three to four mice of each genotype per experiment. **b**, Small intestine sections from naïve *Rorc*(γ)^{gfp/+} (upper panels) and *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} (lower panels) mice showing cryptopatches stained with anti-NKp46 (light blue), anti-CD3ε or anti-B220 (red), anti-CD326 (EpcAM, white) to delineate gut epithelial cells, and endogenous expression of Ror γ t-GFP (green). Inset shows NKp46⁺ staining in Ror γ t⁺ cells. Scale bar, 50μM. Data are representative of at least three independent experiments (*n*=6 per genotype). **c**, Innate lymphocytes from small intestinal lamina propria expressing different levels of Ror γ t-GFP (left panel, gating strategy shown in dot plot) were stained for surface lineage markers, then fixed and permeabilized prior to staining to detect intracellular T-bet (right panels, histograms). Grey histogram shows T-bet specific stain, dotted line indicates control stain. **c**, Gating strategy and analyses of individual innate lymphocyte subsets purified by flow cytometry. *Tbx21* mRNA was analysed by quantitative PCR and show mean $\pm$ s.e.m. normalised to *Hprt* of triplicate samples showing one representative experiment of three. Dot plots are gated on lin⁻CD45⁺ cells. **d**, Intrinsic T-bet expression is required for NKp46⁺ ILC development. Mixed foetal liver chimeric mice were generated by transplanting Ly5.1/5.2⁺ *Rorc*(γ)^{gfp/+} and Ly5.2⁺ *Rorc*(γ)^{gfp/+}*Tbx21*^{-/-} cells into lethally irradiated Ly5.1 mice. After 8 weeks, innate lymphocytes were isolated from the lamina propria of the small intestine and colon and the capacity for *Tbx21*^{+/+} (Ly5.1^{+/5.2}⁺) or *Tbx21*^{-/-} (Ly5.2⁺) foetal liver cells to reconstitute innate lymphocytes was evaluated. Numbers indicate the frequency of each ILC population within the lin⁻CD45⁺ live SYTOX Blue⁻ cell gate which were then examined for expression of α 4 β 7, c-Kit, IL-7R and ROR γ t-GFP. Data show representative profiles from two independent experiments (*n*=8) with similar results. As gated cells in (c) are fixed, some change in Ror γ t-GFP resolution is observed when compared to unfixed cells shown in (a, d).

Figure 2. Notch and T-bet regulate the developmental pathway of innate lymphocyte populations
a, *In vivo* differentiation of ILC subsets in the presence or absence of T-bet. $0.5-1 \times 10^5$ LTi cells or NKp46⁺ ILCs were purified from the small intestinal lamina propria of either *Rorc*(γ)^{gfp/+} or *Rorc*(γ)^{gfp/+} *Tbx21*^{-/-} mice then adoptively transferred into Rag2 γ c-deficient mice. After 14 days, donor lymphocytes were isolated from the small intestinal lamina propria of recipient mice and analysed for the differentiation of innate lymphocyte populations. Data show representative profiles of one of four independent experiments with similar results. Dot plots are gated on lin⁻CD45⁺ live donor cells. **b**, *In vitro* differentiation

of LTi or NKp46⁺ mature ILCs purified from the small intestinal lamina propria of *Rorc*(γ)^{gfp/+} and *Rorc*(γ)^{gfp/+} *Tbx21*^{-/-} mice cultured on OP9 or OP9-DL1 stromal cells in the presence of SCF, IL-7 and Flt3L and analysed on day 9 of culture. Data are representative of at least 2-4 independent experiments with similar results. **c**, T-bet expression in ILC subsets differentiated as in **b**.

Figure 3. CD4⁻ and not CD4⁺ LTi cells give rise to NKp46⁺ ILCs

a. Frequency of CD4⁻ and CD4⁺ LTi subsets isolated from the small intestine of *Tbx21*^{+/+} and *Tbx21*^{-/-} mice. **b.** Total cell number of CD4⁻ and CD4⁺ LTi cells in the small intestine of *Tbx21*^{+/+} compared with T-bet deficient mice. Data show mean ± s.e.m (*n*=4/group). **c.** CD4⁻ LTi, CD4⁺LTi and NKp46⁺ ILCs cells were sorted from *Tbx21*^{+/+} mice and cultured on OP9-DL1 cells in the presence of IL7, SCF and Flt-3L. Differentiated cells were analyzed for NKp46 expression after 5 days. Data are representative of two independent experiments with similar results.

Figure 4. Notch and T-bet regulate the developmental pathway of innate lymphocyte populations
a, Quantitative RT-PCR analyses of *Notch1* and *Notch2* in ILCs isolated from the small intestine of mice of the indicated genotypes. RT-PCR analyses show the mean $\pm$ s.e.m. of samples analysed in triplicate; ILC subsets show mean percent CD45⁺lin⁻ (CD11c⁻Gr-1⁻CD19⁻CD3e⁻CD45⁺) PI⁻ cells $\pm$ s.e.m. isolated from small intestinal lamina propria. Data are representative of at least three similar experiments. **b**, Binding of T-bet within the *Notch1* and *Notch2* genes was analysed using ChIP. Data are the mean enrichment calculated as the percentage enrichment compared to non-immunoprecipitated

input DNA $\pm$ s.d. from 3 independent samples. **c.** Rescue of NKp46⁺ ILC development in LTi cells by retroviral expression of either T-bet or Notch ICS in *Tbx21*^{-/-} LTi cells and coculture on OP9 or OP9-DL1 stromal cells for 5 days. Shown are flow cytometry plots gated on live Cherry⁺ cells. Numbers indicate the proportion of cells within the transduced population (Cherry⁺) that differentiated into NKp46⁺ ILCs.

Figure 5. CD4⁻LTi cells, not CD4⁺LTi cells, give rise to NKp46⁺ ILCs
(a) Flow cytometry of LTi subsets isolated from the small intestine of $Rorc(\gamma t)^{+/GFP}Tbx21^{+/+}$ and $Rorc(\gamma t)^{+/GFP}Tbx21^{-/-}$ mice. Numbers adjacent to outlined areas indicate percent CD4⁻LTi cells (bottom) and CD4⁺LTi cells (bottom). **(b)** Total CD4⁻ and CD4⁺LTi cells in the small intestine of $Rorc(\gamma t)^{+/GFP}Tbx21^{+/+}$ and $Rorc(\gamma t)^{+/GFP}Tbx21^{-/-}$ mice ($n = 4$ per genotype). NS, not significant; * $P = 0.0083$ (unpaired Student's t -test). **(c)** NKp46 expression by CD4⁻LTi cells, CD4⁺LTi cells and NKp46⁺ ILCs sorted from wild-type mice and cultured for 5 d on OP9-DL1 cells in the presence of IL-7, SCF and Flt-3L. Data are representative of at least two experiments (mean and s.e.m. in **b**).