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# Transforming growth factor- $\beta$ signaling guides the differentiation of innate lymphoid cells in salivary glands

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## Summary

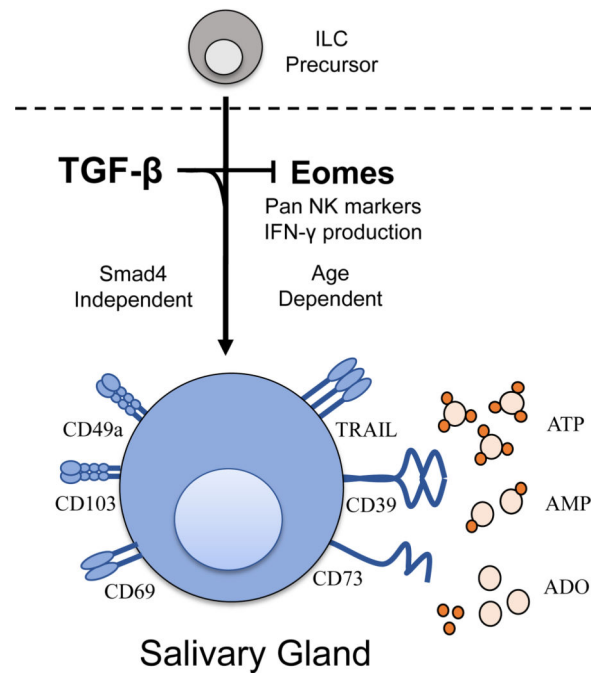
The signals guiding differentiation of innate lymphoid cells (ILCs) within tissues are not well understood. Salivary gland (SG) ILCs as well as liver and intestinal intraepithelial ILC1 have markers that denote tissue residency and transforming growth factor- $\beta$  (TGF- $\beta$ ) imprinting. We deleted *Tgfb2* in cells expressing the ILC and NK marker NKp46 and found that SG ILCs were reduced in number. They lost distinct tissue markers, such as CD49a, and the effector molecules TRAIL and CD73. Expression of the transcription factor Eomes, which promotes NK cell differentiation, was elevated. Conversely, *Eomes* deletion in NKp46<sup>+</sup> cells enhanced TGF- $\beta$ -imprinting of SG ILCs. Thus, TGF- $\beta$  induces SG ILC differentiation by suppressing Eomes. TGF- $\beta$  acted through a JNK-dependent, Smad4-independent pathway. Transcriptome analysis demonstrated that SG ILCs had characteristic of both NK cells and ILC1. Finally, TGF- $\beta$  imprinting of SG ILCs was synchronized with SG development, highlighting the impact of tissue microenvironment on ILC development

## Graphical Abstract

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## Keywords

innate lymphoid cells; salivary gland; TGF- $\beta$ ; development; signaling; transcription factors

## Introduction

Innate lymphoid cells (ILCs) are lymphocytes that lack antigen-specific receptors, and instead recognize cytokines and other soluble mediators released into the tissue microenvironment during pathogen invasion and perturbations of homeostasis (Diefenbach et al., 2014; Eberl et al., 2015). ILCs provide a first line of defense by producing cytokines that activate innate responses. Based on the signature cytokines they produce, ILCs can be divided into three populations: group 1 ILCs produce interferon- $\gamma$  (IFN- $\gamma$ ) in response to interleukin-12 (IL-12), IL-15 and IL-18; group 2 ILCs produce type 2 cytokines such as IL-5 and IL-13 when stimulated by IL-25, thymic stromal lymphopoietin, and IL-33; and group 3 ILCs are prompted by IL-23 and IL-1 $\beta$  to produce IL-22 and/or IL-17. Lymphoid tissue-inducer (LTi) cells are a subset of group 3 ILCs that promote lymphoid organogenesis in the embryo and have a phenotypically similar correlate present in the adult called LTi-like ILC3. Enriched at mucosal surfaces, ILCs are present throughout the adult body and have been implicated in tissue homeostasis, early control of infection, tissue repair, and regulation of adaptive immunity.

ILCs originate from the common lymphoid progenitor, which gives rise to a common innate lymphoid progenitor (CILP) with a restricted differentiation potential that includes all innate lymphocytes (ILC1, ILC2, ILC3 and LTi-like ILC3 cells) as well as natural killer (NK) cells (Yu et al., 2014). The CILP further differentiates into precursors with more restricted potential: the NK cell progenitor (NKP) generates NK cells, while the common helper

lymphoid progenitor (CHILP) gives rise to ILC1, ILC2, ILC3 and LT $\alpha$ -like ILC3 cells (Klose et al., 2014; Xu et al., 2015). Downstream of the CHILP, the innate lymphoid common progenitor (ILCP) can engender ILC1, ILC2 and ILC3 but not LT $\alpha$ -like ILC3 (Constantinides et al., 2014). Specification of distinct ILC lineages from precursors is controlled by transcription factor (TF) networks. Some transcription factors, like Nfil3 and the transcriptional repressor Id2, act early during development at the ILCP and CHILP stages respectively. Other TFs are required for the development of specific subsets. For example, Eomes and T-bet are both required for NK cell development, ROR $\gamma$ t is required for all ILC3, and T-bet is further required for ILC1 and a subset of ILC3 (NKp46<sup>+</sup>ILC3). GATA3 is required early on at the CHILP stage, but is further required for ILC2 lineage specification and ILC3 homeostasis (Diefenbach et al., 2014; Tindemans et al., 2014; Yagi et al., 2014).

Although ILC subsets mainly reside in peripheral tissues, there is limited information regarding the signals that impact ILC differentiation within tissues. In previous studies, we reported the characterization of salivary gland (SG) ILCs (Cortez et al., 2014) and showed that these cells express a unique array of cell-surface molecules thought to contribute to tissue residency. One hallmark of these cells is the integrin CD103, which tethers lymphocytes to epithelial cells by binding E-cadherin. Other markers include CD49a, which promotes lymphocyte homing to non-lymphoid tissues (Meharra et al., 2000), and CD69, which inhibits surface expression of sphingosine-1-phosphate (S1P) receptor 1 (S1PR1), blocking lymphocyte egress from tissues in response to S1P gradients (Shiow et al., 2006). Similar phenotypic features are found in intestinal intraepithelial ILC1 (Fuchs et al., 2013) and liver ILC1 (also called liver resident NK cells) (Daussy et al., 2014; Peng et al., 2013; Sojka et al., 2014). SG ILCs also have special functional features. They are poor producers of IFN- $\gamma$  and cytolytic degranulation (Tessmer et al., 2011), but express TNF-related apoptosis-inducing ligand (TRAIL) (Cortez et al., 2014), a cell surface molecule that induces the death of cells expressing the cognate TRAIL receptor (TRAILR). Moreover, SG ILCs highly express CD39 (Cortez et al., 2014), an ectonucleotidase that degrades ATP into ADP and AMP (Deaglio and Robson, 2011). SG ILCs also have unique developmental requirements: like NK cells, they express both T-bet and Eomes, but, in contrast to NK cells and ILC1, these cells do not require Nfil3 (Cortez et al., 2014). Because of their unique phenotypic, functional and developmental features, SG ILCs do not precisely fit the ILC1 and NK cell paradigm.

Since TGF- $\beta$  induces CD103 (Robinson et al., 2001) and promotes tissue retention of CD8<sup>+</sup> memory T cells (Mackay et al., 2013; Zhang and Bevan, 2013), we hypothesized that this cytokine may play a crucial role in the unique phenotype of SG ILCs as well as intraepithelial intestinal ILC1 and liver ILC1. To test this hypothesis, we generated a mouse strain that lacks TGF- $\beta$  signaling in NKp46<sup>+</sup> cells, which include SG ILCs, NK cells, ILC1 and NKp46<sup>+</sup> ILC3. Here we report that lack of TGF- $\beta$ -signaling markedly reduced SG ILC numbers and the expression of CD49a, CD103 and CD69, while inducing NK cell markers. Lack of TGF $\beta$ R2 also abrogated expression of some SG ILC effector molecules, such as TRAIL and the ectonucleotidase CD73 described here. In contrast, TGF $\beta$ R2 deficiency had minimal impact on the phenotypes of liver ILC1 and gut ILC1, perhaps suggesting that other TGF- $\beta$  superfamily members may guide their differentiation. Mechanistically, TGF- $\beta$  influenced SG ILC differentiation by suppressing Eomes expression and acting in a Smad4-

independent fashion through a non-canonical signaling pathway involving Jun N-terminal kinase (JNK). TGF- $\beta$  imprinting of SG ILCs was synchronized with SG development, which also depends on TGF- $\beta$ , highlighting the impact of tissue microenvironment on ILC development.

## Results

### The unique phenotype of SG ILCs is dependent on TGF- $\beta$ signaling

ILCs within the SG are largely made up of NK1.1<sup>+</sup>CD3<sup>-</sup> cells (SG ILCs), with minor populations of ILC2 and ILC3 (**Figure S1A**). Since NK1.1<sup>+</sup> SG ILCs, intestinal ILC1 and liver ILC1 have markers that reflect TGF- $\beta$  imprinting, we asked whether expression of these markers is paralleled by TGF- $\beta$  expression within tissues. We measured mRNA expression of the three mammalian isoforms of TGF- $\beta$  – *Tgfb1*, *Tgfb2* and *Tgfb3* – in the SG, small intestine, liver and spleen. *Tgfb1-3* transcripts were highly expressed in the SG, with *Tgfb2* and *Tgfb3* being 100- and 10- fold more abundant in the SG than in the spleen, respectively (**Figure 1A**). *Tgfb2* and *Tgfb3* expression in the SG was also higher than in the small intestine, corroborating that the SG environment is very rich in TGF- $\beta$ . The small intestine was second to the SG in *Tgfb1-3* transcript abundance. The spleen contained the most *Tgfb1*, but relatively little *Tgfb2* and *Tgfb3*. Finally, the liver had the lowest expression of all TGF- $\beta$  isoforms.

All TGF- $\beta$  isoforms signal through heterodimeric complexes that share the TGF- $\beta$ -receptor type II receptor (TGF $\beta$ R2) (Massague, 2012). Therefore, to assess the impact of TGF- $\beta$  signaling on the development of NK receptor-expressing ILCs, we generated *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice, which lack TGF $\beta$ R2 in all NKp46<sup>+</sup> cells, including SG ILCs, ILC1, NKp46<sup>+</sup> ILC3 and NK cells. As *Tgfb2* and *Tgfb3* were most highly expressed in the SG and the phenotype of NK1.1<sup>+</sup> SG ILC is rather distinct from that of ILC1 and NK cells (Cortez et al., 2014), we hypothesized that TGF- $\beta$  may substantially influence the characteristics of these cells. The numbers of SG ILCs were reduced by approximately 50% in *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice compared to WT littermate controls (**Figure 1B**). We found no difference in numbers, maturation or function of NK cells in the spleens of *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice in the steady state (**Figure S1B-E**). This result was corroborated in *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice bred at a second university (**Figure S1C and data not shown**). Previously, transgenic mice expressing a dominant negative form of TGF $\beta$ R2 under the control of the *Itgax* promoter were reported to have increased numbers and accelerated maturation of NK cells in the spleen and bone marrow (Marcoe et al., 2012). The discrepancy in NK cell phenotypes may be due to the different approaches used to abrogate TGF- $\beta$  signaling, i.e expression of a dominant negative TGF $\beta$ R2 receptor in CD11c<sup>+</sup> cells versus TGF $\beta$ R2 receptor deletion in NKp46<sup>+</sup> cells.

Lack of TGF- $\beta$  signaling also impacted the markers that distinguish SG ILCs. Whereas most WT SG ILCs expressed CD49a and CD49b (also known as DX5), CD103 and CD69, SG ILCs from *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice lost expression of CD49a (**Figure 1C**) and had reduced expression of CD103 and CD69 (**Figure 1D**). These changes were paralleled by increased expression of CD62L, NKp46, and the NK maturation markers CD27 and CD43 (**Figure 1D**). Thus, TGF- $\beta$  signaling is critical for the differentiation of SG ILCs and maintenance of

their phenotypic features. The lack of TGF- $\beta$  signaling did not impact the numbers of CD3<sup>-</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> cells, which include NK cells and ILC1, within the liver and small intestine (**Figure S1F**). Moreover, expression of CD49a, CD69, and CD62L were unchanged on liver ILC1, while expression of CD49a was minimally reduced on intestinal cells (**Figure S1G**). Examination of NKp46<sup>+</sup> ILC3 in the small intestine of *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* mice revealed normal cell numbers (**Figure S1H**). Thus, TGF- $\beta$  signaling is minimally required for gut ILC1 and dispensable for liver ILC1 and intestinal ILC3.

### TGF- $\beta$ impacts the functional capabilities of SG ILCs

SG ILCs are ineffective at producing IFN- $\gamma$  and releasing lytic granules in comparison to splenic NK cells (Cortez et al., 2014; Tessmer et al., 2011). However, SG ILCs can execute immune functions through alternative effector mechanisms. We previously showed that SG ILCs express the death-inducing ligand TRAIL (**Figure 1E** and (Cortez et al., 2014)). They also highly express CD39, an ectonucleoside triphosphate diphosphohydrolase that hydrolyzes ATP and ADP to AMP (Cortez et al., 2014). We further investigated the expression of nucleotidases and found that SG ILCs also express CD73 (**Figure 1F**), an ecto-5-nucleotidase that degrades extracellular AMP to adenosine (Deaglio and Robson, 2011). CD73 was particularly abundant in cells with high amounts of CD39 (**Figure 1F**). Thus, a considerable subset of SG ILCs can degrade extracellular ATP into adenosine. CD73 was also expressed on intestinal ILC1 and more weakly on liver ILC1, but not on splenic NK cells (**Figure S1I**).

TGF- $\beta$  influenced SG ILCs effector molecules as much as their phenotype. Indeed, *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* SG ILCs no longer expressed TRAIL and CD73, while CD39 was maintained (**Figure 1E, F**). Moreover, SG ILCs from *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* mice acquired the ability to robustly produce IFN- $\gamma$  in response to stimulation with IL-12 and IL-18 or PMA and ionomycin (**Figure 1G and not shown**). *Tgfb2<sup>-/-</sup>* SG ILCs remained poor at releasing lytic granules in comparison to NK cells (**Figure 1H**). Taken together, these data demonstrate that SG ILCs have unique effector capabilities that partially depend on TGF- $\beta$ . Abrogation of TGF- $\beta$  signaling converts SG ILCs into robust IFN- $\gamma$ -producing cells.

### TGF- $\beta$ induces the expression of SG ILC markers by NK cells

We next asked whether TGF- $\beta$  was sufficient to induce SG ILC markers on splenic NK cells. We purified splenic CD49b<sup>+</sup> NK cells from WT or *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* mice, cultured them with TGF- $\beta$  plus IL-2 or IL-2 alone, and analyzed cells on days 3, 5, and 7. Before culture, splenic NK cells expressed CD49b and NKp46, but not CD49a, CD103, TRAIL, or CD73 (**Figure 2A and Figure S2**). Expression of CD49a, CD103, TRAIL, and CD73 became evident on WT cells cultured with TGF- $\beta$ 1 and IL-2 by day 5 and increased through day 7 (**Figure 2A, B**), while CD49b was reduced (**Figure S2**). In contrast, these changes did not occur in *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* cells (**Figure 2A**). Culture of WT cells with IL-2 alone induced CD49a, moderate expression of TRAIL and CD73, but not CD103 (**Figure 2A**). However, these changes were not apparent in *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* cells, suggesting the presence of some TGF- $\beta$  in this culture condition, presumably contributed by the serum supplement. Thus, exposure of splenic NK cells to TGF- $\beta$  is sufficient to induce expression of a SG ILC-like phenotype.

## TGF- $\beta$ signaling directs SG ILCs differentiation through suppression of Eomes

We previously reported that SG ILCs express both Eomes and T-bet, two T-box transcription factors that play essential roles in the development of NK cells and ILC1, respectively (Daussy et al., 2014; Glimcher et al., 2004; Gordon et al., 2012; Klose et al., 2014; Seillet et al., 2014; Sojka et al., 2014). We also have shown that SG ILCs develop independently of Nfil3 (Cortez et al., 2014), a TF required for development of NK cells and ILCs. Corroborating this observation, we found that Eomes and T-bet expression was Nfil3 independent in SG but not spleen (**Figure S3A, B**). To determine if SG ILCs are T-bet and/or Eomes dependent, we assessed SG ILCs in *Eomes<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice, which lack Eomes in all NKp46<sup>+</sup> cells, and *Tbx21<sup>-/-</sup>* mice, which constitutively lack T-bet. In both mouse lines, SG ILCs numbers were comparable to those in WT mice (**Figure 3A**), whereas splenic NK cells were strongly reduced (**Figure S3C**). Thus, SG ILCs, in contrast to NK cells and ILC1, are not strictly dependent on either transcription factor for development.

Further analysis of *Eomes<sup>f/f</sup> x Ncr1<sup>cre</sup>* SG ILCs revealed that deletion of Eomes enhanced the unique phenotypic features of SG ILCs while reducing those shared with NK cells. Surface expression of CD49b was reduced while CD49a remained unchanged (**Figure 3B**). NK1.1 and NKp46 were also reduced (**Figure 3C and Figure S3D**), CD103 and CD73 were increased (**Figure 3C**), and TRAIL expression was not impacted (**Figure S3E**). Moreover, while CD73 expression in WT SG ILCs was primarily localized to CD39<sup>hi</sup> cells, in *Eomes<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice, CD73 became broadly expressed in both CD39<sup>hi</sup> and CD39<sup>lo</sup> populations (**Figure 3D**). In contrast to *Eomes<sup>f/f</sup> x Ncr1<sup>cre</sup>* SG ILCs, *Tbx21<sup>-/-</sup>* SG ILCs showed no major changes in the expression of various markers (**Figure S3F, G**). These data indicate that SG ILC differentiation does not require Eomes, which in fact antagonizes the acquisition of TGF- $\beta$  imprinted features. Corroborating this, we observed that surface expression of CD49a and CD103 on SG ILCs was inversely correlated with intracellular expression of Eomes: CD49a<sup>hi</sup> and CD103<sup>hi</sup> SG ILCs cells had reduced amounts of Eomes compared to CD49a<sup>-</sup> or CD103<sup>-</sup> SG ILCs, which expressed more Eomes (**Figure 3E**).

Given that Eomes is a negative regulator of SG ILC differentiation, we asked whether TGF- $\beta$  may act, at least in part, by suppressing Eomes. Indeed, *Tgfb $\beta$ 2<sup>f/f</sup> x Ncr1<sup>cre</sup>* SG ILCs expressed more Eomes than WT SG ILCs, whereas the amounts of T-bet were similar (**Figure 3F**). We also analyzed Eomes in splenic NK cells from WT and *Tgfb $\beta$ 2<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice cultured with TGF- $\beta$ 1, which is sufficient to induce prototypic SG ILC markers. Culture of WT cells with TGF- $\beta$ 1 plus IL-2 triggered a reduction in Eomes compared to culture in IL-2 alone (**Figure 3G**). In contrast, expression of Eomes was identical in *Tgfb $\beta$ 2<sup>f/f</sup> x Ncr1<sup>cre</sup>* cells cultured with TGF- $\beta$ 1 plus IL-2 or IL-2 alone. Altogether, these data demonstrate that TGF- $\beta$  signaling suppresses Eomes expression in SG ILCs, while Eomes reciprocally attenuates their TGF- $\beta$ -induced signature.

## TGF- $\beta$ signals in SG ILCs through a non-canonical pathway

TGF- $\beta$ -induced activation and dimerization of TGF $\beta$ R2 and TGF $\beta$ R1 is followed by phosphorylation of Smad2 and Smad3, which in turn bind to Smad4. This Smad complex then translocates into the nucleus and triggers transcription of target genes (Massague, 2012). TGF- $\beta$  also transmits intracellular signals through non-canonical pathways that

involve MAPK activation, such as Rho-Rac-JNK, TAK1-p38 and Ras-Erk (Zhang, 2009). To determine whether TGF- $\beta$  signaling in SG ILCs involves the canonical pathway, we generated *Smad4<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice and examined SG ILCs. We found that SG ILC numbers were very similar in *Smad4<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice and WT littermate controls (**Figure 4A**). *Smad4<sup>f/f</sup> x Ncr1<sup>cre</sup>* SG ILCs did not substantially change their characteristic expression of CD49a, CD103, CD69, CD62L, TRAIL, and CD73 (**Figure 4B, C and S4**). To further substantiate that TGF- $\beta$  induction of SG ILC markers is Smad4-independent, we cultured splenic NK cells from *Smad4<sup>f/f</sup> x Ncr1<sup>cre</sup>* mice with TGF- $\beta$ 1 plus IL-2 or IL-2 alone. *Smad4*-deficient cells strongly upregulated CD103, TRAIL, and CD73 in the presence of TGF- $\beta$ 1 (**Figure 4D**), corroborating that TGF- $\beta$  signaling in SG ILCs involves a non-canonical pathway. To test this hypothesis, we cultured WT CD49b<sup>+</sup> splenic NK cells with IL-2 or IL-2 and TGF- $\beta$ 1 in the presence or absence of pharmacological MAPK inhibitors that suppress non-canonical TGF- $\beta$  signaling. The JNK inhibitor SP600125 abrogated TGF- $\beta$  induction of TRAIL and CD73, whereas CD49a was only partially reduced (**Figure 4E**). Thus, TGF- $\beta$  induces SG ILCs phenotype through the JNK pathway, although other Smad4-independent signals may contribute.

### SG ILCs are a unique subset of group 1 ILCs

Although clearly belonging to Group 1 ILCs, given the influence of TGF- $\beta$  on their phenotype and their unique developmental TF requirements and expression profile, SG ILCs do not precisely fit into the ILC1 and NK cell paradigm. We sought to gain a clearer understanding of the relationship of these cells with other ILC populations by gene expression analysis using whole mouse genome microarray. CD49a<sup>+</sup> SG ILCs from 6 week-old C57BL/6 mice were sorted in collaboration with the ImmGen Project in accordance with their standardized protocol for data generation. We then compared these data to the expression profiles of ILC2, ILC3, ILC1, and NK populations from liver, intestine, and spleen, which we previously profiled for ImmGen (Robinette et al., 2015). Unsupervised hierarchical cluster analysis showed that SG ILCs form a separate cluster within the group 1 ILCs, which is distinct from spleen and liver NK cells as well as from liver, spleen, and intestinal intraepithelial ILC1 (**Figure 5A**). Supervised principal-component analysis (PCA) of the top 15% of most variable probesets corroborated that SG ILCs cluster with other group 1 ILCs, rather than with ILC2 or ILC3 (**Figure 5B**).

Comparison of SG ILCs with splenic NK cells confirmed differential expression of previously identified genes such as *Itga1*, *Tnfsf10* and *Cd69* (encoding CD49a, TRAIL and CD69 respectively) in SG ILCs and *Sell* (encoding CD62L) in NK cells (**Figure 5C**). It also revealed several genes significantly upregulated in SG ILCs, such as *Gzmc* and *IL21r* (**Figure 5C**). CD49a<sup>+</sup> SG ILCs shared common expression with ILC1 populations of genes like *Itga1*, *Gzmc*, *Tnfsf10*, *IL21r*, *Tnf*, *Cxcr6*, and *Tcrg* germline transcripts (**Figure 5D and Figure S5**). However, SG ILCs also shared some common transcriptional features with NK cells, most notably the expression of *Eomes* and *Klra8* (encoding Ly49H) and the lack of *IL7ra* (**Figure 5D and S5**). When all transcripts significantly upregulated at least 2-fold between CD49a<sup>+</sup> SG ILCs and spleen NK cells were assessed in other group 1 ILCs, many factors were unique to SG ILCs or only expressed in one ILC1 subset (**Figure 5E**). Thus, SG ILCs express some characteristic transcripts of both ILC1 and NK cells, including those



that have been proposed to define the NK lineage, such as *Eomes*, and the ILC lineage, such as *Cxcr6* and *Tcrp* (**Figure S5**), but also have a unique gene signature likely indicative of SG tissue imprinting.

We next asked whether any differences in gene expression patterns between SG ILCs and NK cells were TGF- $\beta$  dependent. SG ILCs (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>) and NK cells (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>) were sorted from WT and *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> mice and key gene products were evaluated by real time PCR or cell surface staining. *Gzmc* mRNA and IL-21R surface expression were completely dependent upon TGF- $\beta$  (**Figure 5F, G**). Interestingly, other SG ILC phenotypic features such as lack of KLRG1 expression, high expression of Ly49H and glucocorticoid-induced tumor necrosis factor receptor (GITR), were unaltered between WT and *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> mice (**Figure 5G**). These data demonstrate that SG ILCs have a unique gene expression profile with characteristics of both ILC1 and NK cells and that differential expression of at least some of these genes, including Granzyme C and IL-21R, are dependent upon TGF- $\beta$  signaling.

### Circulating cells minimally replace SG ILCs in WT and TGF $\beta$ R2-deficient mice

We next considered why SG ILC were reduced in *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> mice. We hypothesized that lack of TGF- $\beta$  and reduction of tissue residency molecules may facilitate increased recirculation of SG ILCs, reducing the number of cells that reside in the tissue. To test this, we performed parabiosis experiments using congenically marked CD45.1 WT mice and CD45.2 mice that were either WT or *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup>. In these experiments, CD45.1 and CD45.2 mice were surgically joined through the skin, promoting the formation of microvasculature at the site of junction that allows sharing of the cells circulating in the blood (Peng et al., 2013). Analysis of CD45.1 WT and CD45.2 WT parabiont pairs four weeks after joining showed that approximately 90-95% of SG ILCs belonged to the parabiont host and expressed CD49a; the remaining cells belonged to the parabiont donor (**Figure 6A, B**). In contrast, ~40% of splenic NK cells derived from the parabiont donor (**Figure S6A, B**). Thus, SG ILCs are predominantly tissue-resident and only a small percent derive from the blood, consistent with a recently published report (Gasteiger et al., 2015). Similar to naïve mice, parabiotic pairs of CD45.1 WT and *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> CD45.2 showed reduced frequencies of SG ILCs in the *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> parabiont compared to WT parabiont, as well as lack or reduction of CD49a, CD103 and CD69 and partial acquisition of CD62L (**Figure 6C, D**). However, SG ILCs from *Tgfb2*<sup>f/f</sup> *x* *Ncr1*<sup>cre</sup> mice did not show any greater chimerism than WT SG ILCs (**Figure 6E**). As the frequency of SG ILCs in parabiotic pairs was similar to naïve mice of the same genotype and chimerism was identical between genotypes, these data indicate that there is little active replacement of SG ILCs in adult mice by circulating cells, even in the absence of TGF- $\beta$  imprinted tissue-residency markers. We further conclude that the SG niche in adult mice is not actively attracting substantial numbers of recirculating WT NK cells or ILC progenitors to replace *Tgfb2*<sup>-/-</sup> ILCs.

### TGF- $\beta$ promotes survival of tissue resident SG ILCs

Because parabiosis experiments demonstrated no difference in tissue residency between genotypes, we next investigated whether apoptosis contributed to the reduction of SG ILCs

in *Tgfb2<sup>fl/fl</sup>* x *Ncr1-cre* mice. We noticed no major differences in cell death of SG ILCs directly *ex vivo*, likely due to uptake of dying cells within the tissue (**data not shown**). Therefore, we cultured WT or *Tgfb2<sup>fl/fl</sup>* x *Ncr1<sup>cre</sup>* SG single cell suspensions for 1 hour at 37°C in PBS to promote stress-induced death of SG ILCs. We then analyzed cell death by 7-AAD staining. *Tgfb2<sup>fl/fl</sup>* x *Ncr1-cre* SG ILCs showed significantly increased cell death compared to their WT counterparts (**Figure 6F**). Additionally, we found that the inositol phosphatase *Inpp4b* and the transcription factor *Tsc22d1*, which have been implicated in promoting resistance to apoptosis in certain cell types (Rijal et al., 2015; Huser et al., 2010), were highly expressed by SG ILC in a TGF-β dependent manner (**Figure S6C**). This suggests that in addition to promoting the differentiation of SG ILCs, TGF-β may also support their viability, at least in part by controlling the expression of cell survival genes.

### SG ILCs differentiation is linked to SG maturation

We sought to identify the factors responsible for the strong TGF-β influence on SG ILCs. The microbiota can induce a TGF-β-rich environment in the intestine that promotes the differentiation of tolerogenic T cells (Atarashi et al., 2013). We asked whether the microbiota was required for the marked TGF-β signature of SG ILCs. SG ILCs from germ-free mice maintained elevated expression of CD49a and CD103 (**Figure S7A, B**), suggesting that TGF-β expression and signaling in the SG is independent of the microbiota.

The SG differentiates through a process known as branching morphogenesis that requires TGF-β (Jaskoll and Melnick, 1999; Lourenco et al., 2008). Specifically, TGF-β induces the expression of E-cadherin and fibronectin in the clefts that deepen into primordial glandular buds creating branches. Therefore, we asked whether SG ILC differentiation is linked to SG development. It has been shown that the differentiation of SGs in mice is not complete at birth, but continues into adulthood (Larsen et al., 2010). To examine whether ILC differentiation and SG development are linked, we compared *Tgfb1-3* transcripts and ILCs in young (3-4 week-old) and adult (10-16 week-old) mice, in which SG development is ongoing or complete, respectively. We found lower *Tgfb2* and *Tgfb3* transcripts in the SGs of young mice than in those of adult mice (**Figure 7A**). Moreover, SG ILCs in young mice were less abundant than in adult mice (**Figure S7C**), and a sizeable subset further lacked CD49a and CD103 expression (**Figure 7B, C**). SG ILCs of young mice had higher expression of Eomes than those of adult mice (**Figure S7D**), but had similar expression of CD69, CD62L, TRAIL, and CD73 (**Figure 7C**), suggesting an ongoing process of tissue imprinting on SG ILCs during SG development.

We noticed that the intermediately aged 6-week-old mice used for ImmGen sorting protocols almost equally represented CD49a<sup>-</sup> and CD49a<sup>+</sup> SG ILC subsets (**Figure S7E**). To further interrogate the sequence of TGFβ-driven imprinting found between young and old mice, we sorted both populations and analyzed gene expression by whole mouse genome array with ImmGen. Relatively few genes were differentially expressed between CD49a<sup>-</sup> and CD49a<sup>+</sup> SG ILCs. CD49a<sup>-</sup> cells had higher expression of *Klf2* and *S1pr1*, which are indicative of increased tissue egress capacity (Skon et al., 2013), and lower expression of *Gzmc*, *Cxcr6*, and *Tcrγ* germline transcripts (**Figure 7D**). These differences mirror the transcripts differentially regulated between CD49a<sup>+</sup> SG ILCs and splenic NK cells, though with a lesser

fold-change (**Figure 5C, D**, and data not shown). PCA of the top 15% of most variable probesets among ImmGen sorted group 1 ILCs showed that CD49a<sup>-</sup> SG ILCs clustered intermediately between NK cells and CD49a<sup>+</sup> SG ILCs (**Figure 7E**). Furthermore, both CD49a<sup>+</sup> and CD49a<sup>-</sup> SG ILCs expressed transcripts downstream of TGF- $\beta$  signaling while splenic NK cells did not (**Figure S7F**). Altogether, these data suggested that CD49a<sup>-</sup> cells are immature SG ILCs that are to some extent tissue-imprinted and are in the process of maturing under the influence of TGF- $\beta$ . We hypothesized that CD49a<sup>-</sup> cells may be a progenitor with different proliferation kinetics. To test this hypothesis, we evaluated the proliferative capacity of CD49a<sup>-</sup> and CD49a<sup>+</sup> SG ILCs from WT mice by injecting them with 1mg BrdU i.p. for 3 consecutive days followed by analysis of BrdU incorporation. This revealed an enhanced proliferative capacity of CD49a<sup>-</sup> compared to CD49a<sup>+</sup> SG ILCs (**Figure S7G**). These data suggest that CD49a<sup>-</sup> SG ILCs are actively proliferating, and under the influence of TGF- $\beta$ , reduce their expansion and persist as more terminally imprinted CD49a<sup>+</sup> cells.

We also noted that while SG ILCs were present in similar numbers in adult WT and *Nfil3*<sup>-/-</sup> mice, their numbers were markedly reduced in young *Nfil3*<sup>-/-</sup> mice compared to WT littermate controls (**Figure 7F, G**). Altogether these data indicate that SG ILC differentiation is temporally linked with SG development and progressive accumulation of TGF- $\beta$ , which drives the proliferation of CD49a<sup>-</sup> *Nfil3*-dependent immature SG ILCs into CD49a<sup>+</sup> *Nfil3*-independent mature SG ILCs.

## Discussion

We investigated the function of TGF- $\beta$  in ILC differentiation prompted by previous observations that SG ILCs, intraepithelial ILC1 in the oral and intestinal mucosa and liver ILC1 have a signature indicative of TGF- $\beta$  imprinting (Cortez et al., 2014; Fuchs et al., 2013; Peng et al., 2013). This signature includes expression of CD49a, CD103 and CD69, all of which promote retention of lymphocytes within tissues. A similar signature was observed in tissue-resident memory T cells (T<sub>RM</sub>), which are present in the gut mucosa, skin and brain (Mueller and Mackay, 2016; Schenkel and Masopust, 2014) and depend on TGF- $\beta$  for the expression of CD103, CD49a and CD69 and tissue residency (Mackay et al., 2013; Zhang and Bevan, 2013). Our analysis of *Tgfbr2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice demonstrated that SG ILCs require TGF- $\beta$  signaling for the expression of CD49a, CD103 and CD69 as well as effector molecules, such as TRAIL and CD73. Corroborating a role of TGF- $\beta$  in inducing these molecules, NK cells cultured in the presence of TGF- $\beta$  acquired a phenotype similar to that of SG ILCs.

TGF- $\beta$  had a marked influence on the TF profile of SG ILCs, and specifically suppressed Eomes. SG ILCs from *Tgfbr2*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice showed increased Eomes expression. Conversely, SG ILCs from *Eomes*<sup>fl/fl</sup> x *Ncr1*<sup>cre</sup> mice showed enhanced TGF- $\beta$  imprinting. In SG ILCs, expression of Eomes and TGF- $\beta$  dependent markers were inversely correlated. Thus, we envision that TGF- $\beta$ -mediated suppression of Eomes may be largely responsible for the unique phenotype of SG ILCs, including the reduced expression of NK markers (Cortez et al., 2014; Tessmer et al., 2011). However, TGF- $\beta$  may also influence SG ILCs differentiation by controlling other TF, such as Runx3, which has recently been shown to be

important for survival of these cells (Ebihara et al., 2015). TGF- $\beta$  can signal through Smad-dependent and Smad-independent pathways (Massague, 2012; Zhang, 2009). TGF- $\beta$  induction of SG ILC differentiation was Smad4-independent as SG ILCs in *Smad4<sup>fl/fl</sup> x Ncr1<sup>cre</sup>* mice had normal numbers and phenotypes. In contrast, the capacity of TGF- $\beta$  to induce SG ILC molecules in cultured cells was impaired by a pharmacological JNK inhibitor, indicating that TGF- $\beta$  acts through a non-canonical signaling pathway that involves JNK and the downstream c-Jun and AP-1 transcription factors. Similarly, a non-canonical TGF- $\beta$  signaling pathway has recently been shown to induce differentiation of lung-resident memory T cells (Hu et al., 2015) and to skew Th cell differentiation from Th1 to Th17 by suppressing Eomes (Ichiyama et al., 2011).

Consistent with their unique phenotypic and TF features, SG ILCs emerged in transcriptome analysis as a distinct subset of group 1 ILCs with some features between ILC1 and NK cells. Compared to splenic NK cells, SG ILCs shared with ILC1 populations increased expression of CXCR6 and *Tcr $\gamma$*  germline transcripts, both of which are part of the core ILC signature (Robinette et al., 2015), though at lower amounts than ILC1 cells. They also expressed more TRAIL, CD73, Granzyme C and IL-21R. However, SG ILCs also shared some phenotypic features with NK cells compared to ILC1, such as the expression of Eomes, Ly49H and lack of IL7Ra. Similar to the intermediate expression of ILC signature transcripts, Eomes expression was lower in SG ILCs than in NK cells. Remarkably, SG ILCs expressed both T-bet and Eomes, like NK cells, but unlike NK and ILC1 cells, were able to develop in the absence of either TF.

Besides impacting the phenotype and functions of SG ILCs, lack of TGF- $\beta$  signaling caused a reduction in SG ILCs. TGF- $\beta$  may control the SG ILC numbers through multiple mechanisms. First, TGF- $\beta$  signaling may facilitate the retention of SG ILCs, which our parabiosis experiments and a recently published study (Gasteiger et al., 2015) have shown to be tissue resident. Second, we found that *Tgfb $\beta$ 2*-deficient SG ILCs were more susceptible to death than WT SG ILCs. Moreover, TGF- $\beta$  signaling directly impacted the expression of *Inpp4b* and *Tsc22d1* in SG ILC, two genes that have been shown to promote resistance to apoptosis in other cell types (Huser et al., 2010; Rijal et al., 2015). Thus, TGF- $\beta$  may sustain SG ILC numbers by promoting their survival through pathways that will require further studies. It is also possible that TGF- $\beta$  facilitates local proliferation of SG ILCs to replace cells egressing the SG.

The origin of the SG ILCs and their development are yet to be defined. Our parabiosis experiments indicate that in adult mice, the SG niche is not recruiting new cells from the circulation even when there are low numbers of ILCs, as in *Tgfb $\beta$ 2*-deficient mice. In addition, circulating NK cells are unlikely to be major precursors of SG ILCs, as NK cells are strongly reduced in T-bet-, Eomes- and Nfil3-deficient mice, whereas SG ILCs are present in normal numbers in these mice, at least in the steady state. SG ILCs may develop from a progenitor that migrates early on in the developing SG, which may be the immature CD49a<sup>-</sup> ILCs or a cell further upstream. Accordingly, in young mice we identified a clear population of proliferating CD49a<sup>-</sup> ILCs, which was poorly represented in adult mice, showed incomplete TGF- $\beta$  imprinting and was dependent on Nfil3. These data support a linear differentiation model of SG ILC from a CD49a<sup>-</sup> proliferating stage to a CD49a<sup>+</sup> stage

that progressively acquires markers of tissue residency and long-term survival capacity under TGF- $\beta$  imprinting.

Why does TGF- $\beta$  signaling have such a dominant impact on SG ILCs differentiation? All TGF- $\beta$  isoforms are expressed in the SGs during development and have an important role in glandular organogenesis, influencing branching morphogenesis and extracellular matrix deposition (Jaskoll and Melnick, 1999; Lourenco et al., 2008). We postulate that the increasing production of TGF- $\beta$  during development and its persistence in large amounts drives the differentiation and maintenance of ILCs. Repeated episodes of SG inflammation, such as those occurring in Sjögren's syndrome or after tissue damage from radiotherapy treatment of head-and-neck cancer (Martin et al., 2000), cause excessive TGF- $\beta$  production and salivary gland fibrosis, resulting in restriction of secretory components and hyposalivation. It will be important to establish whether SG ILC differentiation is affected in these disorders and contributes to their pathogenesis. Since TGF- $\beta$  is required for branching morphogenesis of SG, TGF- $\beta$  may influence ILCs differentiation in other glands and organs formed by branching morphogenesis, such as mammary glands.

The function of SG ILCs remains poorly understood. Although SG ILCs are only weakly cytolytic and produce little IFN- $\gamma$  (Tessmer et al., 2011), they express TRAIL and may kill target cells that express TRAIL-R. During MCMV infection in BALB/c mice, NKp46<sup>+</sup>CD3<sup>-</sup> cells within the SG have been shown to kill activated CD4<sup>+</sup> T cells through TRAIL-TRAILR interactions, limiting T cell-mediated tissue damage (Schuster et al., 2014). Moreover, we showed that SG ILCs express a set of ectonucleotidases capable of degrading the proinflammatory ATP into the immunosuppressive adenosine. The same ectonucleotidases, CD39 and CD73, are expressed on regulatory T (Treg) cells and contribute to their regulatory function. Similar to Treg cells, we found that SG ILCs also express high amounts of GITR, another protein known to be involved in immunoregulation (Curotto de Lafaille and Lafaille, 2002). We envision that SG ILCs may serve a local regulatory function by limiting the amplitude and/or the duration of immune responses in order to preserve glandular integrity and salivation.

Lack of TGF- $\beta$  signaling had little impact on intestinal intraepithelial and lamina propria ILC1s and no influence on liver ILC1s. We noticed that TGF- $\beta$  superfamily cytokines, such as inhibin C, inhibin E, Bmp2 and Bmp4, which trigger the same intracellular signaling pathways initiated by TGF- $\beta$ , are also highly expressed in the gut and liver (data not shown). It is possible that these cytokines may replace TGF- $\beta$  in the intestinal mucosa and the liver, yet act in a similar fashion by driving the expression of tissue residency markers. We conclude that the impact of TGF- $\beta$  signaling in ILC development is tissue-dependent.

## Experimental Procedures

### Mice

*Tgfbr2<sup>fl/fl</sup>* (B6;129-*Tgfbr2<sup>tm1Karl</sup>/J*) and *Smad4<sup>fl/fl</sup>* (*Smad4<sup>tm2.1Cxd</sup>/J*) mice were obtained from the Jackson Laboratory; *Eomes<sup>fl/fl</sup>* mice were provided by Dr. Takeshi Egawa; *Ncr1<sup>Cre</sup>* mice (Narni-Mancinelli et al., 2011) were bred to *Tgfbr2<sup>fl/fl</sup>*, *Smad4<sup>fl/fl</sup>*, *Eomes<sup>fl/fl</sup>*, mice to generate *Tgfbr2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>*, *Smad4<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>*, and *Eomes<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice respectively and

littermate controls. *Nfil3*<sup>-/-</sup> mice and *Tbx21*<sup>-/-</sup> were provided by Dr. Paul Rothman and Dr. Wayne M. Yokoyama, respectively. Germ Free mice were a kind gift of Dr. Jeffrey Gordon.

### **Antibodies and Flow Cytometry**

The antibodies used for flow cytometric analysis are indicated in Supplemental Experimental Procedures. Stained cells were analyzed on a FACS Canto using Diva software or on a FACS Calibur using CellQuest software. For transcription factor detection cells were first stained for surface markers followed by fixation and permeabilization with the Foxp3 staining buffer set (eBioscience). Cells were then resuspended in 1x permwash solution and stained with antibodies. For detection of intracellular cytokines, cells were seeded in 48 well plates followed by the addition of IL-12 (10 ng/mL, Peprotech) and IL-18 (100 ng/mL, MBLI) or PMA (10<sup>-7</sup> M, Sigma) and ionomycin (1 ug/mL, Sigma). After 1 hr, Brefeldin A (10ug/mL, Sigma) was added to each well and stimulation was allowed to continue for a total of 6 hours. Cells were then washed, surface stained, and fixed using IC fixation buffer (eBioscience). Fixed cells were permeabilized with 1x permwash buffer and stained for intracellular cytokines.

### **Tissues and Cell Isolation**

Salivary gland lymphocytes were enriched as previously described (Cortez et al., 2014). Splenic NK cells were enriched by magnetic cell sorting using CD49b microbeads (Miltenyi Biotec). The isolation of intestinal and hepatic lymphocytes are described in the Supplemental Experimental Procedures.

### **Cell cultures for in vitro differentiation and cell death**

Cells were cultured in complete medium with either IL-2 or IL-2 and 10ng TGF-β1 (Peprotech). After 3, 5, or 7 days in culture, cells were stained for surface markers. SP600125 (Sigma) was diluted in DMSO and added at a final concentration of 10μM per sample every 24 hours at day 1 and 2, followed by staining of surface markers at day 3. To detect cell death, SG were processed to a single cell suspension, then cultured in PBS for 1 hour at 37C and then stained with antibodies followed by addition of 7-AAD.

### **Parabiosis**

Experiments were performed as previously described (Peng et al., 2013). Briefly, skin incisions were made on the flanks of CD45.1 and CD45.2 C57BL/6 or CD45.2 *Tgfb2*<sup>fl/fl</sup> x *Ncr1*<sup>Cre</sup> female mice, followed by joining of the incisions with wound clips. Mice were joined for a total of 4 weeks. Postoperatively mice received pain medication and antibiotics for the duration of the experiment.

### **qRT-PCR**

Spleen, salivary gland, small intestine and liver tissue was dissected and immediately preserved in RNAlater solution (Ambion). RNA was extracted with an RNeasy Mini kit (Qiagen). cDNA was synthesized from RNA with Superscript III first-strand synthesis system for RT-PCR (Invitrogen). RNA expression was analyzed by quantitative PCR using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) and an ABI7000 (Applied

Biosystems). The expression of target mRNA was calculated and normalized to the expression of the house keeping gene *Gapdh* using the  $2^{-CT}$  method.

## Statistical Analysis

All statistical analyses were performed using Prism 5.0 (GraphPad Software). All data were analyzed with a nonpaired Student's *t* test. A p-value of <0.05 was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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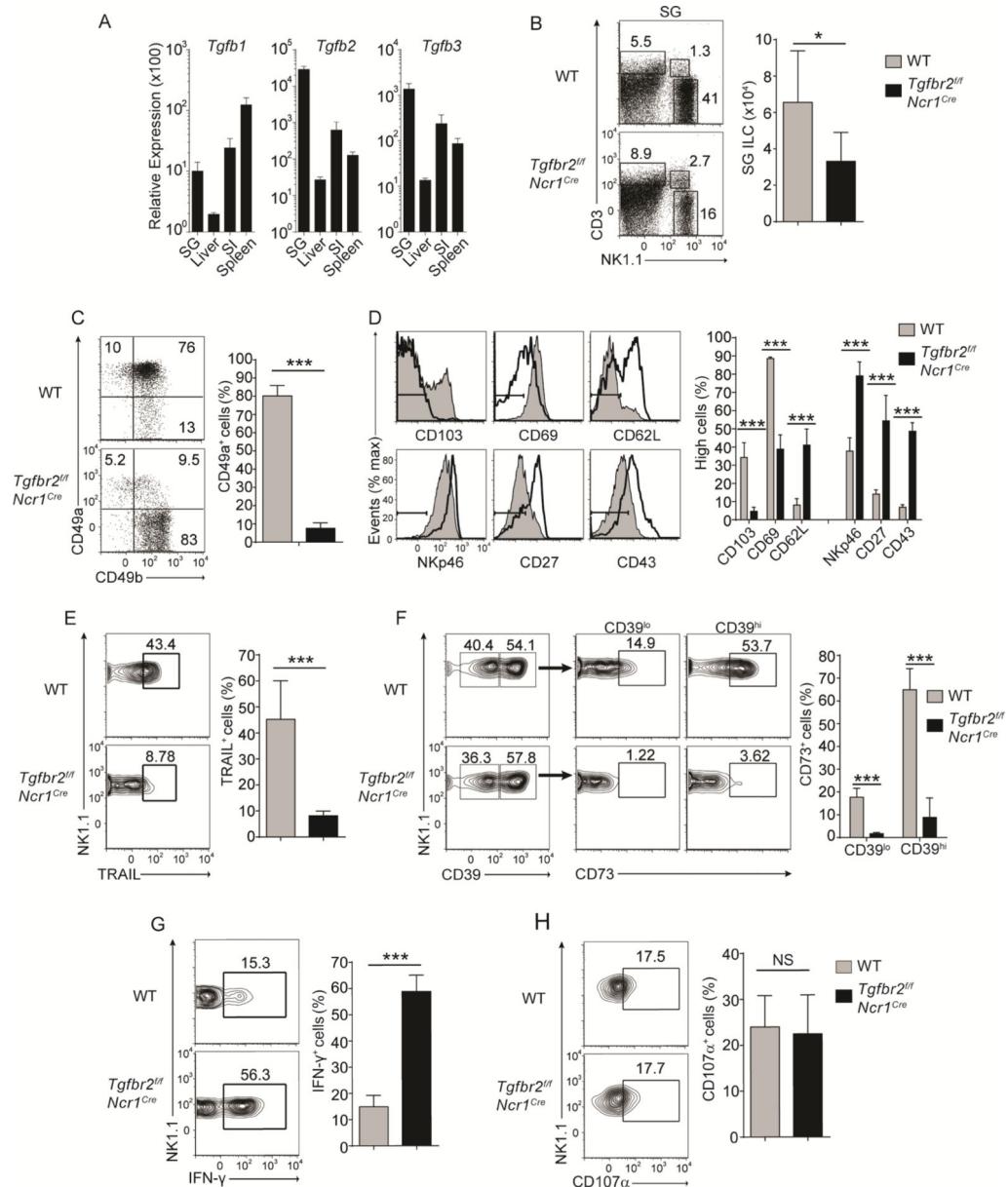
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### **Highlights**

- TGF- $\beta$  is required for SG ILCs differentiation
- TGF- $\beta$  promotes SG ILC differentiation by suppressing Eomes
- TGF- $\beta$  acts through a non-canonical Smad4-Independent pathway
- SG ILCs have features of both ILC1 and cNK cells



**Figure 1. Impact of TGF-βRII deficiency on the distinct phenotype of SG ILCs**

(A) Relative expression of *Tgfb1*, *Tgfb2*, and *Tgfb3* from different tissues of WT mice determined by qPCR. n = 3 mice.

(B) Frequencies and absolute numbers of SG ILCs (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>) from WT and *Tgfb2*<sup>fl/fl</sup> *Ncr1*<sup>Cre</sup> mice. n = 8-10 mice per group.

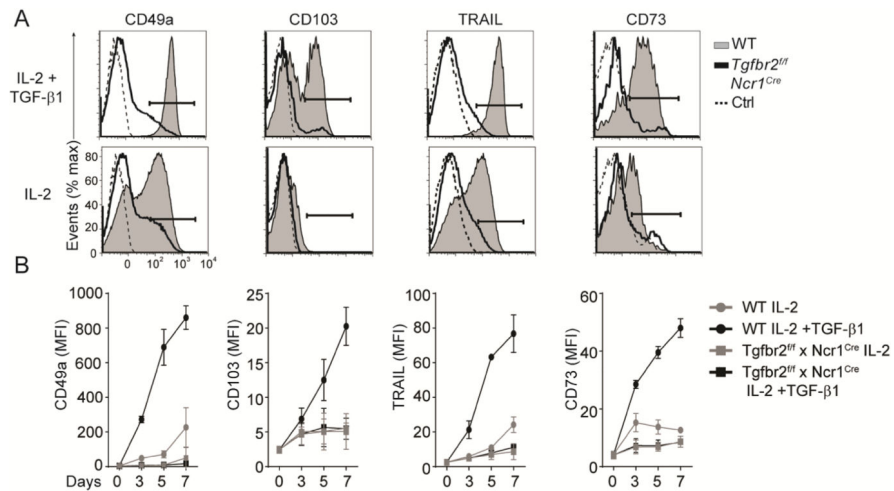
(C) Expression of CD49a and CD49b by SG ILCs from WT and *Tgfb2*<sup>fl/fl</sup> *Ncr1*<sup>Cre</sup> mice. n = 8 mice.

(D) Expression of tissue markers and NK cell markers by SG ILCs from WT and *Tgfb2*<sup>fl/fl</sup> *Ncr1*<sup>Cre</sup> mice (horizontal black bars represent negative staining). n = at least 5 mice per group.

(E-F) Expression of (E) TRAIL and (F) CD39 and CD73 (within CD39 gates) *ex vivo* on SG ILCs from WT and *Tgfb2*<sup>fl/fl</sup> *Ncr1*<sup>Cre</sup> mice. n = at least 6 mice per group.

**(G and H)** Expression of **(G)** intracellular IFN- $\gamma$  and **(H)** surface CD107a after stimulation with IL-12 plus IL-18 or PMA and ionomycin of SG ILC from WT and *Tgfb $\beta$ 2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice. n = 5 mice per group.

**(A)** All gene values were normalized to the expression of the housekeeping gene *Gapdh*. **(B-H)** Data are presented as mean  $\pm$  SD and are representative of at least three independent experiments. Student's t test, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for comparisons between WT control and *Tgfb $\beta$ 2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* groups. See also Figure S1.

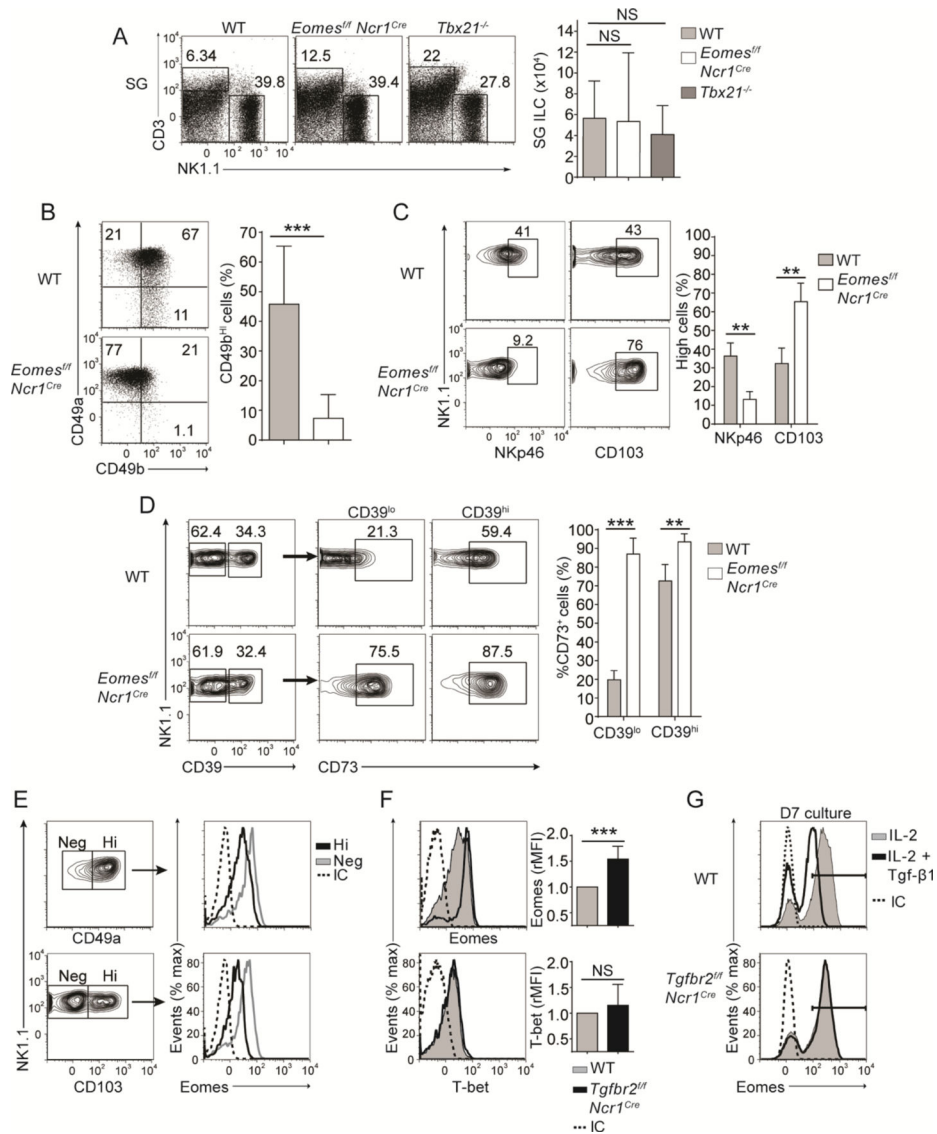


**Figure 2. TGF-β is sufficient to induce SG ILC phenotypic markers on NK cells**

(A) Expression of SG ILC markers on purified splenic NK cells from WT or *Tgfbr2<sup>fl/fl</sup>* x *Ncr1<sup>Cre</sup>* after 7 days in culture with either IL-2 or IL-2 TGF-β1. Control is staining for the indicated markers directly after purification of NK cells on day 0.

(B) MFI of the indicated markers on day 0, 3, 5, and 7 in NK cell cultures from WT or *Tgfbr2<sup>fl/fl</sup>* x *Ncr1<sup>Cre</sup>* in the presence of either IL-2 or IL-2 plus TGF-β1.

(A-B) Data are representative of at least two independent experiments for each time point. See also Figure S2.

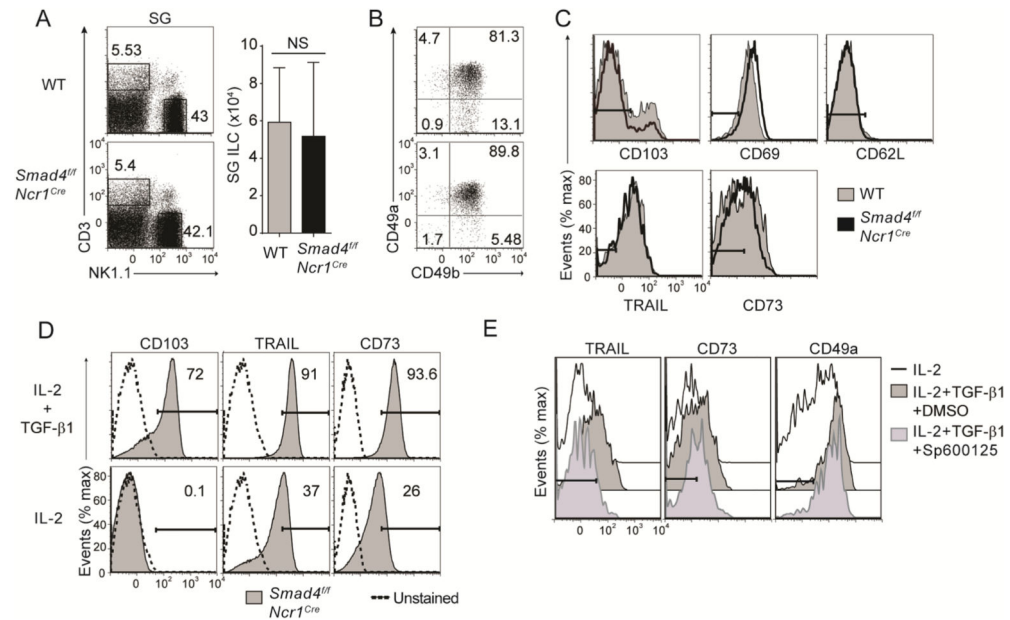


**Figure 3. Reciprocal effects of Eomes and TGF-β on SG ILCs differentiation**

- (A) Frequencies and absolute numbers of SG ILCs from WT, *Eomes<sup>ff</sup> x Ncr1<sup>Cre</sup>*, and *Tbx21<sup>-/-</sup>* mice. n= at least 5 mice per group.
- (B) Expression of CD49a and CD49b by SG ILCs from WT and *Eomes<sup>ff</sup> x Ncr1<sup>Cre</sup>* mice. n = 6 mice.
- (C) Expression of NKp46 and CD103 on SG ILCs from WT and *Eomes<sup>ff</sup> x Ncr1<sup>Cre</sup>* mice. n = 5-6 mice per group.
- (D) Expression of CD39 and CD73 (within CD39<sup>LO-HI</sup> gates) on SG ILCs from WT and *Eomes<sup>ff</sup> x Ncr1<sup>Cre</sup>* mice. n = 4 mice per group.
- (E) Overlay of histograms showing Eomes expression in CD49a<sup>Hi</sup> versus CD49a<sup>-</sup> and CD103<sup>Hi</sup> versus CD103<sup>-</sup> populations of SG ILCs.
- (F) Expression of intracellular Eomes and T-bet from SG ILCs of WT and *Tgfb2<sup>ff</sup> x Ncr1<sup>Cre</sup>* mice. n = 6 mice per group.

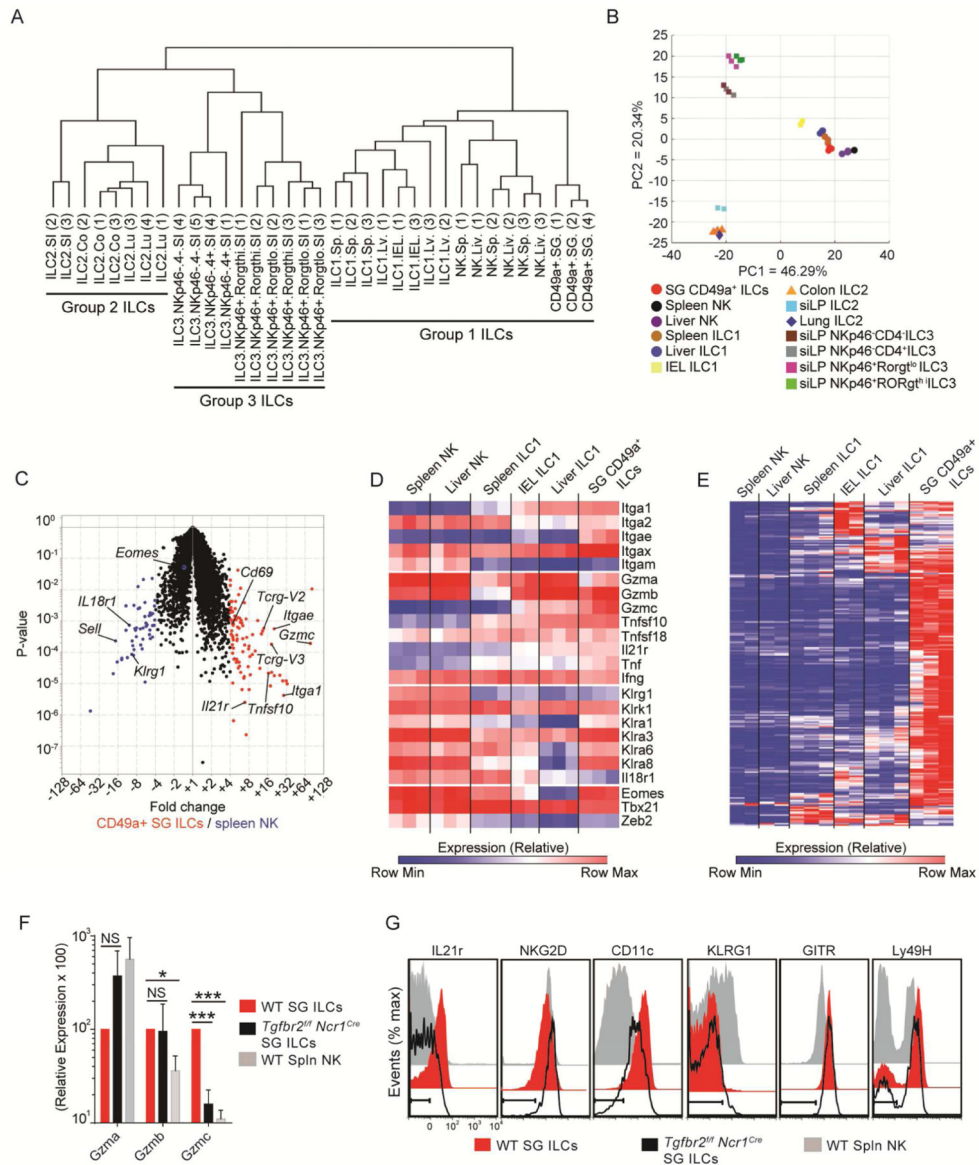
**(G)** Eomes expression in purified splenic CD49b<sup>+</sup> NK cells from WT or *Tgfbt2<sup>ff</sup> x Ncr1<sup>Cre</sup>* mice after 7 days in culture with either IL-2 or IL-2 plus TGF-β1. IC = isotype control.

**(A-D and F)** Data are presented as mean ± SD and are representative of at least two independent experiments. Student's t test, \*\*p < 0.01, \*\*\*p < 0.001 for comparisons between WT control and *Eomes<sup>ff</sup> x Ncr1<sup>Cre</sup>*, *Tbx21<sup>-/-</sup>* groups. **(E and G)** Data are representative of at least two independent experiments with 3 mice per group. See also Figure S3.



**Figure 4. SG ILCs require non-canonical TGF-β signaling for their distinct phenotype**  
**(A)** Frequencies and absolute numbers of SG ILCs from WT and *Smad4<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice. n = 8 mice per group.  
**(B)** Expression of CD49a and CD49b by SG ILCs from WT and *Smad4<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice. n = 8 mice.  
**(C)** Expression of the indicated markers on SG ILCs from WT and *Smad4<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice. n = at least 4 mice per group  
**(D)** CD103, TRAIL, and CD73 expression on splenic NK cells from *Smad4<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice after 7 days in culture with either IL-2 or IL-2 plus TGF-β1.  
**(E)** TRAIL, CD73, and CD49a expression on splenic NK cells from WT mice after 3 days in culture with IL-2 or IL-2 plus TGF-β1 in the presence of DMSO or the JNK inhibitor SP600125. SP600125 or DMSO were added at day 1 and 2 of culture.  
**(A)** Data are presented as mean ± SD. **(B-E)** Data are representative of at least two independent experiments with at least 3 mice per group. See also Figure S4.





**Figure 5. Microarray analysis of CD49a<sup>+</sup> SG ILC**

(A) Unsupervised hierarchical cluster analysis of CD49a<sup>+</sup> SG ILCs and group 1, group 2, and group 3 ILC populations from indicated tissues.

(B) Supervised principal component analysis of CD49a<sup>+</sup> SG ILCs and indicated group 1, group 2, and group 3 ILC populations from tissues.

(C) Comparison of gene expression of splenic NK cells (n=3 replicates) and CD49a<sup>+</sup> SG ILCs (n=3 replicates) by volcano plot; colored dots indicate transcripts significantly upregulated by at least four-fold.

(D) Heatmap of the expression of selected genes normalized by the indicated group 1 ILCs.

(E) Heatmap of the transcripts significantly upregulated at least two-fold between CD49a<sup>+</sup> SG ILCs and splenic NK cells, in comparison to other group 1 ILCs.

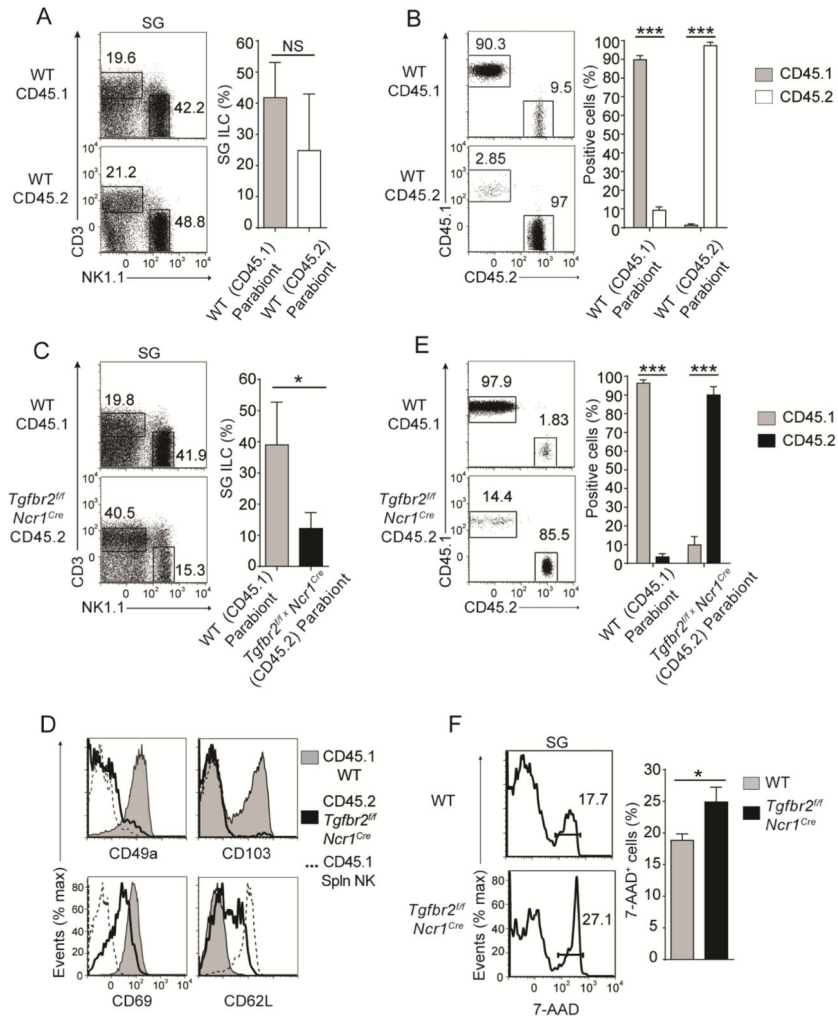
**(F)** Relative expression of *Gzma*, *Gzmb*, and *Gzmc* from sort purified WT SG ILCs (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>), *Tgfb $\beta$ 2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* SG ILCs (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>), and WT splenic NK cells (NK1.1<sup>+</sup>CD3<sup>-</sup>CD45<sup>+</sup>).

**(G)** Staining of indicated markers on WT SG ILCs, *Tgfb $\beta$ 2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* SG ILCs, and WT splenic NK cells (horizontal black bars represent negative staining).

Staining's are representative of at least 2 independent experiments. Student's t test, \*p <0.05,

\*\*\*p <0.001 for comparisons between WT control and *Tgfb $\beta$ 2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* groups.

See also Figure S5.



**Figure 6. Impact of TGF- $\beta$  on tissue residency and survival of SG ILCs**

(A) Frequency of SG ILCs after four weeks of parabiosis between WT (CD45.1) and WT (CD45.2) mice. n = 4 parabiont pairs

(B) Relative frequency of host derived or donor-derived SG ILCs from WT (CD45.1) and WT (CD45.2) parabiont pairs.

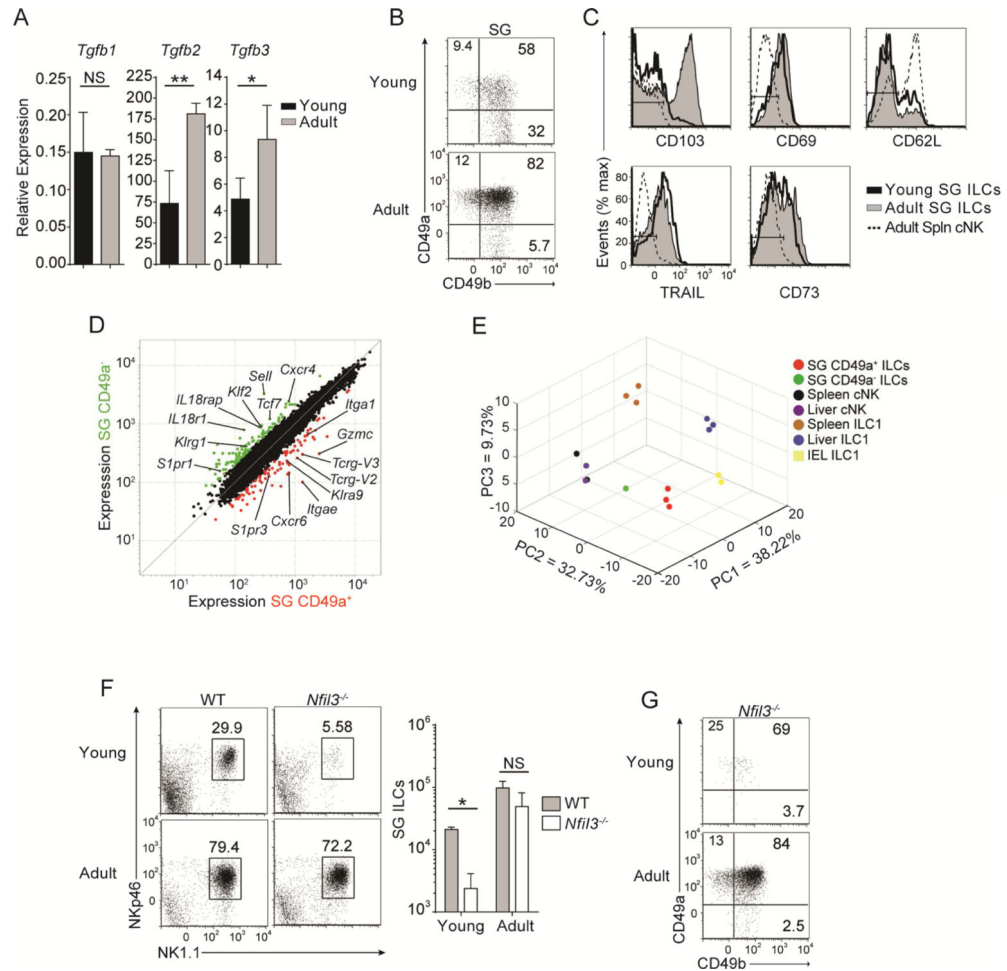
(C) Frequency of SG ILCs after four weeks of parabiosis between WT (CD45.1) and *Tgfb2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* (CD45.2) mice. n = 3 parabiont pairs

(D) Relative frequency of host or donor-derived SG ILCs from WT (CD45.1) or *Tgfb2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* (CD45.2) parabiont pairs.

(E) Expression of indicated markers on host derived SG ILCs from WT (CD45.1) or *Tgfb2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* (CD45.2) parabiont pairs.

(F) Frequency of cell death of SG ILC from WT or *Tgfb2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* mice after 1 hr culture in PBS. n = 4 mice per group.

(A-C and E) Student's t test, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 for comparisons between WT control and *Tgfb2<sup>fl/fl</sup> x Ncr1<sup>Cre</sup>* groups. See also Figure S6.



**Figure 7. TGF- $\beta$  imprinting on SG ILC is age dependent**

(A) Relative expression of *Tgfb1*, *Tgfb2*, and *Tgfb3* from SG of young or adult WT mice determined by qPCR. n = 3 mice.

(B) Expression of CD49a and CD49b by SG ILCs from WT young (3-5 weeks) and WT adult (10 weeks or older) mice. n = 5 mice per group.

(C) Expression of indicated markers on SG ILCs from young mice, adult mice, and splenic NK cells of adult mice. n = 3 mice per group.

(D) Comparison of gene expression of CD49a<sup>+</sup> SG ILCs and CD49a<sup>-</sup> SG ILCs of 6 week old mice; colored dots indicate transcripts upregulated by at least twofold.

(E) Supervised principal component analysis of CD49a<sup>+</sup> SG ILCs and CD49a<sup>-</sup> SG ILCs in comparison to other group 1 ILCs.

(F) Frequencies and absolute numbers of SG ILCs from young or adult WT and *Nfil3*<sup>-/-</sup> mice. n = at least 3 mice per group.

(G) SG ILCs expression of CD49a and CD49b from young and adult *Nfil3*<sup>-/-</sup> mice. n = at least 3 mice per group

All gene values were normalized to the expression of the housekeeping gene *Gapdh*. (A and F) Data are presented as mean  $\pm$  SD and are representative of at least two independent experiments. Student's t test, \*p < 0.05, \*\*p < 0.01, for comparisons between young and

adult WT control and *Nfil3*<sup>-/-</sup> groups. **(B-C)** Data are representative of 3 independent experiments. See also Figure S7.