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Lineage-specific metabolic adaptation of ILC3 in the gut but not central lymphoid tissues is imposed by transcription factor Tox2

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SUMMARY

Group 3 innate lymphoid cells (ILC3) have essential roles in lymphoid organogenesis and tissue repair. Here, we report that Tox2 is highly expressed in ILC3 in the gut. Depletion of Tox2 markedly decreased ILC3 in gut but not at central sites, resulting in defective control of *Citrobacter rodentium* infection. Single-cell transcriptional profiling revealed decreased expression of Hexokinase-2 in Tox2-deficient gut ILC3. Consistent with the requirement for hexokinases in glycolysis, *Tox2*^{-/-} ILC3 displayed decreased ability to utilize glycolysis for protein translation. Strikingly, ectopic expression of Hexokinase-2 rescued *Tox2*^{-/-} gut ILC3 defects. Hypoxia and IL-17A each upregulated Tox2 in ILC3. HIF-1 α -mediated Tox2 induction was specific to CCR6⁺ ILC3 whereas IL-17A-mediated induction of Tox2 and Hk2 occurred in both NCR⁺ and CCR6⁺ ILC3 subsets, suggesting a mechanism by which ILC3 adjust to changing environments by programming glycolytic metabolism. Our results reveal the requirement for Tox2 in the enhanced glycolysis of gut ILC3.

KEYWORDS

ILC3, Tox2, Metabolic adaption, Glycolysis, Tissue residency, HIF-1 α , IL-17

HIGHLIGHTS

- Tox2 is highly expressed in gut ILC3 and not in ILC3 from central lymphoid sites
- Tox2 is required for persistence of gut ILC3
- ILC3 responses are compromised in *Tox2*^{-/-} mice upon *Citrobacter rodentium* infection
- ILC3 in gut are metabolically distinct from ILC3 in central lymphoid sites
- Tox2 regulates Hk2 expression and dependence on glycolysis in gut ILC3
- Ectopic Hk2 expression rescues gut *Tox2*^{-/-} ILC3 defect
- ILC3 in different tissues metabolically adapt to changing environments
- Hypoxia and IL-17A each upregulate Tox2 in ILC3

INTRODUCTION

Innate lymphoid cells (ILC) are immune cells that lack specific antigen receptors but possess similar effector functions as T cells^{1,2}. Group 3 ILCs (ILC3) are heterogenous, comprising CD4 expressing lymphoid tissue inducer (LTi) cells, NKp46 receptor- expressing ILC3 (NCR⁺ ILC3), and CCR6 expressing ILC3 (CCR6⁺ ILC3). Gut-resident ILC3 produce IL-22, IL-17, IFN- γ , GM-CSF and TNF- α that protect the intestinal mucosa against extracellular bacteria and maintain the intestinal barrier³⁻⁵. They also provide protection against colorectal cancers and

enhance antitumor immunity^{6,7}. The development and function of ILC3 require transcription factors ROR- γ t, GATA-3 and Ahr⁸⁻¹⁰. However, other transcription factors implicated in generation and function of ILC3 remain to be identified.

ILC3 are highly enriched at barrier sites such as small intestinal lamina propria, intestinal crypts, and Peyer's patches. They are also present in primary and secondary lymphoid organs, including the thymus, spleen, and lymph nodes (LN)¹¹. Residence in tissues, differentiation and function imposes distinct metabolic requirements^{12,13}, but the nature of these requirements and the transcriptional mechanisms that control them are yet poorly understood. Recently, trained ILC3 have been shown to mount a more robust immune response than naïve ILC3 after *Citrobacter rodentium* rechallenge by rewiring their metabolic pathways¹⁴. Further, transcription factors including Tox, Bhlhe40, Runx3 and Blimp1 are required for persistence of CD8⁺ T cells in tissues and tumors¹⁵⁻¹⁹. For ILC3, mTOR and the hypoxia sensor HIF-1 α are implicated as controllers of ILC3 function in gut, but it is unclear whether requirements for these broadly expressed transcription factors are tissue-specific²⁰⁻²³. Thus, little is known about transcriptional controllers that regulate metabolic adaptations of ILC3 in distinct environments.

Here, we report that the persistence of ILC3 in gut requires the transcription factor Tox2 (thymocyte selection-associated high mobility group box protein family member 2). Tox2 mRNA levels were specifically upregulated in gut ILC3 but not in mesenteric lymph node (mLN) ILC3. ILC3 numbers in the gut were significantly reduced in *Tox2*^{-/-} mice at steady state as well as in *Citrobacter rodentium* infection. We show that Tox2 regulates expression of Hexokinase-2 (Hk2), which is required for glycolysis in gILC3. Ectopic expression of Tox2 was sufficient to increase lactate production and extracellular acidification rate (ECAR) in an ILC3 cell line MNK-3²⁴. Furthermore, ectopic Hk2 expression in *Tox2*^{-/-} bone marrow progenitors rescued *Tox2*^{-/-} gILC3 numbers to levels seen with wild-type Tox2-sufficient bone marrow progenitors. Moreover, hypoxia and IL-17A were each sufficient to induce expression of HIF-1 α , Tox2 and Hk2 in ILC3. Interestingly, in vivo, HIF-1 α -mediated induction of Tox2 and Hk2 was evident only in CCR6⁺ gILC3, whereas IL-17A mediated Tox2 and Hk2 induction was evident in both NCR⁺ and CCR6⁺ ILC3. Thus, ILC3 residing in the gut respond to local signals such as IL-17 and reprogram their metabolic status via upregulated expression of Tox2 and Hk2 to support glycolysis.

RESULTS

Tox2 is specifically required in gut ILC3

Tox2 is a sequence non-specific HMG-box transcription factor in the same family as Tox²⁵. Tox2 contributes to the CD8⁺ T cell exhaustion program and Tox2 deficiency in CD8⁺ cells improve CAR T cell function²⁶. Elevated Tox2 expression is seen in advanced stages of colorectal cancers²⁷. Tox2 is also important for T follicular helper (Tfh) cell development and for the generation and maintenance of memory Tfh cells^{28,29}.

Tox is first expressed at the Early Innate Lymphoid Progenitor (EILP) stage of ILC development, and its expression is maintained in Innate Lymphoid Progenitors (ILCP) and mature ILC^{30,31}. Tox2 is expressed at the next stage of ILC development after EILP, in ILCP³⁰. We found *Tox2* was highly expressed in small intestinal lamina propria (gut) ILC3 (hereafter termed gILC3) in contrast to mesenteric lymph node (mLN) ILC3 (hereafter termed mILC3) (**Fig 1A**). For these experiments measuring Tox2 mRNA, ILC3 were identified using surface markers (Lin⁻EpCAM⁺CD45⁺CD127⁺Kit⁺) and these cells expressed ROR- γ t (**S1A left**). Moreover, we observed co-expression of Kit and ROR- γ t in Lin⁻EpCAM⁺CD45⁺ cells (**S1A right**), confirming past work establishing Kit as a suitable marker for identification of ROR- γ t+ ILC3³²⁻³⁴. Tox2 was also relatively highly expressed in lung ILC2, whereas group 1 ILC (ILC1 and NK cells) isolated from liver expressed low levels of Tox2 (**Fig 1A**). To determine the importance of Tox2, we generated germline *Tox2*^{-/-} mice²⁶. Unlike in Tox-deficient mice, there was no defect in numbers of EILP and ILCP in BM of adult *Tox2*^{-/-} mice (**S1B**). However, ILC3 were significantly reduced in gut of *Tox2*^{-/-} mice (**Fig 1B**). Interestingly, the number of ILC3 was unchanged at other sites such as mLN, spleen, peripheral lymph nodes and thymus (**Fig 1C**). The reduction in numbers was evident among NCR⁺, CCR6⁺ and NCR⁻CCR6⁻ subsets of gILC3 (**Fig 1D, Fig 1E and S1C**). Consistent with the results from gut, we observed a similar defect in ILC3 number in caecum, colon and Peyer's patches (**S1D**). We did not observe defects in cellularity of peripheral lymph nodes such as inguinal, axillary, brachial and mandibular nodes (**S1E**); however, the number of Peyer's patches in *Tox2*^{-/-} mice was slightly but consistently reduced (**S1F**).

To determine when Tox2 is required during ontogeny, we analyzed Tox2 expression and numbers of group 3 ILC in mice at different ages. ILCP from adult bone marrow and E15.5 fetal gut expressed Tox2, as did group 3 ILC3 isolated from both adult and fetal E15.5 embryos (**S1G**). We found that group 3 ILC were significantly reduced in *Tox2*^{-/-} E15.5 intestines (**S1H**). Furthermore, we found both NCR⁺ and CCR6⁺ ILC3 subsets were reduced in *Tox2*^{-/-} mice at all ages analyzed (**S1I**).

The ILC3 defect in gut was evident in mixed stem cell chimeras and so was cell-intrinsic (**Fig 1F**). To assess whether residence in the gut upregulated Tox2 expression, we adoptively transferred mILC3 into immunodeficient NSG (Nod Scid Gamma-c) mice and assessed Tox2 expression in donor ILC3 sorted from gut and spleen one week post transfer (**Fig 1G**). We found that ILC3 recovered from spleen maintained low expression of Tox2 whereas ILC3 recovered from gut upregulated Tox2 expression to levels that were comparable to those in gILC3 (**Fig 1G bottom**). We generated *Rag1*^{-/-}*Tox2*^{-/-} mice and observed that ILC3 number and frequency were

significantly reduced in gut but not mLN when compared to *Rag1*^{-/-} control mice (**Fig 1H**), confirming the ILC3 defect in gut of *Tox2*^{-/-} mice is independent of T cells.

We assessed other ILC populations in small intestine and observed group 1 ILC (ILC1 and NK cells) remained unaltered between WT and *Tox2*^{-/-} mice, whereas ILC2 were reduced in *Tox2*^{-/-} mice (**S1J**). Group 1 ILC numbers at other sites such as in liver, colon and spleen were comparable between WT and *Tox2*^{-/-} mice (**S1K**). Consistent with the defect in gut, ILC2 numbers were reduced by approximately three-fold in lung and colon of *Tox2*^{-/-} mice (**S1L**). B cell, myeloid and T cell numbers were unchanged (**S1M**).

Together, these results established that Tox2 is required in ILC2 at all sites we assessed, and only in gILC3 but not by ILC3 at central sites. We focused our further inquiries on the gut-specific requirement for Tox2 in ILC3.

Tox2 ablation leads to reduced ILC3 response upon *Citrobacter rodentium* infection

We explored the functional consequences of Tox2 ablation using *Citrobacter rodentium* infection, in which production of IL-22 by ILC3 in gut limits infection³⁵⁻⁴². At steady state, we did not observe differences in the frequency of IL-22⁺ and IL-17A⁺ cells in NCR⁺ and CCR6⁺ ILC3 between WT and *Tox2*^{-/-} mice (**S2A**). We infected WT and *Tox2*^{-/-} mice with *C. rodentium* and monitored body weight each day until day 10. In addition, we assessed ILC3 numbers and responses at an early stage of infection (day 6), colon length on day 6 and day 10 post infection (p.i.), as well as bacterial load in feces and translocation to spleen and liver (**Fig 2A**). *Tox2*^{-/-} mice lost more body weight upon *C. rodentium* infection as compared to WT controls (**Fig 2B**). The bacterial load on day 6 p.i. was significantly higher in feces, spleen and liver from *Tox2*^{-/-} mice compared to WT mice (**Fig 2C**). We also measured colon length at day 6 and day 10 p.i.; however, we did not observe any difference between infected WT and *Tox2*^{-/-} mice (**S2B**). On day 6 p.i., there were fewer ILC3 in colonic lamina propria of infected *Tox2*^{-/-} mice as compared to infected WT controls (**Fig 2D**). The remaining ILC3 in gut of *Tox2*^{-/-} infected mice showed lower frequencies of IL-22 producers as compared to WT controls (**Fig 2E**). We did not observe any alteration in T cell frequencies between infected WT and *Tox2*^{-/-} mice on day 6 p.i. (**S2C**). We also infected *Rag1*^{-/-} *Tox2*^{-/-} mice to rule out any contribution of T cells in modulating ILC3 responses. Consistent with the infected *Tox2*^{-/-} mice data, *Rag1*^{-/-} *Tox2*^{-/-} mice lost more body weight (**Fig 2F**), displayed higher bacterial burden (**Fig 2G**), possessed fewer ILC3 (**Fig 2H**) and fewer IL-22 producers (**Fig 2I**) compared to control *Rag1*^{-/-} mice after day 10 p.i. IFN- γ production by group 1 ILC (ILC1 and NK cells) was similar in *Tox2*^{-/-} (**S2D left**) and *Rag1*^{-/-} *Tox2*^{-/-} mice (**S2D right**). Furthermore, IL-22 producers were reduced in NCR⁺, CCR6⁺ and NCR⁻CCR6⁻ gILC3, from both *Tox2*^{-/-} (**S2E**) and *Rag1*^{-/-} *Tox2*^{-/-} (**S2F**) mice compared to their respective *Tox2*^{+/+} controls.

These results confirmed the specific requirement of Tox2 in generating functional gILC3 able to ward off infection with *C. rodentium*.

Tox2 is required by gut ILC3 for their persistence in gut

We generated mice carrying a conditional allele of *Tox2*, with loxP sites flanking exon5 of the *Tox2* gene (*Tox2^{fllox}*) as shown in **S3A**. We crossed *Tox2^{fllox}* mice with mice that express Cre in all *IL7R α* -expressing cells, thus deleting *Tox2* in all lymphoid lineage cells (*IL7R α ^{Cre}Tox2^{fl/fl}*). This mouse recapitulates the phenotype of the *Tox2* germline deficient mouse, thus establishing that the *Tox2^{fllox}* mouse strain efficiently enables conditional deletion of *Tox2* (**S3B**). To assess the role of *Tox2* specifically in ILC3, we crossed *Tox2^{fllox}* mice with *Rorc*-Cre mice. As expected, we observed significant reduction in the frequency and number of gILC3 but not of mILC3 upon *Tox2* ablation in *Rorc*⁺ cells (**Fig 3A**, see **S3C** for ILC3 subsets).

Next, we assessed whether *Tox2* ablation altered CCR7 expression in ILC3 and ILC3 subsets from mLN and gut thereby affecting homing of ILC3. *Tox2* deficiency did not lead to alteration in CCR7 expression by mILC3 and gILC3 (**S3D**, see **S3E** for ILC3 subsets). We observed no defect in proliferation of mILC3 and gILC3 as measured by *in vivo* single pulse bromodeoxyuridine (BrdU) incorporation after 18 hours of labelling (**S3F**). However, gILC3 from *Tox2^{-/-}* mice showed more death as compared to WT controls as measured by cleaved caspase 3 staining whereas mILC3 showed comparable frequency of cells with cleaved caspase 3 between WT and *Tox2^{-/-}* mice (**S3G**).

To assess whether *Tox2* is required for persistence of mature ILC3 in gut, we crossed *Tox2^{fllox}* mice with *ER^{T2Cre}* mice. In this model, *ER^{T2Cre}* is ubiquitously expressed as an inactive cytoplasmic form and is rapidly translocated into nucleus following tamoxifen administration (**Fig 3B**), leading to *Tox2* deletion and decreased *Tox2* mRNA expression in mature ILC3 (**Fig 3C**). Conditional deletion of *Tox2* led to significant reduction of gILC3 and not mILC3, indicating *Tox2* is required for persistence of mature ILC3 in gut (**Fig 3D**).

To determine whether *Tox2* deficiency affected the ILC3-ILC1 transition, we generated *Rorc^{Cre} Rosa-YFP Tox2^{fl/fl}* mice, which allowed tracking *Rorc* expression history in ILC3-ILC1 populations. We observed approximately 30% of ILC1 showed a history of *Rorc* expression in gut (**Fig 3E top**) and the number of ILC1 with a history of *Rorc* expression remained unaltered in the absence of *Tox2* in mLN and gut (**Fig 3E bottom**). We also evaluated solitary intestinal lymphoid tissue structures using the same mouse model to assess if reduced gILC3 in *Tox2^{-/-}* affected the formation of cryptopatches (CPs) and isolated lymphoid follicles (ILFs). In our pilot experiment we assessed the number of CPs and ILFs in duodenum, jejunum and ileum. We observed greater reduction in the number of CPs in duodenum compared to other sections in *Rorc^{Cre} Rosa-YFP Tox2^{fl/fl}* but the number of ILFs remained comparable across all sections analyzed (**S3H**). Hence, we focused in duodenum for quantification of CPs and ILFs. Deficiency of *Tox2* in *Rorc*-expressing cells resulted in smaller CPs and ILFs (**Fig 3F top**). The number of CPs was reduced, however the number of ILFs was only minimally affected in *Rorc^{Cre}.Rosa-YFP Tox2^{fl/fl}* mice compared to *Rorc^{Cre}.Rosa-YFP Tox2^{+/+}* (**Fig 3F bottom**).

Together these results establish that *Tox2* is required for the persistence of mature ILC3 in gut.

Gut ILC3 from *Tox2^{-/-}* mice are transcriptionally distinct from their WT counterparts

To examine whether Tox2 deficiency leads to transcriptional alterations in ILC3, we profiled mILC3 and gILC3 from WT and *Tox2*^{-/-} mice using single-cell RNA-sequencing (scRNAseq). We identified ILC3 clusters in mLN (**S4A top**) and gut (**S4B**) and removed all other contaminating clusters from our analysis. To rule out the possibility of other contaminants, we integrated our mILC3 data with a published mILC3 dataset which used RORγt BAC-transgenic reporter mice to sort ILC3⁴³. We observed the mILC3 subsets overlapped with the published dataset (**S4A**). *Rorc* transcript in RORγt -GFP BAC-transgenic mice was considerably lower in NCR1⁺ ILC3 subset as compared to CCR6⁺ ILC3 subset (**S4A** 4th row; 1st column). This can explain why we detected lower levels of *Rorc* transcripts in wild-type (non-transgenic) NCR1⁺ ILC3 (**S4A** 4th panel; 2nd and 3rd column, violin plot for *Rorc* expression). While *Rorc* expression was low in NCR1⁺ mILC3, it was appreciably higher than in other cells such as Eomes+ NK cells (**S4A** violin plot). Two broad subsets of ILC3: NCR⁺ ILC3 (expressing *Ncr1*, *Tbx21*, *Rorc*^{low} and *Eomes*^{negative}) and CCR6⁺ ILC3 (expressing *Ccr6*, *Cd4*, *Rorc*) were identified based on gene expression in mLN (**S4A**) and gut (**S4B**). Visualization of data using UMAP showed mILC3 from WT and *Tox2*^{-/-} mice overlap with each other suggesting they are transcriptionally quite similar, whereas gILC3 from WT and *Tox2*^{-/-} mice occupy different positions in the manifold, indicating gILC3 from *Tox2*^{-/-} mice are transcriptionally distinct from their WT counterpart (**Fig 4A**, ILC3 subsets in **Fig 4B**). We observed 97 differentially expressed genes (DEG) in CCR6⁺ ILC3 and 95 DEG in NCR⁺ ILC3 in gut as compared to only 14 DEG and 11 DEG in CCR6⁺ ILC3 and NCR⁺ ILC3 in mILC3 respectively (**S4C** and complete gene list in **Supplementary Table 1**).

We assessed the top upregulated and downregulated genes in the absence of Tox2 in gILC3 (**Fig 4C**). We observed that the genes that are upregulated in the absence of Tox2 are associated with effector function (*Il17f*, *Fgl2*) and antigen presentation and processing (*H2-Q4*, *Cd74*) whereas the genes that are downregulated are associated with migration (*Cxcr4*, *Cxcr5*) and metabolism (*Arg1*, *Ldhd*, *Hk2*) (**Fig 4C**, and **Supplementary Table 2**).

Tox2 is required for Hk2 upregulation in gut ILC3

Our scRNAseq data analysis identified *Hk2* as a putative target of Tox2. We focused on Hexokinase-2 (*Hk2*) because it is the first rate limiting for glycolysis, catalyzing the conversion of glucose into glucose-6-phosphate which is then trapped inside the cell^{44,45}. Other Hexokinases such as *Hk1*, *Hk3* were not differentially expressed in our scRNAseq data (**Fig 4D**) and were not impacted by the absence of Tox2 (**Fig 4D and S4D**). Based on the decreased expression of *Hk2*, we examined the expression of genes involved in glycolysis (**Fig 4D**) and oxidative phosphorylation (OXPHOS) (**Fig 4E**). mILC3 from both WT and *Tox2*^{-/-} mice exhibited higher levels of transcripts for genes associated with OXPHOS as compared to gILC3 from WT and *Tox2*^{-/-} mice (**Fig 4E**, see **S4E** for ILC3 subsets), suggesting a differential reliance of mILC3 and gILC3 on OXPHOS and glycolysis, respectively, that was independent of Tox2.

We performed qPCR for *Hk2* in mILC3 and gILC3 and confirmed that *Hk2* expression was upregulated in gILC3 as compared to mILC3 from WT mice, consistent with the scRNAseq data (**Fig 4F**). Importantly, *Hk2* expression was significantly reduced in gILC3 from germline

Tox2^{-/-} mice (**Fig 4F left and see S4F** for ILC3 subsets) as well as from conditional *Tox2* knockout mice (tamoxifen-treated *ER^{T2Cre}Tox2^{fl/fl}*) as compared to WT mice (**Fig 4F right**). We assessed *Hk2* protein expression and found that while *Hk2* levels in mILC3 and CD4 T cells from WT and *Tox2* deficient mice were similar (**Fig 4G**), *Hk2* was markedly elevated in gILC3 as compared to mILC3 (**Fig 4H**). Moreover, *Hk2* expression was significantly reduced specifically in gILC3 from *Tox2*^{-/-} mice (**Fig 4H**). To assess whether residence in the gut upregulated *Hk2* expression by ILC3, we adoptively transferred sorted mILC3 and gILC3 from WT and *Tox2*^{-/-} mice into NSG mice. We observed mILC3 that homed to gut upregulated expression of *Hk2*. As expected, donor mILC3 from *Tox2*^{-/-} mice that homed to gut remained defective in their ability to upregulate *Hk2* (**S4G**).

We also determined whether *Tox2* regulated *Hk2* expression in other cells that needed *Tox2* such as ILC2 and Tfh cells²⁹. We observed that *Tox2* deficiency did not alter *Hk2* expression in ILC2 (**S4H**) or in Tfh cells (**S4I**). *Tox2*-deficient mice lack nearly all ILCs with the exception of gILC3³¹. We examined *Hk2* expression in gILC3 from *Tox2*^{-/-} mice and found *Hk2* expression remained similar between WT and *Tox2*^{-/-} NCR⁺ and CCR6⁺ gILC3 subsets (**S4J**), indicating *Tox* and *Tox2* play distinct roles in gILC3.

In agreement with our earlier data examining cytokine production and proliferation in gILC3 at steady state, the expression of key ILC3 cytokine genes and proliferation genes remained unaltered or minimally altered between WT and *Tox2*^{-/-} ILC3 subsets isolated from mLN and gut (**S4K**). We also assessed expression of T-bet and Bcl-6, that are regulated by *Tox2* in other lineages^{29,46}. We did not observe alteration in T-bet (**Fig 4I**) and Bcl-6 (**Fig 4J**) expression at mRNA and protein levels between WT and *Tox2*^{-/-} gILC3.

To investigate the impact of *Tox2* deficiency on chromatin landscapes, we performed scATAC sequencing using gILC3 from WT and *Tox2*^{-/-}, with WT mILC3 serving as an additional control. To our surprise, our scATACseq analysis revealed that global chromatin accessibility in total gILC3 between WT and *Tox2*^{-/-} mice were similar (**S4L top**). This remained the case when NCR⁺ and CCR6⁺ ILC3 were assessed separately (**S4L bottom**). The single exception was one region at the *Tox2* locus in gILC3 that was deleted in *Tox2*^{-/-} mice (**S4M top**). We looked at the promoter and enhancer regions of *Hk2* locus and did not observe any differentially accessible peaks between WT and *Tox2*^{-/-} gILC3 subsets (**S4M bottom**) suggesting *Tox2* does not alter chromatin landscapes in ILC3 lineage cells.

Together these results indicate that ILC3 upregulate *Hk2* expression in the gut, in a *Tox2*-dependent manner, and *Tox2* does not alter chromatin accessibility in ILC3.

Tox2 deficiency leads to attenuated protein translation in gILC3

The data presented above suggested a dependence on distinct metabolic programs in tissue-resident gILC3 and mILC3. As protein translation is tightly coupled with ATP production^{47,48}, we assessed levels of puromycin incorporation as a surrogate to measure energy generation by glycolysis and OXPHOS pathways in mILC3 and gILC3. To this end, we monitored puromycin incorporation in WT mILC3 and gILC3 following inhibition of glycolysis and OXPHOS with 2-deoxyglucose (2DG) and oligomycin, respectively⁴⁹. We observed much greater reduction in puromycin MFI to near background levels in oligomycin treated mILC3, as compared to 2DG

treated mILC3 (**Fig 5A** see 3rd plot) indicating a very high dependence of mILC3 on mitochondrial energetics and lower glycolytic capacity (**S5A**). However, 2DG treated WT gILC3 displayed greater reduction in levels of puromycin MFI (**Fig 5A** see 2nd plot) compared to oligomycin treated gILC3 indicating that WT gILC3 have higher dependence on glycolysis and lower mitochondrial dependence than WT mILC3 (**S5A**).

Next, we evaluated *in vivo* translation by injecting mice with puromycin⁴⁸. There was no difference in puromycin incorporation in mILC3 or mLN CD4 T cells from WT and *Tox2*^{-/-} mice (**Fig 5B**). Notably, there was significantly reduced incorporation of puromycin in gILC3 from *Tox2*^{-/-} mice as compared to WT controls whereas gut CD4 T cells exhibited similar incorporation (**Fig 5C**; see **S5C** for gILC3 subsets). Thus, *Tox2* is required to maintain global protein translation levels specifically in gILC3.

We assessed the contributions of glycolysis and OXPHOS to protein translation in WT and *Tox2*^{-/-} ILC3. Levels of puromycin incorporation were equivalent in all tested conditions between WT and *Tox2*^{-/-} mILC3 (**Fig 5D**; **S5B**), consistent with low *Tox2* expression in mILC3 and the relative lack of transcriptional alterations in mILC3. However, the levels of puromycin MFI were greatly reduced in *Tox2*^{-/-} gILC3 compared to WT gILC3 (**Fig 5E**). Upon 2DG treatment, WT gILC3 displayed significant reduction in puromycin MFI whereas the *Tox2*^{-/-} gILC3 failed to do so indicating a defect in glycolysis in *Tox2*^{-/-} gILC3 (**Fig 5E** see red boxed groups). These results together indicate that WT gILC3 possess increased glucose dependence and higher glycolytic capacity which is *Tox2*-dependent (**S5D**). These trends were still observed when NCR⁺ and CCR6⁺ ILC3 subsets were assessed (**S5E**). These results are consistent with the notion that upregulated expression of *Tox2* in gILC3 upregulates expression of Hk2, and increased levels of Hk2 in turn confer enhanced glycolytic capacity on WT gILC3.

To support results obtained using puromycin incorporation, we performed a Seahorse assay using the ILC3 cell line, MNK-3²⁴. Because we obtained ~500 *Tox2*^{-/-} gILC3/mouse, it was technically challenging to perform Seahorse assay on primary gILC3. We therefore transduced MNK-3 cells with *Tox2* and Hk2 that served as a positive control (**S5F**). Parental MNK-3 cells did not express *Tox2* (**S5G left**). Interestingly, *Tox2* overexpression in MNK-3 cells increased Hk2 mRNA (**S5G right**) and protein (**S5H**) expression; however, Hk1 protein expression levels remained unaltered (**S5I**). Furthermore, ectopic expression of *Tox2* or Hk2 resulted in increased lactate production (**Fig 5F**) and higher ECAR (**Fig 5G top**) as compared to MNK-3 cells transduced with empty virus. In contrast, *Tox2* or Hk2 expression minimally altered the oxygen consumption rate (OCR) (**Fig 5G bottom**). Basal ECAR was also elevated without significant alteration in basal OCR in *Tox2* or Hk2-overexpressed MNK-3 cells compared to control cells (**Fig 5H**).

Together, these results show mILC3 and gILC3 exhibit distinct metabolic programs with a bias towards glycolysis in gILC3 which is *Tox2*-dependent. These data further indicate that *Tox2* is sufficient to drive glycolysis in ILC3 lineage cells.

Ectopic Hekokinase-2 expression rescues *Tox2*^{-/-} gut ILC3 numbers

The results presented above strongly suggested that *Tox2* promotes glycolysis in gILC3, likely through the upregulated expression of Hk2. To assess the importance of Hk2 regulation by *Tox2* in vivo, we ectopically expressed Hk2 in *Tox2*^{-/-} BM progenitors using lentivirus, and adoptively transferred the transduced BM progenitors into immunodeficient mice (NSG) (**Fig 6A**). This approach was used because transduction of mature ILC3 has not been successfully reported. Ectopic expression of Hk2 in *Tox2*^{-/-} progenitors rescued the ability of *Tox2*^{-/-} ILC3 to persist in the gut (**S6A, S6B, Fig 6B and Fig 6C left**). We observed both NCR⁺ and CCR6⁺ gILC3 subsets were rescued upon Hk2 overexpression in *Tox2*^{-/-} progenitors (**Fig 6D**). Furthermore, this rescue was specific for ILC3 lineage cells since Hk2 overexpression in *Tox2*^{-/-} progenitors did not affect NK cell numbers (**Fig 6C right**) or rescue the ILC2 defect (**S6C**). These results support the conclusion that Hk2 is a critical target of *Tox2* which is essential for persistence of ILC3 in gut.

To further test the importance of Hk2 regulation by *Tox2*, we evaluated whether bypassing Hk2 via supplementation of downstream metabolites would improve the survival of gILC3 in vitro. First, we assessed whether high glucose (25mM), pyruvate (10mM) or acetate (5mM) would enhance the survival of WT and *Tox2*^{-/-} mILC3 cultured for 24 hours on stromal support (irradiated OP9 cells) as shown in **Fig 6E**. Interestingly, the frequency of mILC3 recovered after culture was not significantly modulated by the absence of *Tox2* and survival was also not altered by any of the nutrient supplements we tested (**Fig 6F and Fig 6H left**). Importantly, the frequency of recovered gILC3 was significantly lower than that of mILC3 in standard glucose media (**Fig 6F and Fig 6G top**). Consistent with a high dependence of gILC3 on glucose, high glucose media significantly increased the recovery of WT gILC3 (**Fig 6G and Fig 6H right**). However, this was not the case for *Tox2*^{-/-} gILC3 (**Fig 6G and Fig 6H right**). Notably though, addition of pyruvate or acetate, bypassing Hk2, significantly increased the recovery of *Tox2*^{-/-} gILC3 (**Fig 6G and Fig 6H right**). Thus, even in the absence of *Tox2*-induced Hk2, gILC3 survival can be rescued in vitro by metabolites generated downstream of the enzymatic activity of Hk2.

Our results indicate that Hk2 is a critical target of *Tox2* which is essential for persistence of ILC3 in gut. gILC3 are metabolically distinct from mILC3, requiring high concentrations of glucose to survive in culture. In the absence of *Tox2*, and the concomitant upregulation of Hk2, gILC3 exhibited augmented survival in the presence of metabolites downstream of Hk2. Together, these results suggest that *Tox2*-dependent upregulated expression of Hk2 promotes the survival of gILC3 by increasing their glycolytic metabolism.

Hypoxia and IL-17A each upregulate *Tox2* and Hk2

Our experiments highlighted significant differences in the metabolic programming of ILC3 at different sites as a function of *Tox2* and Hk2 expression. Nevertheless, these experiments did not evaluate potential reasons as to why *Tox2* is upregulated in gILC3 as compared to mILC3. HIF-1 α is a broadly expressed transcription factor that is stabilized under hypoxic conditions, regulating gene expression⁵⁰. HIF-1 α is required for optimal responses by gILC3^{20,21}. gILC3 reside within the gut lamina propria, isolated lymphoid follicles and Peyer's patches⁵¹, and the gut lamina propria is not hypoxic where these ILC3 reside^{52,53}. Nevertheless, the gut lumen and epithelial layers are hypoxic⁵⁴⁻⁵⁶; and gut injury and infection generates hypoxic

environments^{57,58}. Thus, we examined whether HIF-1 α was differentially expressed between mILC3 and gILC3. We detected significantly increased Hif1a expression in gILC3 as compared to mILC3, irrespective of the genotype of the mice, in our scRNAseq data and by antibody measurement of protein levels (**S7A, S7B**), whereas HIF-1 α protein levels were comparable in mLN and gut CD4 T cells in WT and *Tox2*^{-/-} mice (**S7C**). Flow cytometry analyses revealed significant increase in HIF-1 α in both NCR⁺ and CCR6⁺ gILC3, irrespective of whether the cells were from WT or *Tox2*^{-/-} mice (**Fig 7A**). We identified 3 putative binding sites of HIF-1 α near the *Tox2* transcription start site (TSS) (**S7D**).

We therefore examined whether hypoxia would be sufficient to induce HIF-1 α , *Tox2* and *Hk2* expression in ILC3. HIF-1 α levels were upregulated upon exposing mILC3 to hypoxia (5% O₂) for 6 hours; however, HIF-1 α expression in gILC3 did not show further upregulation after incubating gILC3 under hypoxia (**S7E**). In both mILC3 and gILC3, hypoxia resulted in upregulation of *Tox2* expression as assessed by qPCR (**Fig 7B left**). Indeed, a 6-hour exposure of mILC3 to hypoxia induced *Tox2* mRNA expression to levels that were similar to those detected in gILC3 (**Fig 7B left and see Fig 7C** for ILC3 subsets). Significantly, exposure of WT mILC3 to hypoxia also resulted in induction of *Hk2*, to levels similar to those detected in gILC3 (**Fig 7B right**). However, *Hk2* levels were not increased in either *Tox2*^{-/-} mILC3 or gILC3 in response to hypoxic conditions, remaining at the low basal levels present in WT mILC3 (**Fig 7B right**). Hence, acute exposure to hypoxic conditions resulted in the induction of *Tox2* and *Hk2* expression in mILC3, and *Hk2* induction by hypoxia required *Tox2* in vitro.

To determine whether HIF-1 α is required for *Tox2* and *Hk2* induction in both NCR⁺ and CCR6⁺ gILC3, we assessed *Rorc*^{Cre}.*Hif1a*^{fl/fl} mice in which HIF-1 α is deleted from *Rorc*-expressing cells. Consistent with previous work, we saw a 2-fold reduction in gILC3 and no defect in NCR⁺ ILC3 in gut (**S7F, Fig 7D right and 7E right**). Interestingly, we observed CCR6⁺ gILC3 were significantly reduced (**Fig 7E right**), however mILC3 numbers remained unaffected (**Fig 7D left and see Fig 7E left** for NCR⁺ and CCR6⁺ mILC3 subsets). We observed *Tox2* and *Hk2* expression was reduced in CCR6⁺ gILC3 but not in NCR⁺ gILC3 isolated from *Rorc*^{Cre}.*Hif1a*^{fl/fl} mice (**S7G, Fig 7F**) suggesting HIF-1 α -mediated *Tox2* and *Hk2* upregulation is restricted to CCR6⁺ ILC3 in vivo. Thus, the results with HIF-1a deficiency can only partially explain the defects seen in *Tox2*^{-/-} mice where both NCR⁺ and CCR6⁺ gILC3 are affected.

Next, we determined the role of another gut-specific factor, IL-17 which is abundant in small intestinal lamina propria at steady state and plays an important role in gut barrier function at steady state and after injury/infection⁵⁹⁻⁶². Synergy between hypoxia and IL-17 was reported in keratinocytes in a wound-repair model⁶³. We therefore explored the role of IL-17A in regulating *Tox2* expression in ILC3. We observed that IL-17A was sufficient to induce *Tox2* in both ILC3 subsets (**Fig 7G**) as well as in HIF-1 α deficient ILC3 subsets (**Fig 7H**), suggesting IL-17A regulates *Tox2* expression in both ILC3 subsets. We analyzed *Il17a*-germline knockout mice and found that unlike the results with HIF-1 α deficiency, *Il17a* deficiency reduced numbers of both NCR⁺ and CCR6⁺ gILC3 subsets, while mILC3 numbers remained unaltered (**Fig 7I and see 7J** for ILC3 subsets). Furthermore, *Tox2* and *Hk2* expression were reduced in *Il17a*^{-/-} gILC3 (**S7H, see Fig 7K** for ILC3 subsets) compared to the WT control, indicating IL-17 is required for upregulated *Tox2* expression in both ILC3 subsets.

IL-17 stabilizes HIF-1 α in keratinocytes and fibroblasts^{64,65}. To delineate the relationship between HIF-1 α and IL-17 in gILC3, we assessed expression of HIF-1 α in *Il17a*^{-/-} mice. We observed reduction of HIF-1 α expression in gILC3 (**Fig 7L** see **Fig 7M** for ILC3 subsets).

Our results thus establish that the hypoxia sensor HIF-1 α and the cytokine IL-17A can each upregulate Tox2 in gILC3. The results are consistent with the notion that ILC3 sense higher levels of IL-17 in the gut microenvironment at steady state, and respond by upregulated expression of Tox2 and Hk2, which in turn supports glycolysis in gILC3. Together, our data identify Tox2 as a critical regulator of metabolic programming and survival of gILC3.

DISCUSSION

We have identified an essential role for Tox2 in the persistence of ILC3 in gut, but not at central sites. Tox2 expression was upregulated in gILC3 compared to mILC3 counterparts. The numbers of NCR⁺ and CCR6⁺ ILC3 were greatly reduced in gut but not central sites in mice lacking Tox2 in ILC3. Consistently, *Tox2*^{-/-} mice failed to effectively control *C. rodentium* infection.

We observed upregulated expression of Hk2 in gILC3 as compared to mLN ILC3; this upregulation was Tox2-dependent and ILC3-specific as it was not observed in other immune cell types in gut. Because hexokinases are rate-limiting enzymes for glycolysis, we assessed the metabolic dependence of both central and gut ILC3 on glycolysis and mitochondrial respiration. gILC3 showed reduced dependence on oxidative phosphorylation but increased glycolytic ability compared to mILC3. The increased glycolytic ability of gILC3 required Tox2, as it was impaired in *Tox2*^{-/-} gILC3. Ectopic Tox2 expression in the ILC3 cell line MNK-3 significantly increased ECAR and lactate production, further highlighting the role of Tox2 in glycolytic metabolism of ILC3. Ectopic expression of Hk2 in *Tox2*^{-/-} BM progenitors rescued the ability of *Tox2*^{-/-} ILC3 to persist in the gut, supporting the conclusion that Hk2 is a critical target of Tox2 which is essential for persistence of ILC3 in gut. Indeed, *Tox2*^{-/-} gILC3 exhibited defective survival *ex vivo*, even in the presence of high concentrations of glucose. Notably though, their survival could be at least partially restored by supplementing *Tox2*^{-/-} gILC3 cultures with pyruvate or acetate, that are metabolic intermediates downstream of Hk2.

We further explored the rationale underlying this metabolic adaptation of gut resident ILC3. The gut lumen is hypoxic compared to other tissues, but the lamina propria where ILC3 reside is not⁵²⁻⁵⁴. However, gut injury and inflammation generate hypoxic environments within which immune cells must execute their protective functions⁶⁵. Hypoxia stabilizes transcription factor HIF-1 α , and effector responses of gILC3 are reduced in the absence of HIF-1 α ⁶⁶⁻⁶⁹. We observed higher HIF-1 α expression in gILC3 and gILC3 subsets compared to mLN counterparts. Furthermore, we found that hypoxia was sufficient to induce expression of Tox2 and Hk2 in ILC3. Strikingly, we found that HIF-1 α is required in CCR6⁺ ILC3 but not NCR⁺ ILC3 *in vivo*; as in the absence of HIF-1 α , only CCR6⁺ ILC3 showed lower Tox2 and Hk2 expression. Other differences were also evident between NCR⁺ and CCR6⁺ ILC3. Although both subsets upregulated expression of Tox2 in the gut, and required Tox2 for their persistence at this site, the level of expression of Tox2 was consistently lower in NCR⁺ gILC3 than in CCR6⁺ ILC3. Whether these differences are intrinsic to ILC3 subsets or reflect their occupancy of distinct anatomical locations either at steady-state⁷⁰ or during their development *in vivo* requires further investigation.

Because HIF-1 α appeared dispensable in NCR⁺ gILC3 for Tox2 upregulation *in vivo*, and the effects of HIF-1 α ablation in CCR6⁺ gILC3 for Tox2 upregulation were modest, we sought other mechanisms which could upregulate expression of Tox2 distinct from HIF-1 α . IL-17 is abundant in small intestinal lamina propria at steady state^{59,62}. Synergy between hypoxia and the cytokine IL-17 was recently reported in keratinocytes during wound-repair⁶³ as well as in fibroblasts⁶⁴. We found that IL-17A was required for appropriate upregulated expression of HIF-1 α , Tox2 and Hk2 in both NCR⁺ and CCR6⁺ ILC3. Interestingly, IL-17A could upregulate Tox2 expression in ILC3 independently of HIF-1 α *in vitro*.

Because glycolysis is often required for the maintenance of cellular energetics under hypoxic conditions^{71,72}, our results suggest that Tox2 promotes lineage-specific metabolic adaptation that endows ILC3 with the tailored ability to survive and function at IL-17 rich sites, in anticipation of hypoxic conditions that are created by injury and infection. Indeed, alterations in the metabolic status of gILC3 were found to be associated with their function during infection^{14,73}, although these studies did not compare gILC3 with ILC3 at other sites. Our results provide a possible mechanism for how some of these metabolic changes might occur, as they predict gILC3 become more glycolytic in presence of high levels of IL-17 and possibly other gut signals present during infection.

ILC3 are increasingly suggested to play important roles in immune responses to tumors and immune checkpoint blockade. ILC3 were the first cells to infiltrate tumors after cisplatin-based chemotherapy, subsequently recruiting T cells and so improving responses to immune checkpoint blockade⁷. Tumors can be IL-17 rich⁶⁴, and are frequently hypoxic as compared to normal tissues⁷⁴, resulting in a shift in ATP production from OXPHOS to glycolysis⁷⁵. Thus, it is tempting to speculate that tumor-infiltrating ILC3 exhibit metabolic adaptations that are similar to gILC3, and further, that the ability of ILC3 to program glycolytic metabolism in response to IL-17 and hypoxia allows them to pioneer colonization of hypoxic sites such as tumors. In this context, it is interesting that colorectal cancers exhibit upregulated expression of Tox2²⁷. It will therefore be important to evaluate Tox2 and Hk2 expression in ILC3 residing in the hypoxic environments of tumors.

Tox2 may have additional functions in ILC3, and also has essential functions in ILC2 that we have not addressed in the current study. Further, other signals derived from gut and gut-resident host cells may also affect Tox2 expression and guide metabolic adaptation in ILC3. These are appropriate subjects for future studies. Collectively, our present findings demonstrate that metabolic adaptation of gILC3 is under lineage-specific transcriptional control. Tox2 controls persistence of gILC3 by regulating expression of Hk2 and promoting glycolysis. We speculate that enhanced glycolysis in gILC3 is a tissue-specific and lineage-specific adaptation that enables their rapid function under conditions of hypoxia generated by infection or other injury. Thus, ILC3 possess a tailored mechanism involving Tox2 and Hk2, that allows gILC3 to respond in a manner that differs from the response of other lymphocytes residing in the intestinal niche. The results further support the notion that specific metabolites may be exploited to promote or reduce the survival of gILC3⁷⁶, thus allowing the development of strategies to control autoimmune conditions and improve tissue-specific immunity and cancer immunotherapy.

MATERIALS AND METHODS

Mice

B6 (CD45.2), B6-Ly5.1 (CD45.1), B6.129-*Hif1a*^{tm3Rsj0}/J (strain#:007561), B6.129X1-*Gt(ROSA)26Sor*^{tm1(EYFP)Cos}/J (stain#:006148) and *Rag1*^{-/-} mice were purchased from Jackson Laboratory. *Tox2*^{-/-} mice have previously been described. *Tox2* flox mice were generated by using Cas9 protein, two synthetically modified guide RNAs (TCAAGGGCCTCCTGACTAGT and TGGAGATGTGTCCAAAATAG, Synthego) to generate 2 cuts, one on each side of Exon 5, and a single stranded DNA donor template containing this region, with loxP sites on each side, along with ~500 bp of homology. Guide RNA sequences were identified using sgRNA Scorer 2.0 (PMID: 28146356) and single stranded DNA was generated using the Guide-it Long ssDNA production system (Takara). Microinjections to generate knockout mice by CRISPR-Cas9 were performed by Transgenics/Cryopreservation-Laboratory Animal Science Program National Cancer Institute core on a C57BL/6Ncr background. ER^{T2Cre} mouse⁷⁷ (Taconic line 10471), *IL7r-iCre* R26-stop-YFP⁷⁸, *Tcf7*^{GFP79} and *Rorc*^{Cre80} mice have previously been described. *Tox2*^{-/-}.*Tcf7*^{GFP} were generated for EILP quantification in bone marrow. Mice were 6-12 weeks of age and of either sex, except for assessment of thymic ILC3 when 7-day old mice were used. Animal procedures were approved by relevant National Institutes of Health Animal Care and Use Committees.

Isolation of hematopoietic cells

For Lung: Mice were euthanized with CO₂, perfused through the left ventricle of the heart by injecting 10 ml PBS. Lungs were cut into small pieces and digested in Hank's balanced salt solution (HBSS) containing 0.025mg/ml Liberase D (Roche Diagnostics) and 10U/ml DNase I (Roche Diagnostics) for 30 min at 37°C on a shaker (220 rpm). Cells were filtered by using a cell strainer. Leukocytes were enriched by centrifugation (10 min, 650 g) on a 40%/80 % Percoll gradient (GE Healthcare), and RBCs were lysed by ammonium chloride solution.

For Lamina propria: Payer's patches were removed from small intestines. Intestinal tissues were washed, cut into 1-1.5 cm sections, incubated with Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich, St. Louis, MO) containing 5mM EDTA and 1mM DTT at 37°C for 20 mins to remove epithelial cells. For cell isolation from colon, tissue sections were incubated with RPMI media containing Pen/Strep, L-Glutamine, NEAA, Sodium Pyruvate and HEPES (GIBCO). The digested tissue was minced and dissociated in RPMI containing 10% FBS, liberase TL (1 mg/ml; Roche) and DNase1 (100 µg/ml; Roche) with constant stirring at 37°C for 30 min.

Fetal intestines from E15.5 were processed as described earlier⁸¹.

For BM cells: tibia, femur, and pelvic bones were flushed with 10 ml RPMI-containing 2% FBS and penicillin-streptomycin (100 U/ml) using 27.5-gauge needle, the single-cell suspension was filtered through a 70 µm cell strainer and washed once with 5 ml of the same media.

For lymph nodes, spleen and thymus: Tissues were mechanically disrupted. Single cell suspension was filtered through a 70 µm cell strainer and washed once with 5 ml of the same media. This was followed by RBC lysis by ACK lysis buffer (Quality Biological).

Liver tissues were mashed through 70 μm cell strainer and leukocytes were collected at the interface of a 40%/80% Percoll gradient (GE Healthcare).

Antibodies and Flow Cytometry

Bone marrow cell suspensions were incubated with a mix of purified rat, mouse and hamster immunoglobulin G (IgG) before the addition of specific antibodies. Antibodies specific for Ly-6D (49H4), B220 (RA3-6B3), CD19 (1D3), Mac-1 (M1/70), Gr-1 (8C5), CD11c (N418), Ter119 (TER119), NK1.1 (PK136), CD5 (53-7.3), CD3 ϵ (2C11), CD8 α (53-6.72), CD8 β (H35-17.2), CD4 (GK1.5), TCR β (H57), TCR $\gamma\delta$ (GL-3), Kit (2B8), CCR7 (4B12), CXCR5 (2G8), NKp46 (29A1.4), Sca-1 (D7), Thy1.2 (53-2.1), $\alpha_4\beta_7$ (DATK32), IL-7R α (A7R34), ICOS (C398.4A), CD25 (PC61.5), CD45.1 (A20), CD45.2 (104), EpCAM (G8.8), $\alpha_4\beta_7$ (DATK32), Flt3 (A2F10.1), CCR6 (G034E3) were purchased from ThermoFisher Scientific or BD Biosciences or BioLegend. Antibodies specific for transcription factors and cytokines: ROR γt (Q31-378), GATA-3 (TWAJ); T-bet (eBio4B10(4B10)), EOMES (Dan11mag), HIF-1 α (D1S7W), BCL-6 (BCL-DWN), IFN- γ (XMG1.2), IL-22 (IL22JOP) and IL-17 (eBio17b7) from ThermoFisher Scientific, Cell signaling and BD biosciences. HK-I (ab150423) and HK-II (ab209847) were purchased from Abcam.

ILC lineage 'cocktail' used to sort ALP (LinILC) for bone marrow is a mix of the following antibodies: anti-Ly-6D, B220, CD19, Mac-1, Gr-1, CD11c, Ter119, NK1.1, CD5, CD3 ϵ , CD8 α , CD8 β , CD4, TCR β and TCR $\gamma\delta$.

LinILC2: B220, CD19, NK1.1, Gr-1, CD11c, Ter119, CD5, CD3 ϵ , TCR β and TCR $\gamma\delta$.

ILC3 lineage cocktail 'Lin': B220, CD19, NK1.1, Gr-1, CD11c, Ter119, CD5, CD3 ϵ , TCR β and TCR $\gamma\delta$.

All analyses were done on singlet live cells. Gating strategy used for ALP, EILP, ILCP (bone marrow) and ILCP (fetal gut) were described earlier^{30,81}. NK: CD3 e^- B220 $^-$ NK1.1 $^+$ NKp46 $^+$ DX5 $^+$ CD49a $^-$; ILC1: CD3 e^- B220 $^-$ NK1.1 $^+$ NKp46 $^+$ DX5 $^-$ CD49a $^+$; ILC2 (surface stain): LinILC2 $^-$ CD45 $^+$ CD127 $^+$ Thy1 $^+$ ST2 $^+$ CD25 $^+$; ILC2 (intracellular stain): LinILC2 $^-$ CD45 $^+$ Thy1 $^+$ CD127 $^+$ GATA-3 $^+$; ILC3 (surface stain): Lin $^-$ CD45 $^+$ EpCAM $^-$ Kit $^+$ CD127 $^+$; ILC3 (intracellular stain): Lin $^-$ CD45 $^+$ EpCAM $^-$ ROR- γt $^+$ CD127 $^+$; For intracellular staining to detect cytokines and transcription factors, cells were first stained for cell surface molecules and permeabilized using the eBioscience's transcription factor staining buffer set (cat: 00-5523-00) according to the manufacturer's instructions. FITC Active Caspase-3 Apoptosis kit was used to measure cleaved Caspase-3 (BD Biosciences, cat: 550480). Live/dead discrimination was performed by staining with DAPI or LIVE/DEAD Fixable Blue Dead Cell stain kit (L-34962 Invitrogen). Samples were acquired using a flow cytometer (LSRFortessa; BD) and analyzed using FlowJo software (Tree Star). Cell populations were sorted using an Aria flow cytometer (BD). All cell sort purities were >95%. Absolute cell numbers were obtained using an Accuri C6 PLUS flow cytometer (BD).

Bone Marrow Transplantation

For competitive bone marrow chimeras⁸², 5,000 sorted LSK cells from donor mice (CD45.2) were mixed with 2,500 competitor WT LSK cells (CD45.1 or CD45.1 $^+$ CD45.2 $^+$) and intravenously transferred into lethally irradiated (900 rads) WT recipient mice (CD45.1).

Plasmids, cell culture and transduction

Full length mouse Tox2 cDNA (Cat# MR212216; Origene) was cloned into MSCV-IRES-GFP (MIGR1) and retroviral supernatant preparation were performed as described⁸³.

Hk2 cDNA (Cat# MC202280; Origene) along with a synthetic gene fragment containing 2A-GFP (Twist Biosciences) was cloned together into BamHI/EcoRI digested FUGW plasmid (Gift from David Baltimore, 14883, Addgene). Generation of infective lentiviral particles were performed in HEK293T (American Type Culture Collection) cells as described⁸⁴.

Cell culture and transduction

MNK-3 cells were cultured as described²⁴. MNK-3 cells were transduced using control GFP virus, Tox-2 virus and Hk2 virus with 4 µg/ml of polybrene (Cat# 7711/10; R&D SYSTEMS) at 900 g for 2 hours, following which viral supernatant was removed after 16 hours and GFP+ cells were sorted after 72 hours culture with 10 ng/ml of IL-7 and IL-15 (217-17, 210-15; Peprotech). Sorted GFP+ cells were rested for additional 2 days before performing Seahorse assay.

Bone marrow LSK and ALP were transduced using Retronectin (Takara). 24-well plates were coated with 50 µg/ml Retronectin according to the manufacturer's instructions. High-titre GFP or Hk2-GFP lentiviral supernatants along with sorted bone marrow LSK and ALP in IL-3, IL-6 (10 ng/ml), SCF (100 ng/ml), Flt3-ligand, IL-7 (30 ng/ml) and polybrene (6 µg/ml) were added into wells and centrifuged at 25°C for 2h, following which viral supernatants were removed after 16h incubation. Fresh medium with cytokines (IL-7, SCF and Flt3-ligand (30 ng/ml)) was added and transduced cells were sorted 24 hours post-infection.

Seahorse assay

Oxygen consumption rate (OCR) and Extracellular acidification rate (ECAR) were measured using the XFe96 Extracellular Flux Analyzer (Seahorse Biosciences, Agilent). Agilent XFe96 Extracellular Flux Assay cartridges were hydrated overnight in pure water, then water was replaced by 200 µL of Seahorse Calibrant and incubated at least 1h at 37°C (non-CO₂). MNK3 cells (1e5/well) were harvested 2 days post sort after resting in media. Murine T cells from C57Bl6 mice were selected from mice spleens (EasyStep mouse T cell isolation kit #1951A) and cultured with IL2 only (1e5/well) for 3 days. For titration experiments, MNK3 parental cells were harvested and resuspended at the indicated number of cells/well. Cells were resuspended in 80 µL Agilent XF media (buffered RPMI pH7.4) in the presence of glucose (11 mM) and L-glutamine (2 mM), and pyruvate (1 mM) and seeded in 96-well Seahorse XF96 cell culture microplates (Seahorse Biosciences, Agilent) coated with CellTak (Corning 354240) 20 min at room temperature. Cells were incubated at 37°C (non-CO₂) for 30 minutes, then the total volume of each well was set to 180 µL by adding 100 µL of XF media. Oligomycin (Sigma-Aldrich #04876, 2 µM), FCCP (Sigma-Aldrich #C2920, 1 µM), Rotenone (Sigma-Aldrich R8875, 1 µM), Antimycin A (Sigma-Aldrich A8674, 1 µM) were injected as indicated.

Adoptive transfer of ILC3 and transduced progenitors

Sorted 2000 gILC3 or 5000 mILC3 were adoptively transferred into NSG mice by intravenous injection. The donor population was sorted from spleen and gut one week post transfer.

20,000-50,000 transduced BM LSK and ALP were adoptively transferred into NSG mice by intravenous injection. Mice were analyzed 4 weeks after reconstitution for donor chimerism in blood and donor-derived ILC3 in gut were assessed 12 week post-reconstitution.

ILC3 *in vitro* cultures

ILC3 culture with metabolites: Sorted 2000 gILC3 or 5000 mILC3 were cultured on irradiated OP9 stromal layers in α -MEM supplemented with 20% FBS, glutamine, penicillin, streptomycin, IL-7, IL-2 (30ng/ml) were added without or with glucose (25 mM; G8270), acetate (5 mM; S5636) from Sigma-Aldrich and pyruvate (10 mM; 11360-070) from ThermoFisher Scientific.

ILC3 culture with metabolic inhibitors: mLN and gut lymphocytes were treated for 30 min with Control, 2-Deoxy-D-Glucose (2DG, final concentration 100mM), Oligomycin (final concentration 1 μ M) or both. Puromycin (10 μ g/ml) was added for another 30 min. After incubation cells were stained for puromycin as described below.

For PMA/Ionomycin stimulation: mLN and gut lymphocytes were stimulated with 50 ng/ml PMA (cat: P1585) and 500 ng/ml Ionomycin (I9657) from Sigma-Aldrich for 2.5 hours and Brefeldin A (ThermoFisher Scientific; B7651) was added in the last 2 hours of stimulation.

For hypoxia experiments: Sorted gILC3 (2000 cells) and mILC3 (5000 cells) were incubated for 6h in 5% O₂ to mimic hypoxic condition in the gut⁶⁷ or maintained in control atmospheric oxygen conditions (21% O₂). *Tox2* and *Hk2* expression was then evaluated by qRT-PCR.

Puromycin staining

The antibiotic puromycin (PMY) is an aminoacyl-tRNA analog that binds to the ribosome A-site and is covalently incorporated onto the 3' end of the nascent polypeptide resulting in chain termination. After fixation and permeabilization, the puromycylated polypeptides can be detected by an intracellular stain with an antibody specific to puromycin. The intracellular RPM signal from PMY treated mice minus the RPM signal from PBS treatment generates a measure of the total amount of translation⁸⁵. Mice were injected intravenously with 100 μ l of puromycin (PMY) (10 mg/ml, Calbiochem) in PBS (Life Technologies) that was warmed to 37 °C. Mice were sacrificed 30 min post PMY injection and thymi processed as stated above. Samples were stained with LIVE/DEAD Fixable Blue Dead Cell Stain Kit (ThermoFisher Scientific) on ice for 10 min, then fixed and permeabilized in fixation/permeabilization buffer (1% paraformaldehyde, ThermoFisher Scientific; 0.0075% digitonin, Wako) in PBS for 20 min at 4 °C. Cells were then labeled with a directly conjugated (using Life Technologies protein labeling kit as per the manufacturer's instructions) anti-PMY Ab (clone PMY-2A4) for 1 hour.

BrdU staining

Mice were injected intraperitoneally (i.p) with BrdU (1.5 mg) 18 hour prior to sacrifice, and mLN and gut were processed as stated above. Cell suspensions were intracellularly stained for

BrdU using the BD bioscience APC BrdU flow kit (552598), according to the manufacturer's instructions. Control mice received vehicle alone injections.

Tamoxifen treatment

Tamoxifen (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich C8267-500ml) at a concentration of 25 mg/ml by shaking 6 h at 37 °C. Adult mice were administered with 200 µl tamoxifen (5 mg per mouse) via intraperitoneal injection daily, 5 times on alternate days. Mice were sacrificed 3 weeks post treatment⁸⁶.

SILT quantification

Small intestines were transversally cut into three equal size segments (duodenum, jejunum, and ileum). After removal of contents by using forceps and performing washes with PBS, they were cut longitudinally. The opened segments were flattened and incubated in 4% paraformaldehyde/PBS for 30 min. Then, tissue sections were swiss-rolled⁸⁷ and incubated in 4% paraformaldehyde/PBS at 4 °C overnight. Fixed intestines were firstly washed with PBS and dehydrated performing an incubation with 15% sucrose for 24 hours, followed by another incubation in 30% sucrose for also 24 hours. Then, swiss-rolls were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA). After discarding approximately, the first 800 µm of the embedded tissue by using the cryostat HM525 (Thermo Scientific), five sections of 12 µm in thickness were cut, each section were 100 µm apart and stained using antibodies against the following mouse proteins: B220 (RA3-6B2, 1:50, eBioscience), CD3e (500A2, 1:50, eBioscience). Detection of YFP expression was indicative of RORγt⁺ cells. Sections were counterstained with DAPI and mounted using Prolong Glass Antifade Mountant (Invitrogen)⁸⁸. Whole stained tissue sections were imaged at 20x magnification by using the SoRa CSU-W1 spinning disk microscope (Nikon) with implemented motorized accessories. NSI-Elements software (Nikon) was used to stitch approximately 150-175 image frames by applying the blending method to form the whole tissue section. To perform quantification of CP and IFL, we focused on duodenum because in our pilot experiment, the difference in CP number was prominent in duodenum than other sections analyzed however the number of ILF was comparable across the sections analyzed between WT and *Tox2*^{-/-} mice. CPs and ILFs were counted in stiched images in 5 sections taken at 100µm intervals through the depth of the duodenum.

Quantification of glucose and mitochondrial dependence

Glucose and Mitochondrial dependencies along with glycolytic and FAO and AAO capacities are calculated as previously described⁴⁷

Glucose dependence (%) = 100*((Puromycin MFI of Control group - Puromycin MFI of 2-DG treated group)/(Puromycin MFI of Control group - Puromycin MFI of both inhibitors treated group))

Mitochondrial dependence (%) = $100 * ((\text{Puromycin MFI of Control group} - \text{Puromycin MFI of Oligomycin treated group}) / (\text{Puromycin MFI of Control group} - \text{Puromycin MFI of both inhibitors treated group}))$

Glycolytic capacity (%) = $100 - \text{Mitochondrial dependence}$

FAO and AAO capacity (%) = $100 - \text{Glucose dependence}$

Puromycin MFI for each group is calculated by subtracting values of puromycin MFI of each group from the control MFI (without puromycin).

Citrobacter infection

Citrobacter rodentium (strain ICC169) was cultured overnight in Luria-Bertani (LB) broth at 37°C as previously described⁸⁹. Mice were infected orally by gavage with 10^8 colony-forming units (CFU) resuspended in sterile PBS. The mice were assessed on day 6 for ILC3 function. To determine the bacterial load in spleen, liver and feces, tissues were homogenized, serially diluted and plated onto LB-agar plates containing nalidixic acid, and the colonies were counted after incubation overnight at 37°C.

Quantitative RT-PCR

A total of 5×10^3 sorted mILC3, gILC3 along with ILC3 subsets, ALP, ILC2, ILC1 and NK cells were lysed in RLT buffer, and total RNA was isolated using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) using TaqMan probes designed against *Tox2* (Mm01241014; ThermoFisher scientific) and *Gapdh* (Mm9999915; ThermoFisher scientific), *Hk2* (Mm00443385). Results were analyzed using the $\Delta\Delta$ cycle threshold method and plotted as relative expression normalized to β -actin.

scRNA-Seq and analysis

Cells from mLN and gut were isolated by sorting from WT or *Tox2*^{-/-} mice by gating for Lin⁻ CD5⁻ CD45⁺ kit⁺ to mainly enrich for ILC3. Sorted cells were loaded on the 10X Chromium platform (10X genomics). Libraries were constructed using the Chromium Single Cell 3' kits according to the manufacturer's instructions (v3 chemistry, 10x Genomics). Libraries were sequenced with a NextSeq (v3 chemistry, Illumina). A primary analysis was performed with the Cell Ranger (version 6.0.0) software using the default parameters. Data set in Fig. 4: WT mLN (2585 cells), *Tox2* KO mLN (2816 cells), WT gut (4134 cells), *Tox2* KO gut (3985 cells), expressing a median of 1500–4000 genes per sample and all captured at the same time. The median number of UMI counts ranged between 4000 and 9500 per cell. Single-cell analysis was performed using Seurat (version 4.1.0)⁹⁰, applying default settings unless otherwise stated. We excluded cells with >10% mitochondrial gene content and >5000 genes per cell. Mitochondrial content and cell-cycle related genes were regressed out. We performed UMAP dimensional reduction using 20 principal components. Cells were clustered using Seurat's FindClusters function. Contaminants were identified based on gene expression signature determined using the

function FindAllMarkers with a minimum Log2 fold change threshold of 0.25 and the Wilcoxon Rank Sum test. After removing contaminant cells, the remaining cells were clustered using Seurat's FindClusters function. Seurat's FindMarkers function was used to obtain the DEGs between clusters considering those genes expressed in at least 12% of cells in the clusters to be compared. P-values were determined using the Wilcoxon ranked sum test and genes with a Bonferonni-adjusted p -value of <0.05 are reported here. Violin plots shown in the figures were made using Seurat's normalized data. Average gene expression from cells of the same cluster was generated using the Seurat's Average Expression function. Gene ontology pathway analysis of DEGs was carried out with the ClusterProfiler R package (version 4.0.5).

scATAC-Seq and analysis

The libraries obtained from different experimental conditions were aggregated using the cellranger-atac aggr pipeline. Subsequently, the fragment files were loaded into R using Signac.

Only high-quality cells were retained, based on the following parameters: having more than 3000 and less than 30000 number of peaks, the percentage of reads in peaks being higher than 15, the transcription start site enrichment being higher than 15 and having a nucleosome signal less than 4. The data was analyzed using a standard Signac workflow, using a q_0 cutoff for variable features and using excluding the first latent semantic indexing dimension (LSI). Gene activity was predicted based and leveraged to isolate clusters containing ILC3s. The ILC3s were reanalyzed using the same workflow, and annotations were transferred from the scRNAseq data using the FindTransferAnchors, TransferData and AddMetaData functions. Specifically, canonical correlation analysis was used to identify "anchors" and the first LSI dimension was excluded from the TransferData function. Annotated heatmaps were generated using the DimPlot function and the coverage plots were generated using the CoveragePlot functions.

Statistics

Statistical significance was performed with GraphPad Prism. Differences between groups were determined by a two-tailed unpaired Student's t test or by one-way ANOVA with multiple comparisons. For statistical significance, asterisks indicate the level of significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Data Availability

scRNA-seq and scATAC-seq data are deposited to GEO and a GSE accession number will be provided when it is available.

Supplementary Table 1. List of differentially expressed genes (DEGs) in mILC3 and gILC3 subsets isolated from WT and *Tox2*^{-/-} mice.

Supplementary Table 2. GO analysis of the DEGs in gILC3 from WT and *Tox2*^{-/-} mice

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AUTHOR CONTRIBUTIONS

A.D. and A.B. conceived and designed the research. A.D. performed most experiments and analyzed data with the help from G.U.M-R, N.B., A.S., J.M., M.L., Y.Z., A.M.S., Y.D., M.V-S. and S.R.M.. J.M. and N.T. performed and analyzed Seahorse assays, and provided expertise to interpret assays of protein synthesis. R.C. and P.A. generated reagents. M.O.S., J.Y., V.S.S and Y.B. provided essential reagents and expertise. T.I., N.M. and W.C. provided *Il17a*^{-/-} mice and expertise. G.U.M-R., A.B. and A.D. analyzed scRNAseq data. M.L. analyzed scATACseq data. G.U.M-R. performed and analyzed microscopic analysis with help from A.D., J.Z., and A.B.. C.H., N.T., V.S.S., S.M.R. and Y.B. provided essential conceptual insights. A.D., N.T., V.S.S. and A.B. wrote the manuscript. All authors read and commented on the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. *Tox2* is specifically required for gut ILC3 and not ILC3 at central sites

(A) The abundance of *Tox2* mRNA relative to b-actin was measured by quantitative reverse-transcription PCR (qPCR) in sorted All Lymphoid Progenitors (ALP) from bone marrow (BM), NK cells and ILC1 from liver, ILC2 from lung and NCR⁺ and NCR⁻ ILC3 from mesenteric lymph nodes (mLN) and small intestinal lamina propria (gut). n=4-5 mice per group.

(B) Flow plots show a representative analysis of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ ROR- γ t⁺ ILC3 in the gut in WT and *Tox2*^{-/-} mice (left). Bar graph shows cellularity of gILC3 gated as in WT and *Tox2*^{-/-} mice (right). n=7 mice per group.

(C) Flow plots show representative analyses of Lin⁻ EpCAM⁻ CD45⁺ ROR- γ t⁺ ILC3 from mesenteric lymph node (mLN), spleen, peripheral lymph nodes (pLN) and thymus in WT and *Tox2*^{-/-} mice (left). Bar graph shows cellularity of ILC3 in WT and *Tox2*^{-/-} mice from mLN, spleen, pLN and thymus (right). n=6-9 mice per group.

(D-E) Bar graph showing absolute number of NCR⁺, CCR6⁺ and NCR⁻CCR6⁻ ILC3 subsets from gut (D) and mLN (E) of WT and *Tox2*^{-/-} mice. n=9 mice per group.

(F) We mixed 5,000 LSK cells from WT and *Tox2*^{-/-} mice (CD45.2) with 2,500 WT competitor LSK cells (CD45.1) and together injected them into lethally irradiated (900 rads) WT recipient (CD45.1). Reconstitution of ILC3 from gut and mLN along with B cells and NK cells from spleen were examined at 12 weeks post reconstitution. Bar graph shows percentage chimerism. n=6 mice per group.

(G) Experimental plan showing FACS sorted Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ ILC3 from mesenteric lymph nodes (mILC3) were adoptively transferred into NSG recipients and a week later donor mILC3 (CD45.2) were sorted from gut and spleen of NSG mice (CD45.1) (top). *Tox2* expression was analyzed by qRT-PCR from sorted donor ILC3 that homed into spleen and gut. mILC3 and ILC3 from gut (gILC3) shown as controls (bottom). n=4 mice per group.

(H) Flow plots show a representative analysis of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ ILC3 in the gut from *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice. Plots were gated on Lin⁻ CD45⁺ EpCAM⁻ gut cells (left). Bar graph shows cellularity of ILC3 in *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice from mLN and gut (right). n=5-9 mice per group. Data are representative of at least three independent experiments. Error bars are SEM. See also Figure S1.

Figure 2. *Tox2* ablation leads to reduced ILC3 response upon *Citrobacter rodentium* infection

(A) Experimental plan showing WT and *Tox2*^{-/-} as well as *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice were infected orally with 10⁸ CFU *Citrobacter rodentium* and immune responses were assessed at indicated time points.

(B) Weight loss of WT and *Tox2*^{-/-} mice after infection with *C. rodentium*. Data are representative of 3 independent experiments. n=8-10 mice per group.

- (C) Bacterial load measured in feces and translocation to peripheral organs, spleen and liver, on day 6 post infection (p.i.) in WT and *Tox2*^{-/-} mice. n=4-5 mice per group.
- (D) Flow plots show a representative analysis of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ ROR- γ t⁺ ILC3 isolated from colonic lamina propria of WT and *Tox2*^{-/-} mice on day 6 p.i. (top). Bar graph shows absolute number of colon lamina propria ILC3 in WT and *Tox2*^{-/-} mice on day 6 p.i. (bottom). n=4-5 mice per group.
- (E) Representative flow plots showing cytokine production by ILC3 in colon of WT and *Tox2*^{-/-} mice on day 6 p.i. (left). Bar graph shows frequencies of IL-22⁺ and IL-17A⁺ ILC3 on day 6 p.i. in WT and *Tox2*^{-/-} mice (right). n=4-5 mice per group.
- (F) Weight loss of *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice after infection with *C. rodentium*. n= 8-10 mice.
- (G) Bacterial load measured in feces and translocation to peripheral organs, spleen and liver, on day 7 post infection (p.i.) in *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice. n=4-5 mice per group.
- (H) Flow plots show a representative analysis of ILC3 isolated from colonic lamina propria of *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice on day 7 p.i. (top). Bar graph shows absolute number of ILC3 in colon of *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice on day 7 p.i. (bottom). n=4-5 mice per group.
- (I) Representative flow plots showing cytokine production by ILC3 in colon of *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice on day 6 p.i. (left). Bar graph shows frequencies of IL-22⁺ and IL-17A⁺ ILC3 on day 6 p.i. in *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice (right). n=4-5 mice. Data are representative of at least three experiments. Error bars are SEM. See also Figure S2.

Figure 3. *Tox2* is required by gut ILC3 and for their persistence in gut

- (A) Flow plots show a representative analysis of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ ILC3 from mLN (top) and gut (bottom) of *Rorc*^{Cre} *Tox2*^{+/+} and *Rorc*^{Cre} *Tox2*^{fl/fl} mice. Bar graph shows cellularity of ILC3 from mLN (mILC3) and gut (gILC3) in *Rorc*^{Cre} *Tox2*^{+/+} and *Rorc*^{Cre} *Tox2*^{fl/fl} mice (right). n=5 mice per group.
- (B) Schematic representation showing ER^{T2Cre} *Tox2*^{+/+} and ER^{T2Cre} *Tox2*^{fl/fl} mice were treated with 5mg/ml tamoxifen on days 0, 2, 4, 6, and 8. mILC3 and gILC3 were isolated by cell sorting three weeks after tamoxifen injection.
- (C) *Tox2* deletion was examined by qPCR after tamoxifen treatment in sorted Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ mILC3 and gILC3 from ER^{T2Cre} *Tox2*^{+/+} and ER^{T2Cre} *Tox2*^{fl/fl} mice. n=5 mice per group.
- (D) Flow plots show a representative analysis of ILC3 from mLN (top) and gut (bottom) in ER^{T2Cre} *Tox2*^{+/+} and ER^{T2Cre} *Tox2*^{fl/fl} mice three weeks after tamoxifen treatment. Plots were gated on Lin⁻ CD45⁺ CD5⁻ cells. Bar graph shows cellularity of ILC3 from mLN and gut ER^{T2Cre} *Tox2*^{+/+} and ER^{T2Cre} *Tox2*^{fl/fl} mice (right panel). n=5 mice per group.
- (E) Representative histograms showing *Rorc* expression history in NK cells (top); ILC1 (middle) and Lin⁻ NK1.1⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ ILC3 (bottom) from *Rorc*^{Cre} Rosa-YFP *Tox2*^{+/+} and *Rorc*^{Cre} Rosa-YFP *Tox2*^{fl/fl} mice. Quantification of *Rorc*⁺ILC1 from mLN and gut show in bar graph. n=4 mice per group.

(F) Visualization of CPs identified as YFP⁺ CD3e⁻ B220⁻ and ILFs identified as YFP⁺ CD3e⁻ B220⁺ in duodenum of *Rorc*^{Cre} Rosa-YFP *Tox2*^{+/+} and *Rorc*^{Cre} Rosa-YFP *Tox2*^{fl/fl} mice. Quantification of CPs and ILFs from duodenum (bottom). n=3 mice per group. All data are from or are representative of three independent experiments. Error bars are SEM. See also Figure S3.

Figure 4. Gut ILC3 from *Tox2*^{-/-} mice are transcriptionally distinct from their WT counterparts

scRNA-seq analysis performed on sorted as Lin⁻ EpCAM⁻ CD5⁻ kit⁺ ILC3 from mLN (mILC3) and gut (gILC3) isolated from WT and *Tox2*^{-/-} mice (A-C)

(A) A UMAP representation of mILC3 (top) and gILC3 (bottom) isolated from WT and *Tox2*^{-/-} mice, colored and clustered by original identity.

(B) A UMAP representation of 2 broad ILC3 subsets, NCR⁺ and CCR6⁺ ILC3 in mILC3 (top) and gILC3 (bottom) isolated from WT and *Tox2*^{-/-} mice, colored and clustered by original identity.

(C) A heatmap of averaged RNA normalized Z scores shows expression of selected upregulated and downregulated genes in gILC3 and mILC3 as controls from WT and *Tox2*^{-/-} mice, where each column represents an average of 2585 cells from WT mILC3, 2816 cells from *Tox2*^{-/-} mILC3, 4134 cells from WT gILC3, 3985 cells from *Tox2*^{-/-} gILC3. Hk2 expression is indicated in red box.

(D) Glycolysis pathway showing the requirement of specific enzymes at each step (left). A heatmap of averaged RNA normalized Z scores shows the gene expression of glycolytic enzymes in mILC3 and gILC3 from WT and *Tox2*^{-/-} mice (right), where each column represents an average of 2585 cells from WT mILC3, 2816 cells from *Tox2*^{-/-} mILC3, 4134 cells from WT gILC3, 3985 cells from *Tox2*^{-/-} gILC3.

(E) Heatmap of averaged RNA normalized Z scores showing gene expression of enzymes that are involved in oxidative phosphorylation pathway in mILC3 and gILC3 from WT and *Tox2*^{-/-} mice, where each column represents an average of 2585 cells from WT mILC3, 2816 cells from *Tox2*^{-/-} mILC3, 4134 cells from WT gILC3, 3985 cells from *Tox2*^{-/-} gILC3.

(F) Hexokinase-2 (*Hk2*) expression relative to b-actin was examined by qPCR in mILC3 and gILC3 sorted from WT and *Tox2*^{-/-} mice (left panel) and from ER^{T2Cre} *Tox2*^{+/+} and ER^{T2Cre} *Tox2*^{fl/fl} mice (right panel) three weeks after tamoxifen treatment. n=4-5 mice per group.

(G-H) Hk2 expression was analyzed by flow cytometry (left) in Lin⁻ CD5⁻ ROR-γt⁺ CD127⁺ ILC3 and CD4 T cells as control from mLN (G) and gut (H) of WT (black line) or *Tox2*^{-/-} (red line) isolated, overlaid against an isotype control (gray line). Bar graph represents the MFI of Hk2 staining on ILC3 and CD4 T cells (right). n=5 mice per group.

(I) Violin plot of *Tbx21* gene expression in gILC3 from WT and *Tox2*^{-/-} mice assessed in scRNA seq data and histogram showing T-bet protein expression in gILC3 from WT and *Tox2*^{-/-} mice and WT gut ILC1 as control. Bar graph represents the MFI of T-bet staining on gILC3 from WT and *Tox2*^{-/-} mice. n=5 mice per group.

(J) Violin plot of *Bcl6* gene expression in gILC3 from WT and *Tox2*^{-/-} mice in scRNA seq data and histogram showing BCL-6 protein expression in gILC3 WT and *Tox2*^{-/-} mice. Bar graph

represents the MFI of BCL-6 staining on gILC3 from WT and *Tox2*^{-/-} mice. n=5 mice per group. Data are representative of three independent experiments. Error bars are SEM. See also Figure S4.

Figure 5. *Tox2* deficiency leads to an attenuated protein translation in gut but not LN ILC3 with a decreased dependence on glycolysis

(A) Representative histograms of puromycin antibody staining (solid lines) on mILC3, gILC3 overlaid against a without puromycin control (black dotted line) following a 30 minute *in vitro* exposure to glycolytic (2DG), oxphos (oligomycin) and dual inhibitors (2DG+Oligomycin). Control MFI in the absence of puromycin staining are presented in black line in each plot. Bar graph represents the MFI of puromycin gated on mILC3 and gILC3 from WT mice. n=5 mice per group.

(B) Representative histograms of anti-puromycin antibody staining on Lin⁻ EpCAM⁻ CD45⁺ ROR- γ ⁺ ILC3 and CD4 T cells as control from mLN of WT and *Tox2*^{-/-} mice treated with either puromycin (solid lines) or PBS (dotted lines). Bar graph represents the MFI of puromycin gated on ILC3 and CD4 T cells from mLN of WT and *Tox2*^{-/-} mice. n=5-6 mice per group.

(C) Representative histograms of anti-puromycin antibody staining on ILC3 and CD4 T cells as control from gut of WT and *Tox2*^{-/-} mice treated with either puromycin (solid lines) or PBS (dotted lines). Bar graph represents the MFI of puromycin gated on ILC3 and CD4 T cells from mLN of WT and *Tox2*^{-/-} mice. n=4-6 mice per group.

(D) Quantification of MFI of puromycin incorporation in indicated groups from WT and *Tox2*^{-/-} mice gated on mILC3. n= 5 mice per group.

(E) Representative histograms of puromycin antibody staining (solid lines) on gILC3 from WT and *Tox2*^{-/-} mice following a 30 minute *in vitro* exposure to glycolytic (2DG), oxphos (oligomycin) and dual inhibitors (2DG+Oligomycin). Control histograms showing fluorescence in the absence of puromycin staining are presented (dotted lines). Quantification of MFI of puromycin incorporation in indicated groups from WT and *Tox2*^{-/-} gILC3. n= 5 mice per group.

(F) Bar graph represents concentration of lactate measured in supernatant from 3 day culture of sorted GFP⁺ transduced MNK-3 cells with empty virus (+GFP), *Tox2* virus (+*Tox2*.GFP) and Hk-2 virus (+Hk2.GFP) along with untransduced MNK-3 cells (UT). n=9-10

(G) ECAR (top) and OCR (bottom) levels were assessed using the XFe96 analyzer in MNK3 cells transduced with *Tox2*.GFP, Hk2.GFP or GFP vectors and in unactivated murine T cells (negative control), before and after sequential injections with oligomycin, FCCP and Rotenone-Antimycin-A. n=12-15 replicates from 2 independent experiments.

(H) Basal ECAR and OCR levels in MNK3 cells transduced with Hk2.GFP, *Tox2*.GFP or GFP control vectors or in unactivated murine T cells. n=12-15 replicates from 2 independent experiments.

Data are representative of three independent experiments otherwise state. Error bars are SEM. See also Figure S5.

Figure 6. Bypassing Hexokinase-2 rescued survival of *Tox2*^{-/-} gut ILC3 *in vitro*

- (A) Experimental schema showing lentiviral transduction and adoptive transfer of transduced bone marrow (BM) progenitors, LSK and ALP, from WT and *Tox2*^{-/-} mice with empty virus (GFP) or Hk2.GFP.
- (B) Representative flow plots showing Lin⁻ CD45⁺ EpCAM⁻ Kit⁺ CD127⁺ ILC3 recovered from gut after 12 weeks reconstitution of GFP and Hk2.GFP transduced WT and *Tox2*^{-/-} BM progenitors.
- (C-D) Bar graph showing quantification of recovered ILC3 (left) and NK cells (right) from NSG gut in indicated groups (C) and ILC3 subsets (D). n=4-9 mice per group. Error bars are SEM.
- (E) Experimental schema assessing ILC3 survival on sorted Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺ mILC3 and gILC3 following a 24h culture in IL-2/IL-7 cytokine support on OP9 stroma in the presence of low glucose, or following addition of high concentrations of glucose, pyruvate or acetate.
- (F) Representative flow plots showing recovery of WT and *Tox2*^{-/-} CD45⁺ sorted mILC3 24h following culture in low glucose (11mM), low glucose + high glucose (additional 25mM), low glucose + pyruvate (10 mM), and low glucose + acetate (5 mM). n= 4-5 mice per group.
- (G) Representative flow plots showing recovery of WT and *Tox2*^{-/-} CD45⁺ gILC3 24h following culture in low glucose (11mM), low glucose + high glucose (25mM), low glucose + pyruvate (10 mM), and low glucose + acetate (5 mM). n=5 mice per group.
- (H) Bar graph quantification of the recovered frequencies of mILC3 (left) and gILC3 (right) from WT and *Tox2*^{-/-} mice following a one day culture in the indicated conditions as shown in panel F. n=5 mice per group. Data are representative of three independent experiments. Error bars are SEM. See also Figure S6.

Figure 7. Hypoxia upregulates *Tox2* and its target *Hk2*

- (A) HIF-1 α expression on NCR⁺ and CCR6⁺ Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ ROR- γ t⁺ ILC3 from mLN and gut of WT and *Tox2*^{-/-} mice. Quantification of HIF-1 α staining in indicated populations. n=5 mice per group.
- (B-C) *Tox2* (left panel) and *Hk2* (right panel) mRNA levels were measured by qPCR in Lin⁻ CD45⁺ EpCAM⁻ Kit⁺ CD127⁺ mILC3 and gILC3 (B) and ILC3 subsets from mLN and gut (C) from WT and *Tox2*^{-/-} mice were measured following a 6h incubation in atmospheric (21% O₂, Nor) or hypoxic (5% O₂, Hyp) by qRT-PCR. n=5-7 mice per group.
- (D-E) Quantification of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ ROR- γ t⁺ ILC3 from mLN and gut (D) and ILC3 subsets (E) from *Rorc*^{Cre} *Hif1a*^{+/+} and *Rorc*^{Cre} *Hif1a*^{fl/fl} mice. n= 4-7 mice per group.
- (F) *Tox2* and *Hk2* mRNA levels were measured by qPCR in Lin⁻ CD45⁺ EpCAM⁻ Kit⁺ CD127⁺ ILC3 subsets from mLN and gut of *Rorc*^{Cre} *Hif1a*^{+/+} and *Rorc*^{Cre} *Hif1a*^{fl/fl} mice. Data are from two independent experiment with n=3 mice per group.
- (G-H) *Tox2* mRNA levels were measured by qPCR in NCR⁺ and CCR6⁺ mILC3 after 6h of indicated cytokine treatment from WT mice (G) and *Rorc*^{Cre} *Hif1a*^{+/+} and *Rorc*^{Cre} *Hif1a*^{fl/fl} mice (H).

(I-J) Flow plots showing Lin⁻CD45⁺CD127⁺ROR- γ ⁺ ILC3 from mLN and gut of WT and *Il17a*^{-/-} mice (I left) and quantification of total ILC3 (I right) and ILC3 subsets (J) from mLN and gut. n= 6-8 mice per group.

(K) *Tox2* and *Hk2* mRNA expression were assessed by qPCR in indicated cells from WT and *Il17a*^{-/-} mice. Data are from three independent experiment with 6-8 mice per group. Error bars are SEM.

(L-M) HIF-1 α antibody expression analyzed by flow cytometry and quantification of HIF-1 α expression on mILC3 and gILC3 (L) and ILC3 subsets (M) isolated from WT and *Il17a*^{-/-} mice. n= 4 mice per group. Data are representative of three independent experiments otherwise stated. Error bars are SEM. See also Figure S7.

Figure S1, refers to Figure 1. *Tox2* requirement in immune cells

(A) ROR- γ t expression on Lin⁻ CD45⁺ EpCAM⁻ CD127⁺ Kit⁺ ILC3 from WT gut (left) was analyzed by flow cytometry. Flow plots showing co-expression of Kit and ROR- γ t in Lin⁻ EpCAM⁻ CD45⁺ cells from WT gut (right).

(B) Quantification of EILP and ILCP from WT.TCF-1.GFP (WT) and *Tox2*^{-/-}.TCF-1 GFP reporter mice (*Tox2*^{-/-}). n= 4 mice per group.

(C) Flow plots show gating strategy for ILC3 subsets based on surface expression of NKp46 and CCR6 on ILC3 from gut (left) and mLN (right) from WT *Tox2*^{-/-} mice. ILC3 expressing NKp46 (NCR⁺), CCR6 (CCR6⁺) and without NKp46 and CCR6 (NCR⁻CCR6⁻) are shown in the plot.

(D) Bar graph shows ILC3 frequency in live cells from caecum, colon lamina propria and Peyer's patches in WT and *Tox2*^{-/-} mice. n= 4-6 mice per group.

(E) Quantification of cells from peripheral lymph nodes; inguinal, axillary, brachial and mandibular lymph nodes from WT and *Tox2*^{-/-} mice. Data are from 4 mice per group.

(F) Quantification of Peyer's patches from adult WT and *Tox2*^{-/-} mice. n= 7 mice per group.

(G) The abundance of *Tox2* mRNA relative to b-actin was measured by qPCR in indicated populations isolated from adult bone marrow (BM), mLN (m) and gut (g) and E15.5 fetal gut. n= 5 mice per group.

(H) Flow plots show a representative analysis of group 3 ILC (Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ Kit⁺) from E15.5 WT and *Tox2*^{-/-} fetal gut (left). Quantification of group 3 ILC in E15.5 fetal from WT and *Tox2*^{-/-} fetuses (right). n= 9 mice per group.

(I) Quantification of Lin⁻ EpCAM⁻ CD45⁺ CD127⁺ ROR- γ t⁺ ILC3 (top) and NCR⁺ (middle) and CCR6⁺ (bottom) ILC3 from gut of WT and *Tox2*^{-/-} mice at each indicated time point. n= 4-8 mice per group.

(J) Flow plots show a representative analysis of ILC1, NK cells, ILC2 and ILC3 in small intestinal lamina propria (SI LP) from WT and *Tox2*^{-/-} mice using intracellular markers (top). Quantification of NK cells, ILC1, ILC2 and ILC3 in SI LP from WT and *Tox2*^{-/-} mice (bottom). n= 4-6 mice per group.

(K) Quantification of NK cells from spleen and NK and ILC1 from liver and colon of WT and *Tox2*^{-/-} mice. n=4-5 mice per group.

(L) Quantification of ILC2 from lung and colon of WT and *Tox2*^{-/-} mice. n=4-6 mice per group.

(M) Quantification of B cells, myeloid cells and CD4 T, CD8 T cells from spleen of WT and *Tox2*^{-/-} mice. n= 5 mice per group.

Data are representative of at least three independent experiments. Error bars are SEM.

Figure S2, refers to Figure 2. Reduced ILC3 response in *Tox2*-deficient mice post *C. rodentium* infection is T-cell independent

(A) Bar graph shows frequencies of IL-22⁺ and IL-17A⁺ in NCR⁺ ILC3 (left) and CCR6⁺ ILC3 (right) from uninfected gut of WT and *Tox2*^{-/-} mice after 3 hrs PMA/Ionomycin stimulation. n=4 mice per group.

(B) Bar graph shows colon length on day 6 and day 10 p.i. in WT and *Tox2*^{-/-} mice. n= 4-8 mice per group.

(C) Bar graph shows frequency of CD4 T, CD8 T, and $\gamma\delta$ T cells among live cells on day 6 p.i. from colon of WT and *Tox2*^{-/-} mice. n= 4-5 mice per group.

(D) Bar graph shows frequencies of IFN- γ ⁺ ILC1 and NK cells from colonic lamina propria on day 6 p.i. of WT and *Tox2*^{-/-} mice (left panel) and day 7 p.i. from *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice (right panel). n= 4 mice per group.

(E-F) Representative flow plots showing frequencies of IL-22⁺ and IL-17⁺ cells (top) by NCR⁺ (left), CCR6⁺ (middle) and NCR⁻CCR6⁻ (right) ILC3 in colonic lamina propria of WT and *Tox2*^{-/-} mice on day 6 p.i. (E) and *Rag*^{-/-} and *Rag*^{-/-}*Tox2*^{-/-} mice on day 7 p.i. (F). Bar graph shows quantification of frequencies (bottom panel) of IL-22⁺ and IL-17A⁺ producers in indicated ILC3 subsets. n= 4-5 mice per group.

Data are representative of at least three independent experiments. Error bars are SEM.

Figure S3, refers to Figure 3. *Tox2* is not needed for homing of ILC3 into gut and proliferation of ILC3

(A) Strategy of generating *Tox2* flox mice. Two loxP sites were added flanking exon 5 of the *Tox2* gene.

(B) Flow plots show a representative analysis of ILC3 (left) from mLN (top) and gut (bottom) in *IL7R α* ^{Cre/+} *Tox2*^{+/+} and *IL7R α* ^{Cre/+} *Tox2*^{fl/fl} mice. Plots were gated on Lin⁻CD45⁺ EpCAM⁻ cells. Bar graph shows cellularity of ILC3 from mLN and gut in *IL7R α* ^{Cre/+} *Tox2*^{+/+} and *IL7R α* ^{Cre/+} *Tox2*^{fl/fl} mice (right). n=5 mice per group.

(C) Bar graph shows absolute count of NCR⁺ and CCR6⁺ ILC3 from mLN (left) and gut (right) of *Rorc*^{Cre} *Tox2*^{+/+} and *Rorc*^{Cre} *Tox2*^{fl/fl} mice. n=5 mice per group.

(D) Representative histograms of CCR7 staining on ILC3 and CD4 T cells as control from mLN (left) and gut (right) in WT and *Tox2*^{-/-} mice, overlaid against an isotype control.

(E) Representative histograms of CCR7 staining on NCR⁺ ILC3 and CCR6⁺ ILC3 from mLN (top panel) and gut (bottom panel) in WT and *Tox2*^{-/-} mice, overlaid against an isotype control.

(F) The proliferation status of WT and *Tox2*-deficient ILC3 from mLN and gut as measured by BrdU incorporation assay after 18 hours of a single pulse of BrdU injection. Bar graph shows data from 4-5 mice group.

(G) The survival status of WT and *Tox2*^{-/-} ILC3 from mLN and gut was examined by cleaved Caspase 3 staining. Bar graph shows data from 4-5 mice per group.

(H) Quantification of CPs (top) and ILFs (bottom) from duodenum, jejunum and ileum of small intestine of *Rorc*^{Cre} Rosa-YFP *Tox2*^{+/+} and *Rorc*^{Cre} Rosa-YFP *Tox2*^{fl/fl} mice. Data from 1 experiment.

Data are representative of at least three independent experiments otherwise stated. Error bars are SEM.

Figure S4, refers to Figure 4. Single-cell transcriptome analysis of ILC3 from mLN and gut

(A-B) UMAP representation of mILC3 from three samples, PMID: 36071169, our WT and *Tox2*^{-/-} mILC3 (top). Feature plots showing the expression of individual genes indicated in published mILC3 dataset⁴³(left), WT (middle) and *Tox2*^{-/-} (right) mILC3 in our dataset (A) and in gILC3 (B) from WT and *Tox2*^{-/-} mice. Violin plot showing expression of *Rorc* expression in NCR⁺ and CCR6⁺ ILC3.

(C) Table displaying the number of differentially expressed genes in each subset of ILC3 (NCR⁺ and CCR6⁺) isolated from mLN and gut of WT and *Tox2*^{-/-} mice.

(D) Hexokinase-1 (Hk1) expression in Lin⁻ CD5⁻ ROR- γ ⁺ CD127⁺ ILC3 and CD4 T cells as control from mLN (top) and gut (bottom) of WT and *Tox2*^{-/-} mice, overlaid against an isotype control (gray line).

(E) Violin plot of OXPHOS gene score in NCR⁺ and CCR6⁺ ILC3 from mLN and gut of WT and *Tox2*^{-/-} mice.

(F) A heatmap of averaged RNA normalized Z scores from scRNA-seq data showing glycolytic gene expression including *Hk2* mRNA levels in NCR⁺ and CCR6⁺ ILC3 from mLN and gut of WT and *Tox2*^{-/-} mice.

(G) Donor ILC3 were sorted from NSG mice post one week transfer as shown in Fig1G and *Hk2* expression was measured by qPCR. Expression of *Hk2* was measured in sorted ILC3 from mLN and gut of WT and *Tox2*^{-/-} mice as controls. Data are from two independent experiments with n=4 mice per group.

(H) Hk2 staining on Lin⁻ CD45⁺ GATA-3⁺ CD127⁺ ILC2 from mLN (left) and gut (right) of WT and *Tox2*^{-/-} mice was analyzed by flow cytometry, overlaid against an isotype control. Bar graph represents the MFI of Hk2 staining on ILC2 from mLN and gut. n=5 mice per group.

(I) Representative flow plots showing Tfh cells (CXCR5⁺ BCL-6⁺) pre-gated on CD4⁺CD44⁺ cells in gut of WT and *Tox2*^{-/-} mice and histogram showing Hk2 staining on gut Tfh from WT and *Tox2*^{-/-} mice, overlaid against an isotype control.

(J) Hk2 staining on total ILC3, NCR⁺ and CCR6⁺ ILC3 from gut of WT and *Tox2*^{-/-} mice analyzed by flow cytometry, overlaid against an isotype control.

(K) A heatmap of scaled average expression of indicated genes for cytokine and proliferation in NCR⁺ and CCR6⁺ ILC3 from mLN and gut of WT and *Tox2*^{-/-} mice.

(L) UMAP of scATAC-seq data visualizing total ILC3 (top) and ILC3 subsets (bottom) from WT mLN, WT gut and *Tox2*^{-/-} gut.

(M). Coverage plots for the *Tox2* (top) in ILC3 and *Hk2* (bottom) loci, split according to cell type and sample. Red box in *Tox2* locus indicate the region deleted in *Tox2*^{-/-} mice.

Data are representative of at least three independent experiments otherwise stated. Error bars are SEM.

Figure S5, refers to Figure 5. *Tox2* deficiency leads to reduced protein translation and decreased dependence on glucose in both ILC3 subsets in the gut

(A) Bar graph showing a quantification of the glucose and mitochondrial dependence along with glycolytic and FAO and AA capacity of WT mILC3 and gILC3. n=5 mice per group.

(B) Representative histograms of puromycin antibody staining (solid lines) on mILC3 from WT and *Tox2*^{-/-} mice following a 30 minute *in vitro* exposure to glycolytic (2DG), oxphos (oligomycin) and dual inhibitors (2DG+Oligomycin). Control histograms showing fluorescence in the absence of puromycin staining are presented (dotted lines). n=5 mice per group.

(C) Representative histograms of anti-puromycin antibody staining on NCR⁺ and CCR6⁺ ILC3 from gut of WT and *Tox2*^{-/-} mice treated with either puromycin (solid lines) or PBS (dotted lines). Bar graph represents the MFI of puromycin gated on both gILC3 subsets from WT and *Tox2*^{-/-} mice. n=4 mice per group.

(D) Bar graph showing a quantification of the glucose and mitochondrial dependence along with glycolytic and FAO and AA capacity between WT and *Tox2*^{-/-} gILC3. n=5 mice per group.

(E) Representative histograms of puromycin antibody staining under indicated conditions (solid lines) on NCR⁺ and CCR6⁺ ILC3 from mLN (1st and 2nd row) or from gut (3rd and 4th row). Control histograms showing fluorescence in the absence of puromycin staining are presented (dotted lines).

(F) Representative flow plots showing transduction efficiency and post sort profiles of transduced MNK-3 cells with empty virus (GFP) control and *Tox2* (*Tox2*.GFP) and *Hk2* (*Hk2*.GFP) virus or left untransduced (UT) for 3 days.

(G) *Tox2* and *Hk2* expression were measured by qPCR in untransduced MNK-3 cells and sorted GFP⁺, *Tox2*.GFP⁺ and *Hk2*.GFP⁺ MNK-3 cells. n=5-6 per group.

(H-I) Histogram showing staining of *Hk2* (H) and *Hk1* (I) in indicated sorted populations and control MNK-3 cells (left). Bar showing quantification of MFI of *Hk2* and *Hk1* in indicated populations (right). n=5 per group.

Data are representative of at least three independent experiments otherwise indicated. Error bars are SEM.

Figure S6, refers to Figure 6. Transduction of bone marrow progenitors

(A) Representative flow plots showing transduction efficiency and post sort profiles of WT and *Tox2*^{-/-} bone marrow progenitors (LSK+ALP) transduced with empty virus (GFP) and Hk2 (Hk2.GFP) virus or left untransduced (UT).

(B) Flow plots showing gating strategy of ILC3 shown in Fig 6B.

(C) Quantification of gut Lin⁻CD45.2⁺CD127⁺GATA-3⁺ ILC2 from NSG gut after 12 weeks reconstitution of transduced progenitors. n=4 mice per group.

Data are representative of three independent experiments. Error bars are SEM.

Figure S7, refers to Figure 7. HIF-1 α and IL-17A upregulated *Tox2* in gut ILC3

(A) A violin plot of scRNA-seq data shows *Hif1a* mRNA levels in mILC3 and gILC3 from WT and *Tox2*^{-/-} mice.

(B) Bar graph represents the MFI of HIF-1 α staining on mILC3 and gILC3 from WT and *Tox2*^{-/-} mice. n=6-7 mice per group.

(C) HIF-1 α expression (left) and quantification (right) on CD4 T cells from mLN and gut of WT and *Tox2*^{-/-} mice. n=4 mice per group.

(D) Upper panel, the *Tox2* promoter visualized in the UCSC genome browser. The picture illustrates the location of putative Hif1 α binding sites predicted by and stored in the JASPAR core collection. Lower panel, summary table of indicated features of the predicted Hif1 α binding sites in the *Tox2* promoter. TSS, transcription start site

(E) HIF-1 α expression and quantification on mILC3 and gILC3 isolated from WT and *Tox2*^{-/-} mice incubated either under normoxia or hypoxia were analyzed by flow cytometry. Data are representative from two independent experiments with n=3 mice per group.

(F) Flow plots showing Lin⁻ CD45⁺ EpCAM⁻ CD127⁺ ROR- γ ⁺ ILC3 in mLN and gut from *Rorc*^{Cre} *Hif1a*^{+/+} and *Rorc*^{Cre} *Hif1a*^{fl/fl} mice.

(G-H) *Tox2* and *Hk2* mRNA levels were measured by qPCR in mILC3 and gILC3 from *Rorc*^{Cre} *Hif1a*^{+/+} and *Rorc*^{Cre} *Hif1a*^{fl/fl} (G) and WT and *Il17a*^{-/-} (H) mice. n=3-6 mice per group.

Data are from three independent experiments otherwise indicated. Error bars are SEM.

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