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Shared dependence on TOX for development of lymphoid tissue inducer cell and NK cell lineages

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Abstract

Thymocyte selection-associated HMG box factor (TOX) is a DNA-binding factor required for development of CD4 T cells, natural killer T cells, and T regulatory cells. Here we document that both NK cell development and lymphoid tissue organogenesis are inhibited in the absence of TOX. We find that development of lymphoid tissue inducer cells, a rare subset of specialized cells that plays an integral role in lymphoid tissue organogenesis, requires TOX. *Tox* is highly upregulated in immature NK cells in the bone marrow, consistent with the loss of mature NK cells in the absence of this nuclear protein. Thus, multiple cell lineages in the immune system share a TOX-dependent step for development.

Introduction

The immune system is comprised of primary lymphoid organs, including thymus and bone marrow, which support the complex developmental programs necessary for differentiation of precursor cells into distinct immune mediators, and secondary lymphoid organs, including spleen, lymph nodes and Peyer's patches, which focus antigens and promote cellular interactions needed for the initiation of the adaptive immune response as well as the subsequent generation of effector functions.

The development of lymph nodes and Peyer's patches, the latter consisting of lymph node-like structures localized on the small intestine, occurs during embryogenesis through a complex series of interactions between hematopoietic and stromal cells involving both soluble and membrane bound mediators1. Cell-bound lymphotoxin (LT) α1β2 interacting with LTβ receptor is important for this process2, such that ablation of lymphotoxin α (LTα) results in complete lack of lymph nodes, Peyer's patches as well as disorganized B and T cell areas in the spleen3. In addition, mice deficient in the chemokine CXCL13 or its receptor CXCR5 exhibit a defect in Peyer's patches and have compromised lymph node formation4. Absence of interleukin 7 (IL-7) signaling through deletion of IL-7 receptor

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(IL-7R, CD127) also inhibits secondary lymphoid tissue organogenesis, although to variable levels5-7.

Key to the development of lymph nodes and Peyer's patches is a distinct subset of cells called lymphoid tissue inducer (LTi) cells, which have been identified in both mice and humans8, 9. LTi cells are hematopoietic non-T cells (and thus express CD45 but lack T cell receptor (TCR) gene rearrangements or expression of CD3) that nonetheless express cell surface CD4 in mice, while CD4 expression is heterogeneous in humans8-10. LTi cells also express CD127, CXCR5, and LTα, consistent with the disruption of lymphoid tissue development in gene knockouts encoding these molecules8, 11, 12.

During fetal development, LTi cells concentrate as clusters in the lymph node anlagen8, 10, 13. Transfer of fetal $CD45^+CD3^-CD4^+$ cells into mice with severe defects in lymphoid tissue formation rescues these defects11, 12. IL-7 can promote *de novo* differentiation and proliferation of LTi cells from bone marrow in adult mice, indicating that some precursors are retained after birth14. Indeed, LTi-like cells can be found in several lymphoid tissues in the adult mouse, including the thymus, spleen and gut. Adult and fetal LTi cells share most cell surface markers14 as well as a similar transcriptome15, and adult LTi cells can promote development of $VCAM1^+$ intestinal lymphoid tissue when transferred into CXCR5-deficient mice14, 15. However, whether adult LTi cells can support true lymph node organogenesis remains to be formally demonstrated. Adult LTi cells play other roles in the immune system, including production of inflammatory cytokines interleukin 17 (IL-17) and interleukin 22 (IL-22)16 regulation of the autoimmune regulator Aire expression by medullary thymic epithelial cells17, 18 and organization of B and T cells areas in secondary lymphoid organs18.

Differentiation of LTi cells is known to be dependent on several nuclear factors, such as Ikaros19, the E-protein inhibitor Id220, 21 and a splice variant of the Retinoid-Related Orphan Receptor γ (RORγ(t))10. Consequently, gene knockouts of these factors result in loss of LTi cells and the absence of peripheral lymph nodes and Peyer's patches10, 20-22. Interestingly, gene knockouts of two of these transcriptional regulators also affect the differentiation of natural killer (NK) cells. Thus, mice deficient in Ikaros or Id2 lack NK cells19-21, as well as lymph nodes, suggesting the possibility of a common development pathway between these distinct cell types.

Development of NK cells has been shown to be dependent on the expression of interleukin 15 receptor (IL-15R)23 and a downstream IL-15-regulated transcription factor E4BP4 (NFIL3)24, 25. However, the block in NK cell development observed in IL-15R-deficient or E4BP4-deficient mice occurs at an earlier stage than that seen in Id2-deficient mice21, 24. Overexpression of E4BP4 resulted in increased expression of *Id2* in hematopoietic progenitor cells, suggesting that expression of Id2 could be directly regulated by E4BP424. Indeed, reconstitution of E4BP4-deficient mice with ectopically expressed Id2 rescues the development of NK cells, indicating that in this context E4BP4 functions primarily as an Id2 inducer. Whether development of secondary lymphoid tissue is also inhibited in E4BP4-deficient mice was not addressed yet. Thus, whether Id2 is similarly regulated during LTi development remains unclear.

In the current study we demonstrate that an additional nuclear protein, TOX (thymocyte selection-associated HMG box factor), plays a dual role in development of the NK and LTi lineages. The *Tox* gene was initially identified based on its upregulation during thymocyte differentiation and is expressed during specific stages of T cell development in the thymus26. TOX-deficient mice have a severe block at a transitional stage of positive selection in the thymus, leading to loss of the CD4 T lymphocyte lineage27. As shown here, TOX is also expressed in LTi cells and at specific stages in the development of NK cells. Consistent with this, $Tox^{-/-}$ mice have few LTi cells leading to failure of lymph node organogenesis and a drastic reduction in the frequency and size of Peyer's patches. In addition, we demonstrate a cell intrinsic defect in the ability of $Tox^{-/-}$ hematopoietic cells to differentiate into NK cells. These data add another common link between the LTi and NK cell lineages and reveal TOX as a key player in multiple aspects of immune system development.

Results

Defective lymphoid organogenesis in the absence of TOX

$Tox^{-/-}$ mice have a defect in the development of CD4 T lineage cells27. In addition, all peripheral lymph nodes, including mesenteric lymph nodes, were absent in $Tox^{-/-}$ mice (Fig. 1a). This phenotype was independent of the role of TOX in T cell development, as lymph nodes structures were present in mice with a conditional T cell deletion of *Tox*, while the T cell defect phenotype was recapitulated (Supplementary Fig. 1 and J.K., unpublished observations).

Peyer's patches could be identified in the small intestine of some, but not all, $Tox^{-/-}$ mice. However, even when present, Peyer's patches were drastically reduced in both size and frequency **(**Fig. 1b,c). Histological analysis of the remaining Peyer's patches in $Tox^{-/-}$ mice demonstrated the presence of lymphocytes in these structures (Fig. 1d) and their organization in distinct B and T cells areas (Fig. 1e). However, some reduction in the density of T cells was noted (Fig. 1e, low magnification), likely due to the T lymphopenia in animals that lack TOX27. These results suggested that while the development of Peyer's patches was severely compromised in the absence of TOX, remaining structures could still be colonized by $Tox^{-/-}$ lymphocytes.

To address if the loss of secondary lymphoid organs was caused by the abnormal complement of lymphocytes in $Tox^{-/-}$ mice, we generated bone marrow chimeras. Lymph nodes and Peyer's patches were present in all wild-type chimeric mice, whether reconstituted with $Tox^{-/-}$ (n=6) or $Tox^{+/-}$ (n=5) donor bone marrow cells. Moreover, like their $Tox^{+/-}$ counterparts, $Tox^{-/-}$ cells colonized both lymph nodes and Peyer's patches, arguing against a severe migration defect in cells that lack TOX (Fig. 2a,b). In addition, a T cell intrinsic block in thymic positive selection27 was evident in wild-type mice reconstituted with $Tox^{-/-}$ bone marrow, because reduced numbers of CD4 T cells were found in lymph nodes and Peyer's patches in these chimeric mice (Fig. 2a,b). In contrast, $Tox^{-/-}$ mice reconstituted with wild-type bone marrow were still devoid of lymph nodes and we could not detect any Peyer's patches in these mice (n=8, data not shown).

Lack of lymph node in *Tox*$^{-/-}$ mice could also be due to the inability of TOX-deficient cells to maintain lymph node primordia. For example, both aly/aly and *Il2rγ*$^{-/-}$*Rag2*$^{-/-}$ mice have defects in lymph node formation, however, the former lack lymph nodes due to absence of lymphotoxin signaling during embryogenesis, while the latter exhibit a defect in the maintenance of the anlage due to the absence of mature lymphocytes required for crosstalk with these structures during the post-natal period28. Transfer of wild-type lymphocytes to neonates rescues lymph nodes in *Il2rγ*$^{-/-}$*Rag2*$^{-/-}$ mice28, although the mechanism is unclear29. To address this possibility, total lymph node cells derived from wild-type mice were injected into *Tox*$^{-/-}$ mice within 24 h of birth. Donor wild-type T cells were detected in the spleen of *Tox*$^{-/-}$ host animals 4 weeks post-injection, and at similar frequency to those found in *Tox*$^{+/-}$ control host animals (Fig. 2c). However, we failed to detect lymph nodes in *Tox*$^{-/-}$ hosts (Supplementary Fig. 2). Thus, we conclude that the absence of lymph nodes and loss of Peyer's patches in *Tox*$^{-/-}$ mice was likely caused by a defect in the generation of the primordia in the absence of TOX, rather than due to the abnormal cellular environment these mice.

Tox$^{-/-}$ mice lack LTi cells

Development of lymph nodes occurs during fetal development in both mice and humans and is dependent on CD4^{+} (in mice) LTi cells. LTi cells home to specific areas in the mesentery during embryogenesis and, through interaction with mesenchymal cells, initiate organogenesis of rudimentary lymph node structures. We asked if LTi cells were present in *Tox*$^{-/-}$ mice. Despite the fact that lymph node organogenesis is an embryonic process, LTi cells are still detected in the spleen of neonatal animals15, 30, 31 (Fig. 3a). However, in the absence of TOX, the frequency and absolute numbers of LTi cells was reduced over 10-fold **(**Fig. 3a-c). We also looked for the presence of fetal LTi cells in day E18 small intestines. Consistent with observations in neonatal spleens, fetal LTi cells were significantly reduced, and in many cases undetectable, in the absence of TOX (Fig. 3d,e). Similar results were obtained from E18 fetal spleen, even when CD4 was not used as a marker (data not shown).

We next asked whether *Tox* was normally expressed in populations of LTi cells. To facilitate isolation of LTi, we used splenocytes from RAG1-deficient mice, which lack T and B cells, to minimize contamination from these lymphocyte subsets. We isolated CD45^{+}CD11c^{-}CD3^{-}B220^{-}CD4^{+}CD127^{+} splenic LTi cells, previously reported to be functionally indistinguishable from embryonic LTi cells30, and used them for gene expression analysis. These cells express *Id2* **(**Fig. 3f) and *Rorc* (encoding RORγ(t)) **(**Fig. 3g**)**, identifying them as LTi cells10, 21. *Tox* mRNA was detected in this subset **(**Fig. 3h), consistent with a cell intrinsic defect in LTi cell development in the absence of TOX. Fetal LTi cells also expressed *Rorc* (Fig. 3i) and *Tox* (Fig.3j). Together, these data indicate that the absence of lymph nodes is likely due to a cell-intrinsic defect in the production of LTi cells in *Tox*$^{-/-}$ mice.

Impaired NK cell development in *Tox*$^{-/-}$ mice

Because RORγ(t) plays a role in the thymus as well as in LTi development10, 22, similar to TOX, we considered the possibility that RORγ(t) expression might be influenced by TOX. However, we did not observe any change in the expression of RORγ(t) in *Tox*$^{-/-}$ thymocytes

compared to their wild-type counterparts by protein intracellular staining (Supplementary Fig. 3a). This is consistent with the fact that mice deficient in RORγ(t) do not phenocopy *Tox*$^{-/-}$ mice in terms of the T cell developmental defect22. In addition, we assessed *Rorc* gene expression in *Rorc*^{GFP} knock-in reporter mice10 and found similar *Rorc* gene expression in thymocytes from TOX-deficient and TOX- expressing cells (Supplementary Fig. 3b).

Because LTi and NK cells share many commonalities in their development program, including dependence on Ikaros and Id2 factors, we investigated whether NK cells development was dependent on TOX. In the bone marrow, approximately 15% of lineage-negative (Lin^{-}) cells are mature NK cells (mNK) expressing the interleukin 2 receptor (IL-2R)β chain (CD122) and DX5 cell surface markers (Fig. 4a, **top**). In the absence of TOX, the frequency of these cells was reduced approximately 50-fold compared to the wild-type (Fig.4a, **top**). To eliminate the possibility that expression of CD122 was influenced by TOX, we additionally used NK1.1 and DX5 or NKp46 and CD122 to stain Lin^{-} cells in the bone marrow (Fig. 4a, **bottom** and data not shown) and spleen (Supplementary Fig. 4a). Similar results were obtained when using these various staining strategies (Fig.4a and Supplementary Fig. 4a,b).

Within the Lin^{-} CD122^{+} bone marrow cell population, these same markers can also be used to identify NK cell developmental stages21, 32, 33, including NK1.1^{-}DX5^{-} precursors (NKp), NK1.1^{+}DX5^{-} immature cells (iNK) and NK1.1^{+}DX5^{+} mature NK cells (mNK). While both the NKp and iNK subsets were still present (with a trend towards a decrease in iNK), the mNK subpopulation was severely depleted in the absence of TOX **(**Fig. 4a,b). The inhibition of NK cell development was also apparent in the spleens of *Tox*$^{-/-}$ mice **(**Fig. 4c), where there was approximately a 40-fold decrease in frequency and absolute numbers of NK cells (Fig. 4d).

To determine if the inhibition of NK cell development could be due to a cell-intrinsic defect, we purified NK cell developmental subsets from the bone marrow and spleen of wild-type mice and analyzed the expression of *Tox* . Splenic B cells, which do not express TOX nor are developmentally affected by loss of TOX 27, were used as a biological negative control. Low amounts of *Tox* mRNA were detected in both bone marrow NKp and splenic NK subsets **(**Fig. 4e), while both iNK and mNK from bone marrow highly expressed *Tox* **(**Fig. 4e). The upregulation of *Tox* in iNK and mNK stages is consistent with the observed block in NK cell development in the absence of TOX **(**Fig. 4a,b). The fact that *Tox* is further downregulated in splenic compared to bone marrow mNK cells, may be an indication of continued maturation of these cells or additional micro-environmental or cell subset differences.

We isolated a small number of mature bone marrow NK cells from *Tox*$^{-/-}$ mice and assessed if they expressed *Id2*, an essential gene for the development of the NK cell lineage that is expressed subsequent to the NKp stage21. Interestingly, *Tox*$^{-/-}$ bone marrow mNK cells expressed less *Id2* than their wild-type counterparts (Fig.4f). This suggests that either induction or maintenance of *Id2* expression is influenced by TOX or that the NK cell lineage development block in *Tox*$^{-/-}$ mice occurs prior to full upregulation of the *Id2* gene.

As shown above (Fig. 4c), some mature NK cells can be detected in the spleens of *Tox*$^{-/-}$ mice. To determine if these remaining NK cells were functional, we performed a cytolytic assay for NK activity *in vivo*, taking advantage of the fact that NK cells but not CD8^{+} T cells would kill otherwise syngeneic major histocompatibility complex (MHC) class I negative targets34. Thus, control or *Tox*$^{-/-}$ mice were injected with a differentially CFSE-labeled mix of wild-type and β2 microglobulin-deficient target cells, and the ratio of recovered target cells from the spleen was analyzed 16 hours later. Specific killing of the β2 microglobulin-deficient target cells by *Tox*$^{-/-}$ hosts was reduced by approximately two-thirds when compared to the killing activity of *Tox*$^{+/-}$ control hosts (Fig. 4g). This is consistent with the severe loss of mature NK cells from TOX-deficient mice. However, these data also suggest that NK cells that escape the developmental block maintain at least some effector function even in the absence of TOX.

Cell intrinsic requirement for TOX in NK cell development

To further study the cell-intrinsic role of TOX in developing NK cells, we made use of a stromal cell-free cell culture system that can support NK cells development from hematopoietic progenitor cells35. In this two-step system, a cytokine cocktail induces CD122^{+} NKp cells after 6-day of culture, which further develop into NK1.1^{+} DX5^{+} mNK cells upon 6 days of culture with IL-1535. Purified *Tox*$^{+/-}$, but not *Tox*$^{-/-}$, bone marrow Lin^{-} Sca-1^{+}cKit^{+} (LSK) produced NK cells in these cultures **(**Fig. 5a). These results recapitulated the *in vivo* phenotype of *Tox*$^{-/-}$ mice.

Both *Tox*$^{+/-}$ and *Tox*$^{-/-}$ bone marrow precursor cells produced progeny with similar expression of CD122 (Fig. 5b), arguing against a defect in differentiation or proliferation in *Tox*$^{-/-}$ progenitor cells in the primary culture. To further test the proliferative capacity of *Tox*$^{-/-}$ progenitors, we mixed highly purified phenotypically-marked wild-type and *Tox*$^{-/-}$ LSK cells at a ratio of 1:1 and analyzed their expansion after the 6 days of culture (Fig.5c). *Tox*$^{-/-}$ cells underwent greater expansion than wild-type cells in these cultures, arguing against an initial proliferative defect in the absence of TOX. Together, these data are consistent with a requirement for TOX subsequent to the progenitor stage of NK cell development in the bone marrow, and are also consistent with the peak of *Tox* expression in iNK cells.

We also considered the possibility that *Tox*$^{-/-}$ bone marrow progenitors had undergone irreversible lineage commitment prior to isolation, possibly as a result of altered cellular niches. However, *in vitro* NK cell development was restored upon retroviral-mediated expression of TOX in *Tox*$^{-/-}$ progenitors (Fig. 6a). In addition, TOX expression boosted the NK cell output from *Tox*$^{+/-}$ LSK cells, when compared to *Tox*$^{+/-}$ LSK cells infected with a control recombinant virus expressing the H-2K^{b} MHC molecule. This may suggest either that TOX is limiting in TOX heterozygous knockout mice or that sustained or increased TOX expression can promote the NK cell fate.

The E protein activity inhibitor Id2, like TOX, is required for both LTi and NK cell development20, 21, 36. In addition, the basic leucine zipper transcription factor E4BP4, which is also required for NK cell development, is upregulated, directly or indirectly, as a consequence of IL-15-mediated signaling and is an upstream regulator of Id224. Because

mNK cells isolated from *Tox*$^{-/-}$ bone marrow had decreased expression of *Id2* (Fig. 4f) we tested if expression of Id2 could compensate for loss of TOX, as it does for loss of E4BP424. Expression of Id2 was not able to rescue NK cell development from *Tox*$^{-/-}$ bone marrow progenitors (Fig. 6b). However, Id2 expression was able to efficiently inhibit the modest level of B cell production in these cultures, as expected due to the requirement for E protein activity during B cell development37 (Fig. 6c). These data demonstrate that the requirement for TOX in NK cell development cannot be solely attributed to control of Id2, and may indicate the presence of an additional regulatory pathway required for the development of both NK and LTi cell subsets.

Discussion

We have demonstrated an essential role for TOX in the formation of peripheral lymph nodes and Peyer's patches. The development of secondary lymphoid tissue is a complex process, involving both mesenchymal and hematopoietic cells. In the case of Peyer's patch development, two cell types of hematopoietic origin, the LTi cells and the CD4^{-}CD11c^{+} cells (the latter distinct from splenic dendritic cells)38 are involved. Whether these CD11c^{+} cells also play a role in lymph node development is not known. Here we show that in the absence of TOX LTi cells are severely depleted both from fetal and neonatal animals. In addition, both fetal and adult LTi cells normally express *Tox*. Thus, these results suggest that a defect in LTi cells production in the absence of TOX leads to the failure of normal secondary lymphoid tissue development. We have also shown that TOX is required for the development of NK cells. TOX is thus the third nuclear factor described to play a role in the development of both cell lineages, along with Ikaros and Id2.

TOX and Id2 deletion results in strikingly similar phenotypes regarding NK cell and secondary lymphoid tissue development. In terms of the latter, both TOX and Id2 appear to regulate a developmental stage subsequent to pNK differentiation21. However, some Peyer's patch structures, albeit scarcer and smaller than normal, can be detected in the absence of TOX, while *Id2*$^{-/-}$ mice lack Peyer's patches completely20, 21. Whether this difference relates to detection sensitivities or a true difference in the 'leakiness' of the two phenotypes remains to be determined.

Differences exist between embryonic development of Peyer's patches and lymph nodes, including earlier embryonic development and reliance on TRANCE signaling for the latter39, 40, while loss of IL-7R leads primarily to absence of Peyer's patches41, 42. A narrowed developmental window or a particularly stringent requirement for a full complement of LTi cells in lymph node development could explain the complete loss of these structures in *Tox*$^{-/-}$ mice, compared to presence of some small Peyer's patches in these animals.

Despite the lack of lymph nodes, a small number of LTi-like cells can be identified in adult *Tox*$^{-/-}$ mice (J.K., unpublished observations). Adult LTi-like cells have other functions in the immune system, including production of IL-17 and IL-22, development of Aire^{+} epithelial cells in the thymic medulla and maintenance and organization of secondary

lymphoid organs16-18. It will thus be of interest to determine if lack of TOX also impacts these functions.

NK cell development is highly dependent on IL-15 signaling and thus on expression of the IL-15 receptor23. However, the *in vitro* development of $CD122^+$ NKp from TOX-deficient hematopoietic precursors demonstrates that the inhibition of NK cell development in the absence of TOX is not caused by failure to express CD122. In the thymus, TOX expression is dependent on TCR-mediated activation of calcineurin43. It has been argued that expression of E4BP4, and thus Id2, is downstream of IL-15 signaling24. Whether TOX is similarly regulated in this cell context remains to be determined.

Id2 is also upregulated following positive selection in the thymus, and TOX expression precedes Id2 expression during thymocyte progression through transitional stages of development (J.K., unpublished observations). However, $Id2^{-/-}$ mice do not display the defect in T cell development seen in mice that lack TOX, although compensatory effects by the related factor Id3 cannot be ruled out36. Although $Tox^{-/-}$ bone marrow NK cells expressed lower levels of *Id2* mRNA, we found that ectopic expression of Id2 was not able to rescue NK cell development in the absence of TOX, as had been observed for the NK lineage factor E4BP424. While the exact relationship between TOX and Id2, if any, remains to be further elucidated, it is clear that the role of TOX in NK cell development is not solely as an upstream regulator of Id2.

The NK and LTi lineages have some interesting commonalities, although the exact developmental relationship between these cell types is still unclear. It is known for over a decade that neonatal $CD45^+CD4^+CD3^-$ mesenteric lymph nodes cells cultured in limiting dilution conditions in the presence of IL-2 and stromal cells produce $NK1.1^+$ colonies with cytolytic activity8. This result is particularly interesting in relation to recent data characterizing NK-like and LTi-like cells in the gut. So-called LTi-like Lin^- $CD4^+CD127^+CD3^-$ cells can be found in the spleen and intestinal lamina propria in mice, and these cells produce IL-17 and IL-22 and express RORγ(t)16, 44. Similarly, there is a $CD127^+$ subset of LTi-like cells that can give rise to an IL-15-independent but RORγ(t)- and Id2-dependent $NKp46^+CD3^-$ population found in the gut that also produces IL-2245-47. Cell subsets with similar properties are also found in human9, 48.

We have observed that some $NKp46^+CD3^-ROR\gamma(t)^+$ cells can still be found in the small intestine of $Tox^{-/-}$ mice (data not shown), consistent with data showing that these cells are distinct from both LTi cells and the major NK cell lineage9, 46-48. However, given the abnormal lymphocyte environment in the $Tox^{-/-}$ mice, which could influence the expansion of these cells, comparative quantification with normal animals is problematic, and earlier progenitor cell populations have not been well defined. Thus, we cannot formally rule out a role for TOX in the development of these distinct NK-like cell subsets. While the exact relationships between LTi and NK cell subpopulations and their precursors awaits additional clarification, the addition of TOX to the small list of known transcriptional regulators of LTi and NK cell lineages provides a new tool to address these issues.

TOX seems to play a non-redundant function in three aspects of development of the immune system- CD4 T cell development, NK cell development and LTi development. Somewhat analogous to the pattern of TOX expression during positive selection of thymocytes, expression of the *Tox* gene in the NK lineage is transiently upregulated during a key developmental transition. Indeed, the highest expression of TOX is predictive of the observed developmental block. In thymocytes, *Tox* is highly expressed by $CD4^{+}8^{lo}$ thymocytes and is required for their development26, 27; in the bone marrow, *Tox* is highly expressed in iNK cells and is required for their continued development. Whether a similar pulse of expression can also be observed during a specific stage of LTi development in the fetus is thus of interest.

In sum, our data reveal TOX as an essential regulator of the development of multiple facets of the adaptive immune system, and add another commonality to the developmental pathways of NK cells and LTi cells.

Methods

Mice

All mice were bred at Cedars-Sinai Medical Center or The Scripps Research Institute and kept under specific pathogen-free conditions. Experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees at Cedars-Sinai Medical Center or The Scripps Research Institute. Production of $Tox^{-/-}$ mice has been described previously27 and mice used in these experiments were on a mixed C57BL/6J and 129S6/SvEv genetic background. $Tox^{fl/fl}$ conditional knockout mice were produced similarly with the exception that following gene targeting and subsequent transient Cre expression, CMTI-1 mouse ES cells were selected for elimination of the selectable marker cassettes but maintenance of floxed exon 1. Unless stated otherwise, studies included $Tox^{+/-}$ littermates as experimental controls. Wild-type mice used were either C57BL/6J or B6.SJL-$Ptprc^{a}$ ($CD45.1^{+}$) mice, or $Tox^{+/+}$ littermates. B6.129P2(Cg)-$Rorc^{tm2Litt}$/J ($Rorc^{GFP}$ knock-in reporter mice)10, 49 were purchased from The Jackson Laboratory.

Bone marrow chimera

Recipient mice ($CD45.1^{+}$ wild-type or $CD45.2^{+}$ $Tox^{-/-}$) were lethally irradiated (10 Gy) to deplete hematopoietic cells. Donor wild-type ($CD45.1^{+}$) or $Tox^{-/-}$ ($CD45.2^{+}$) Lin^{-} bone marrow cells, enriched for hematopoietic progenitors by magnetic bead depletion (StemCell Technologies), were injected intravenously and chimeras were analyzed 6–8 weeks later.

Adoptive transfer of lymph node cells in neonates

Neonatal mice were given an intraperitoneal injection of 4×10^{6} total lymph node cells from $CD45.1^{+}$ wild-type mice. After 4 weeks, chimerism was analyzed by the presence of donor T cells in the spleen. Presence of lymph nodes was performed by visual inspection.

In vivo NK killing assay

Adult mice were given by intravenous injection (tail vein), a 1:1 mix of $CFSE^{lo}$-labeled wild-type and $CFSE^{hi}$-labeled β_2 microglobulin-deficient concanavalin A-activated (48 hr) spleen cell blasts as targets. Exact ratios of the two target populations was determined by flow cytometry of the mixed cells prior to injection. Ratios of CFSE-labeled cells were analyzed in the spleens of host animals by flow cytometry 16 hours post-injection. The percentage of specific lysis was determined by the following formulas: ratio = (percentage of $CFSE^{hi}$/percentage of $CFSE^{lo}$); percentage of specific lysis = [1– (ratio post-injection/ ratio pre-injection) × 100].

Antibodies

All antibodies were purchased from eBioscience or BD Biosciences and staining was performed as per the manufacturer's instructions. Flow cytometry was performed as described previously43.

Visualization of lymph nodes

Mice were injected with a solution of 1% Chicago Sky Blue (Sigma) and analyzed 5 days later for dye uptake by peripheral lymph nodes. For visualization of Peyer's patches, small intestines were submerged in a 7% solution of acetic acid in PBS for 5 minutes.

Histology and immunofluorescence

Serial 5 μm frozen sections from small intestines were stained with hematoxylin and eosin, DAPI to visualize nucleated cells (data not shown), or immunostained for expression of B220 and CD3ε and analyzed on Leica TCS SP spectral confocal microscope with white light laser and CytoView Software (Infinicyte, LLC).

Quantitative real-time PCR

Real-time quantitative RT-PCR analysis was performed using the standard curve method, where samples were normalized based on *Gapdh* expression, and analyzed using SDS 2.1 software (Applied Biosystems). Primers for real-time RT-PCR were purchased from QIAGEN, except for *Rorc* 5′-CCGCTGAGAGGGCTTCAC and 5′-TGCAGGAGTAGGCCACATTACA.

In vitro NK culture

Hematopoietic progenitor cells were isolated from total bone marrow, either by enrichment using magnetic bead-based negative selection with the mouse hematopoietic progenitor enrichment kit (StemCell Technologies) or by FACS sorting for LSK cells. Cells were cultured in a previously described two-step system35, where the first culture contained 20 ng/ml of IL-6, 0.5 ng/ml of IL-7, 50 ng/ml of SCF and 5 ng/ml of Flt3-L in complete RPMI for 6 days with a media change at day 3. Cells were then cultured in 20 ng/ml of IL-15 for an additional 6 days, before analysis for NK cell lineage markers.

Retroviral gene transduction

Recombinant retroviruses encoding H-2K^b, TOX or Id2 (in the Mig-R1 vector, which contains an IRES-GFP cassette) were produced from transfected Plat-E packaging cells50 and used to infect hematopoietic progenitor cells. Cells were incubated with supernatant containing virus for 3 days in the initial cytokine cocktail described above. At day 3, cells were washed and continued culturing, also as described above.

Statistics

The probability (*P*) associated with a Student's t-Test using a two-tailed distribution of equal variance is shown in some Figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Randall TD, Carragher DM, Rangel-Moreno J. Development of secondary lymphoid organs. Annu Rev Immunol. 2008; 26:627–650. [PubMed: 18370924]
2. Koni PA, et al. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. Immunity. 1997; 6:491–500. [PubMed: 9133428]
3. De Togni P, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science. 1994; 264:703–707. [PubMed: 8171322]
4. Ansel KM, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature. 2000; 406:309–314. [PubMed: 10917533]
5. Cao X, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity. 1995; 2:223–238. [PubMed: 7697543]
6. Luther SA, Ansel KM, Cyster JG. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. J Exp Med. 2003; 197:1191–1198. [PubMed: 12732660]
7. Yoshida H, et al. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. Immunity. 2002; 17:823–833. [PubMed: 12479827]
8. Mebius RE, Rennert P, Weissman IL. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity. 1997; 7:493–504. [PubMed: 9354470]
9. Cupedo T, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. Nat Immunol. 2009; 10:66–74. [PubMed: 19029905]
10. Eberl G, et al. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol. 2004; 5:64–73. [PubMed: 14691482]
11. Fukuyama S, et al. Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(−)CD4(+)CD45(+) cells. Immunity. 2002; 17:31–40. [PubMed: 12150889]

12. Finke D, Acha-Orbea H, Mattis A, Lipp M, Kraehenbuhl J. CD4+CD3− cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. Immunity. 2002; 17:363–373. [PubMed: 12354388]
13. Cupedo T, et al. Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. J Immunol. 2004; 173:2968–2975. [PubMed: 15322155]
14. Schmutz S, et al. Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma+ lymphoid tissue inducer cells. J Immunol. 2009; 183:2217–2221. [PubMed: 19635901]
15. Kim MY, et al. Neonatal and adult CD4+ CD3− cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). J Immunol. 2006; 177:3074–3081. [PubMed: 16920944]
16. Takatori H, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med. 2009; 206:35–41. [PubMed: 19114665]
17. Rossi SW, et al. RANK signals from CD4(+)3(−) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. J Exp Med. 2007; 204:1267–1272. [PubMed: 17502664]
18. Scandella E, et al. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. Nat Immunol. 2008; 9:667–675. [PubMed: 18425132]
19. Wang JH, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity. 1996; 5:537–549. [PubMed: 8986714]
20. Yokota Y, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature. 1999; 397:702–706. [PubMed: 10067894]
21. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. J Exp Med. 2007; 204:1119–1130. [PubMed: 17452521]
22. Sun Z, et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science. 2000; 288:2369–2373. [PubMed: 10875923]
23. Suzuki H, Duncan GS, Takimoto H, Mak TW. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. J Exp Med. 1997; 185:499–505. [PubMed: 9053450]
24. Gascoyne DM, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol. 2009; 10:1118–1124. [PubMed: 19749763]
25. Kamizono S, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J Exp Med. 2009; 206:2977–2986. [PubMed: 19995955]
26. Wilkinson B, et al. TOX: an HMG box protein implicated in the regulation of thymocyte selection. Nat Immunol. 2002; 3:272–280. [PubMed: 11850626]
27. Aliahmad P, Kaye J. Development of all CD4 T lineages requires nuclear factor TOX. J Exp Med. 2008; 205:245–256. [PubMed: 18195075]
28. Coles MC, et al. Role of T and NK cells and IL7/IL7r interactions during neonatal maturation of lymph nodes. Proc Natl Acad Sci U S A. 2006; 103:13457–13462. [PubMed: 16938836]
29. Chappaz S, Finke D. The IL-7 signaling pathway regulates lymph node development independent of peripheral lymphocytes. J Immunol. 2010; 184:3562–3569. [PubMed: 20207995]
30. Kim MY, et al. Heterogeneity of lymphoid tissue inducer cell populations present in embryonic and adult mouse lymphoid tissues. Immunology. 2008; 124:166–174. [PubMed: 18205791]
31. Meier D, et al. Ectopic lymphoid-organ development occurs through interleukin 7-mediated enhanced survival of lymphoid-tissue-inducer cells. Immunity. 2007; 26:643–654. [PubMed: 17521585]
32. Rosmaraki EE, et al. Identification of committed NK cell progenitors in adult murine bone marrow. Eur J Immunol. 2001; 31:1900–1909. [PubMed: 11433387]
33. Kim S, et al. In vivo developmental stages in murine natural killer cell maturation. Nat Immunol. 2002; 3:523–528. [PubMed: 12006976]

34. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986; 319:675–678. [PubMed: 3951539]
35. Williams NS, et al. Generation of lytic natural killer 1.1+, Ly-49- cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. J Exp Med. 1997; 186:1609–1614. [PubMed: 9348320]
36. Ikawa T, Fujimoto S, Kawamoto H, Katsura Y, Yokota Y. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. Proc Natl Acad Sci U S A. 2001; 98:5164–5169. [PubMed: 11296270]
37. Bain G, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell. 1994; 79:885–892. [PubMed: 8001125]
38. Veiga-Fernandes H, et al. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. Nature. 2007; 446:547–551. [PubMed: 17322904]
39. Kim D, et al. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. J Exp Med. 2000; 192:1467–1478. [PubMed: 11085748]
40. Kong YY, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature. 1999; 397:315–323. [PubMed: 9950424]
41. Yoshida H, et al. IL-7 receptor alpha+ CD3(−) cells in the embryonic intestine induces the organizing center of Peyer's patches. Int Immunol. 1999; 11:643–655. [PubMed: 10330270]
42. Adachi S, et al. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. Int Immunol. 1998; 10:1–6. [PubMed: 9488150]
43. Aliahmad P, et al. TOX provides a link between calcineurin activation and CD8 lineage commitment. J Exp Med. 2004; 199:1089–1099. [PubMed: 15078895]
44. Ivanov II, Diehl GE, Littman DR. Lymphoid tissue inducer cells in intestinal immunity. Curr Top Microbiol Immunol. 2006; 308:59–82. [PubMed: 16922086]
45. Tsuji M, et al. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. Immunity. 2008; 29:261–271. [PubMed: 18656387]
46. Satoh-Takayama N, et al. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. J Immunol. 2009; 183:6579–6587. [PubMed: 19846871]
47. Satoh-Takayama N, et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. J Exp Med. 207:273–280. [PubMed: 20142427]
48. Crellin NK, Trifari S, Kaplan CD, Cupedo T, Spits H. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. J Exp Med. 207:281–290. [PubMed: 20142432]
49. Eberl G, Littman DR. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. Science. 2004; 305:248–251. [PubMed: 15247480]
50. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. 2000; 7:1063–1066. [PubMed: 10871756]

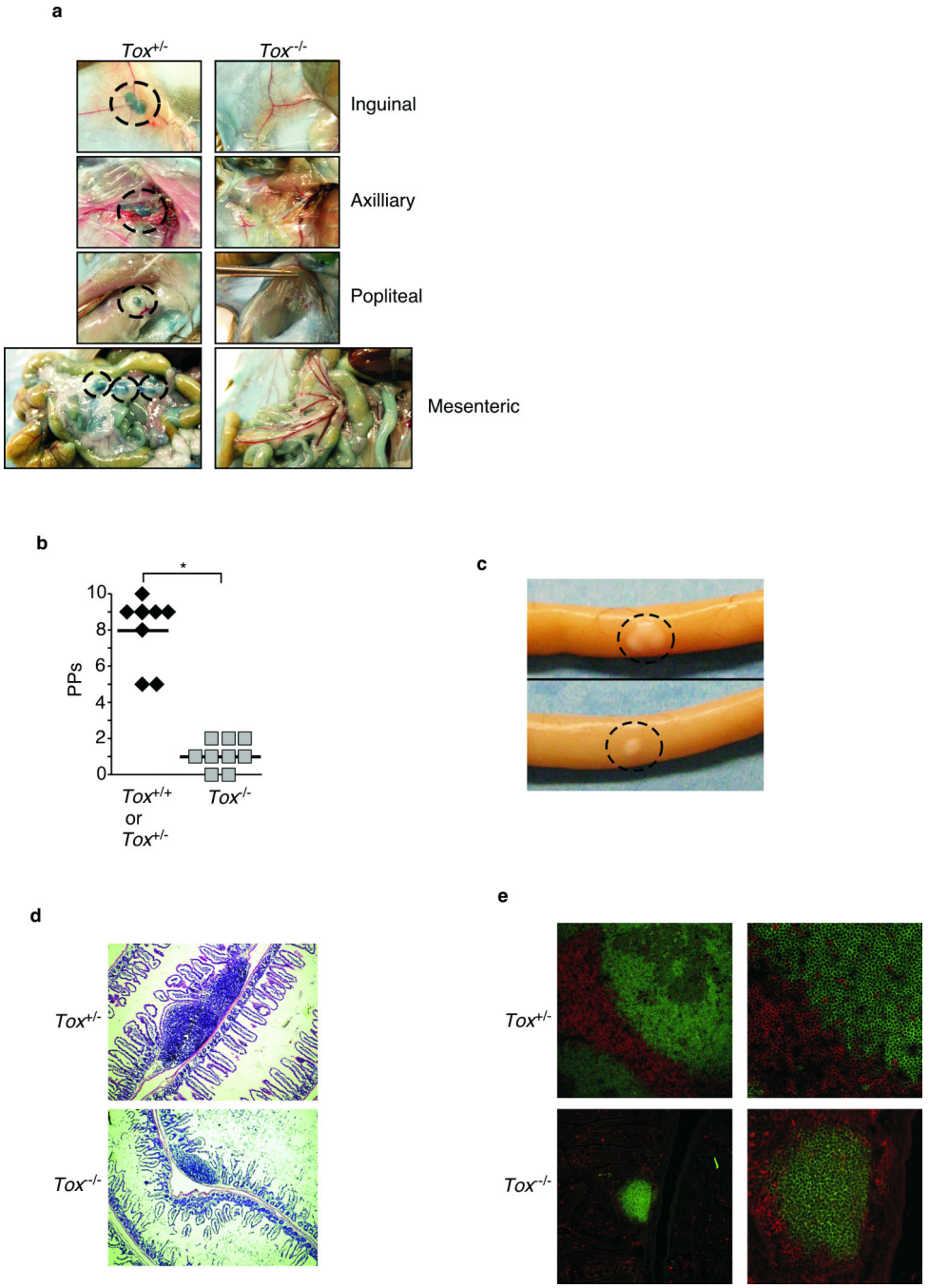

Figure 1.

Absence of lymph nodes in $Tox^{-/-}$ mice. **(a)** Analysis of the gross anatomy of lymph nodes visualized by Chicago sky blue dye injection in $Tox^{+/-}$ and $Tox^{-/-}$ mice. Lymph nodes have never been observed in >100 $Tox^{-/-}$ mice ranging from 4–12 weeks of age (analyzed in the absence of dye). **(b,c)** Quantification of Peyer's patches by visual inspection was performed in adult control (wild-type or $Tox^{+/-}$) and $Tox^{-/-}$ mice. Each symbol represents an individual mouse, and a Peyer's patch from a representative mouse of each genotype is shown in the photograph; horizontal bars are the mean. *$P = 1.4\times10^{-8}$ **(d)** Hematoxylin and eosin staining

of frozen sections of small intestine that included a Peyer's patch from *Tox*$^{+/-}$ and *Tox*$^{-/-}$ mice. Magnification, 25X. **(e)** Immunofluorescence staining of Peyer's patches in *Tox*$^{+/-}$ and *Tox*$^{-/-}$ mice. B cell and T cell areas are identified by B220 (green) and CD3 (red) staining. Magnification, 20X and 63X. Data (d,e) is representative of at least three animals of each genotype.

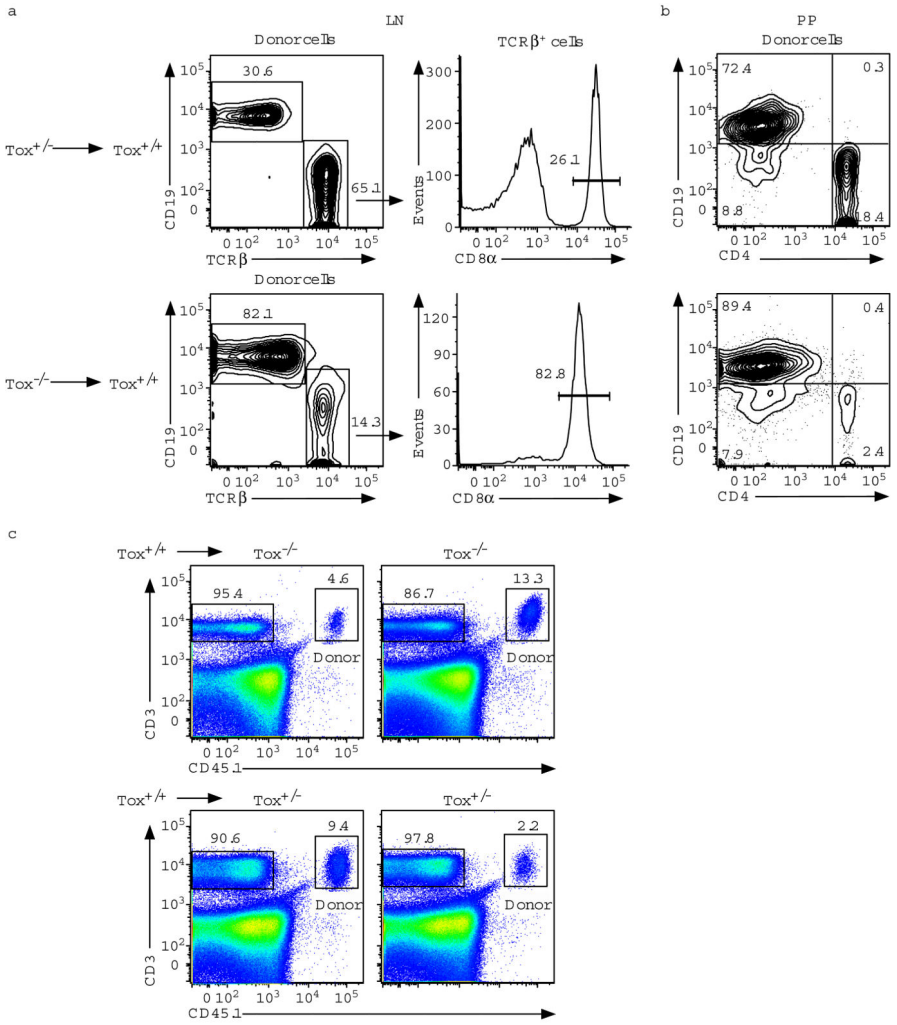

Figure 2.

Defect in formation of lymph node structures in *Tox*$^{-/-}$ mice. **(a)** Representative plots displaying donor *Tox*$^{+/-}$ (n=5) and *Tox*$^{-/-}$ (n=6) B (CD19^{+}) and T (TCRβ^{+}) cells in lymph nodes of reconstituted *Tox*$^{+/+}$ recipient mice. Frequencies refer to percentages of gated populations. The frequency of CD8^{+} T cells is also shown. **(b)** Analysis of Peyer's patches as in **(a)** except that the frequency of CD4^{+} T cells is shown. **(c)** Presence of CD45.1^{+} donor wild-type cells in the spleen of chimeric *Tox*$^{-/-}$ (n=2) or *Tox*$^{+/-}$ (n=2) mice, four weeks post adoptive transfer.

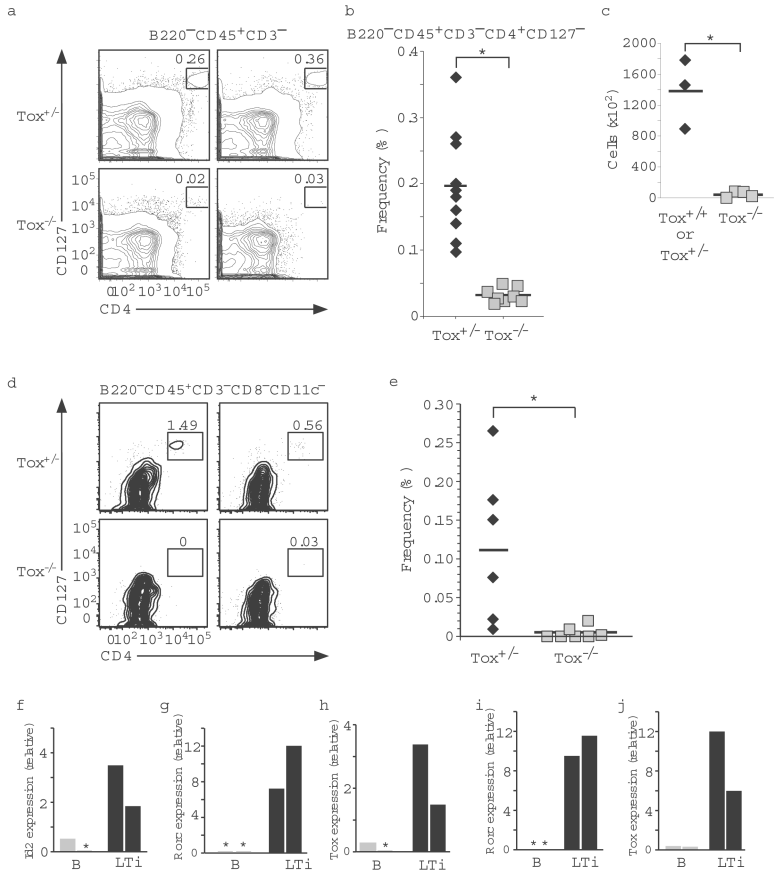

Figure 3.

TOX is required for the development of lymphoid tissue inducer (LTi) cells. **(a)** Representative analysis of LTi cells in neonatal (day 0) spleen. Frequencies refer to percentages of gated populations. **(b,c)** Quantification of the frequency (n=10 and 8 for $Tox^{+/-}$ and $Tox^{-/-}$, respectively, $*P = 3.2\times10^{-5}$) and absolute cell numbers (n=3 and 4 for $Tox^{+/-}$ and $Tox^{-/-}$, respectively, $*P = 0.002$) of LTi cells from neonatal spleen. **(d)** Analysis of fetal LTi cells (day E18) from small intestines. Frequencies refer to percentages of gated populations. **(e)** Quantification of the frequency of LTi cells from fetal (day E18) small intestines of $Tox^{+/-}$ (n=6) and $Tox^{-/-}$ (n=7) mice. $*P = 0.012$ **(f,g,h)** Quantitative RT-PCR gene expression analysis in $Tox^{+/+}$ LTi cells ($CD45^{+}CD3^{-}B220^{-}CD11c^{-}$ $CD4^{+}CD127^{+}$) purified from $Rag1^{-/-}$ spleens compared to wild-type splenic B cells. Two independent experiments are shown. *<0.01 **(i,j)** Quantitative RT-PCR gene expression analysis in wild-type LTi cells, identified as in (f), purified from embryonic day E17 spleens and compared to wild-type adult splenic B cells. Two independent experiments using three to four pooled mice per experiment are shown.

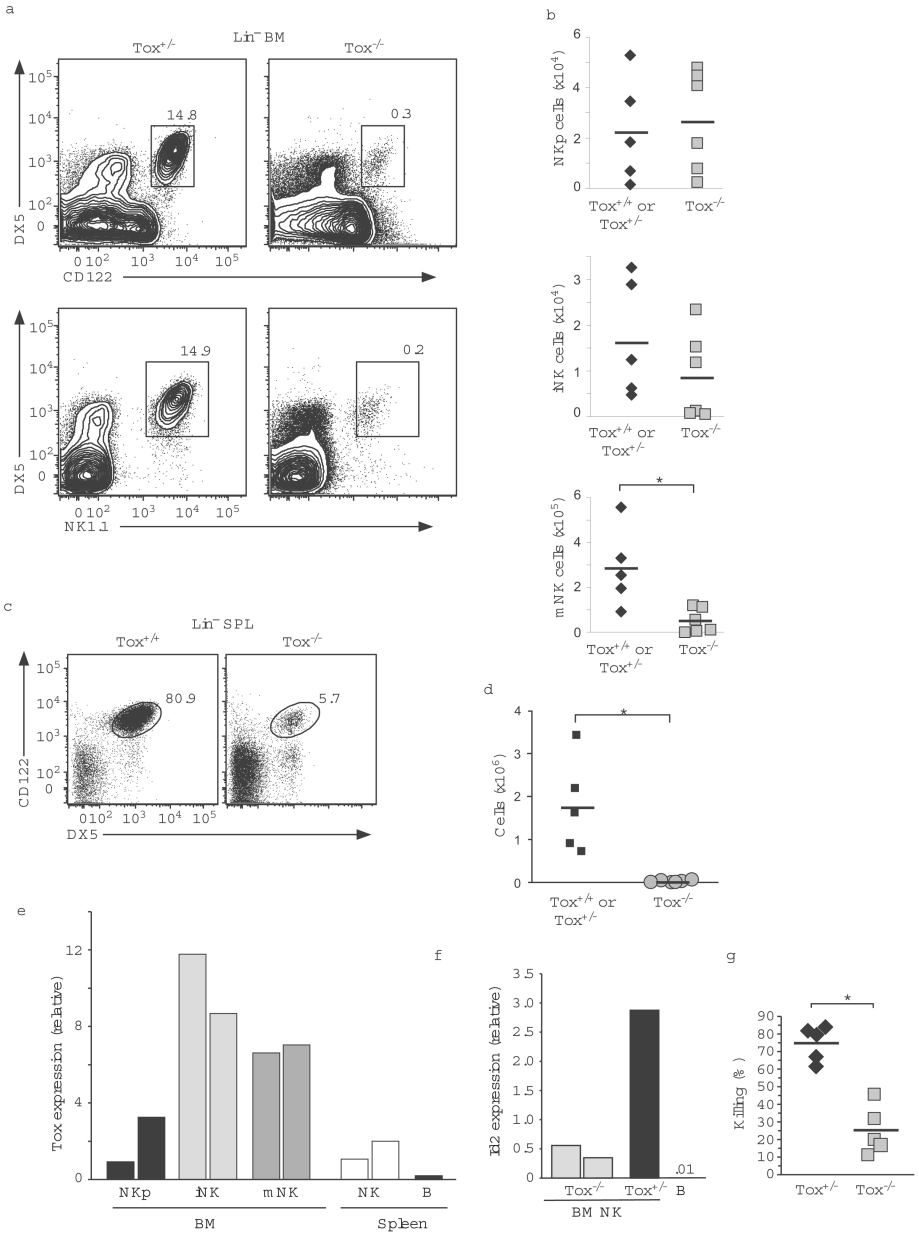

Figure 4.

Impaired development of NK cells in the absence of TOX. **(a)** Analysis of bone marrow NK cell populations. IL-2Rβ (CD122) or NK1.1 and DX5 expression is shown on Lin$^-$ (CD3$^-$CD4$^-$CD8$^-$CD19$^-$Gr1$^-$Ter119$^-$) cells. Frequencies refer to percentages of gated populations. Data is representative of 6 mice for each genotype. **(b)** Absolute cell numbers of bone marrow NK cell subsets: NK progenitors (NKp), Lin$^-$ CD122$^+$NK1.1$^-$ DX5$^-$; immature NK (iNK), Lin$^-$ CD122$^+$NK1.1$^+$DX5$^-$; mature NK (mNK), Lin$^-$ CD122$^+$NK1.1$^+$DX5$^+$ subpopulations (*P = 0.012). Each symbol represents an individual mouse; horizontal lines indicate the mean. **(c)** Analysis of CD122 and DX5 expression on Lin$^-$ splenic cells. Data is representative of five mice. **(d)** Absolute cell numbers of splenic NK cells. *P = 3.3×10^{-3} **(e)** *Tox* expression in isolated *Tox*$^{+/+}$ bone marrow NK cell

subpopulations, $Tox^{+/+}$ splenic NK cells, and B cells. Two independent experiments are shown. **(f)** Expression of *Id2* in bone marrow mNK cells. Two biological replicates are shown for $Tox^{-/-}$ cells. **(g)** *In vivo* NK cell killing assay. Each symbol represents an individual mouse; horizontal lines indicate the mean. $*P = 1.8\times10^{-4}$.

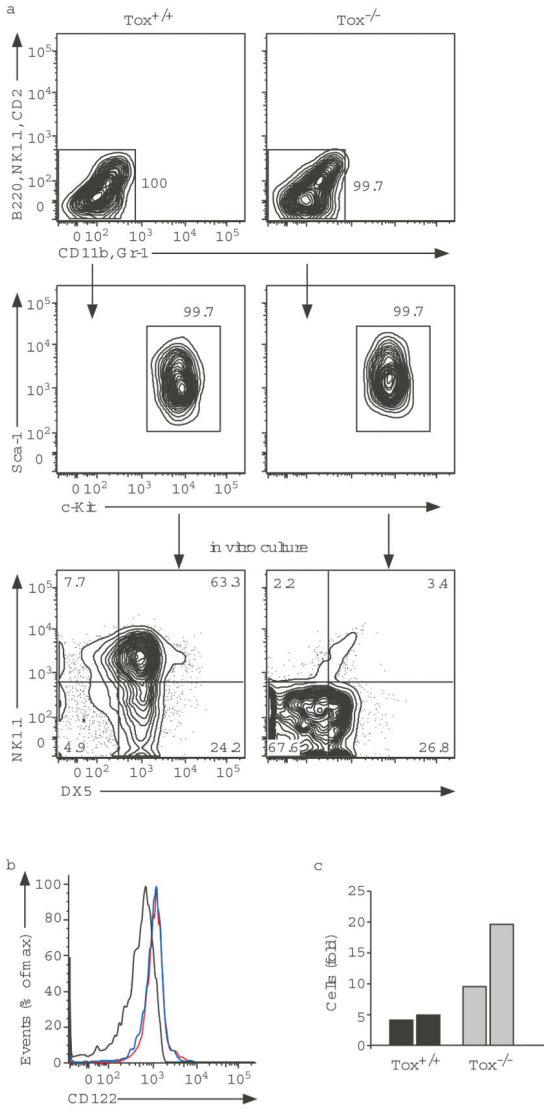

Figure 5.

Defect in NK cell development in the absence of TOX is cell-intrinsic. **(a)** *Tox*$^{+/-}$ and *Tox*$^{-/-}$ bone marrow LSK cells were purified and cultured *in vitro* to generate NK cells. Shown is staining of the LSK cell population at the initiation of culture and at day 12 (gated for Lin^{-} cells, except for NK lineage markers). Data is representative of four experiments. **(b)** Expression of CD122 on Lin^{-} *Tox*$^{+/-}$ (red) and *Tox*$^{-/-}$ (blue) enriched bone marrow progenitor cells cultured for six days and compared to Lin^{-} *Tox*$^{+/-}$ cells at day 0 (black). Data is representative of four experiments. **(c)** Expansion of allelically marked 1:1 mixtures of *Tox*$^{+/+}$ and *Tox*$^{-/-}$ LSK cells was assessed by cell counts and flow cytometry after six days of culture (n=2).

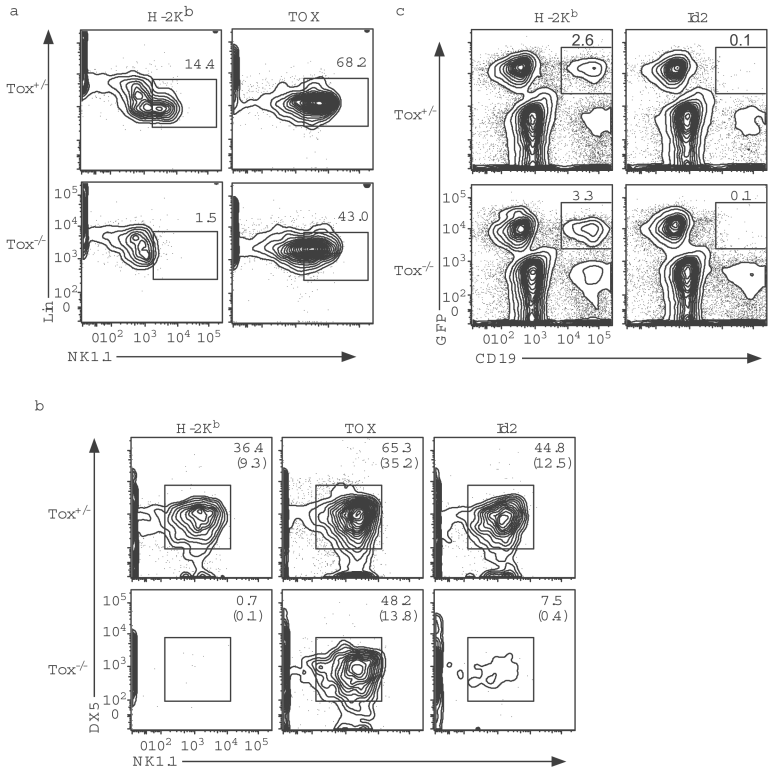

Figure 6.

Expression of *Tox* but not *Id2* can rescue the defect in NK cell development. **(a)** Retroviral gene transduction was used to express TOX, or the H-2K^b molecule as a control, in developing bone marrow progenitor *Tox*$^{+/-}$ and *Tox*$^{-/-}$ cells in NK-promoting cultures. Expression of NK1.1 versus Lin is shown on GFP$^+$ gated cells. Data is representative of four experiments. **(b)** Retroviral gene transduction was used to express TOX, Id2, or the K^b molecule, in developing bone marrow progenitor cells in NK-promoting cultures. Expression of NK1.1 *versus* DX5 is shown on Lin$^-$GFP$^+$ gated cells. Frequencies of the gated populations are shown, and the frequency of the gated population within the total GFP$^+$ subset is indicated in parentheses. Data is representative of four experiments. **(c)** Expression of CD19 *versus* GFP is shown on *Tox*$^{+/-}$ and *Tox*$^{-/-}$ cultured cells expressing H-2K^b or Id2. Data is representative of four experiments.