Supplemental data

Supplemental figure legends

Figure S1. Generation of *Stx11*$^{-/-}$ mice. (A) Structure of the targeted region of the murine *Stx11* gene, the targeting vector, and the deleted gene construct resulting from homologous recombination. The murine *Stx11* gene consists of one coding exon which was completely deleted. The two homology regions are indicated as gray dashed boxes adjacent to the coding exon. The probe used for Southern blot analysis of genomic DNA digested with HindIII is indicated as a black line. The location of the neomycin resistance gene (Neo) and one copy of the diphteria toxin gene (dta) in the targeting vector are shown. (B) Southern blot analysis of genomic DNA from different *Stx11* genotypes. Genomic DNA was digested with HindIII, separated electrophoretically, blotted onto a nylon membrane, and probed with the radioactively labelled probe indicated in A. The detected bands represent the WT, or the knockout allele. (C) Western blot analysis for the STX11 protein of membrane fractions isolated from peripheral blood mononuclear cells confirms the different *Stx11* genotypes. Membrane enriched fractions were separated by SDS-PAGE, blotted and probed with an antibody directed against the N-terminus of the STX11 protein. SNAP23 was used as a loading control. (D) Histochemistry of WT and *Stx11*$^{-/-}$ mice. Spleen, lymph node and thymus sections from four to six mice of each genotype were stained with hematoxylin and eosin. Photos were taken with a light microscope under 100x magnification. One representative experiment is shown.

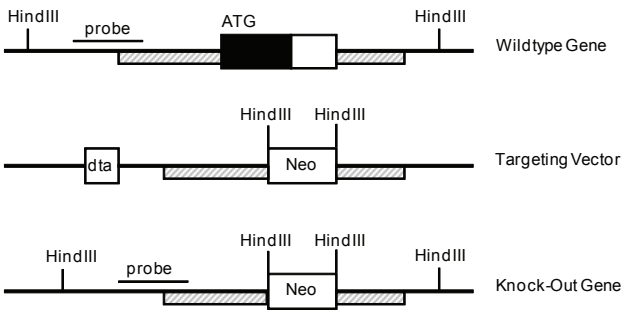
Figure S2. The cytotoxic impairment in *Stx11*$^{-/-}$ NK cells is not reversed after IL-2 stimulation. Specific lysis was assessed by release of cytoplasmic lactate dehydrogenase *in vitro* from YAC-1 cells co-incubated with WT or *Stx11*$^{-/-}$ LAK cells at various effector/target ratios

(E:T). Results are expressed as mean + SD of three independent cultures derived from three mice of each genotype. Two independent experiments were performed.

Figure S3. *Stx11*$^{-/-}$ NK and CD8^{+} T cells display a phenotype comparable to WT counterparts. (A, B) Flow cytometry analysis of WT or *Stx11*$^{-/-}$ NK and CD8^{+} T cells freshly isolated from spleen. Plots are gated on SSC and NKp46^{+} (A) or SSC and CD8^{+} T cells (B). Numbers in plots indicate percent of positive cells. Shaded histograms show marker expression as indicated, bold lines show the corresponding isotype control. One representative experiment out of two performed is shown (n=5-6 mice).

D'Orlando et al., Supplementary Figure 1

A



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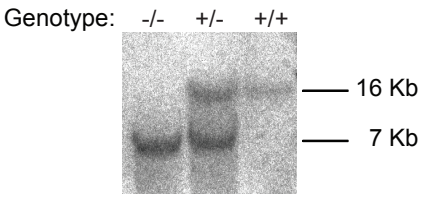

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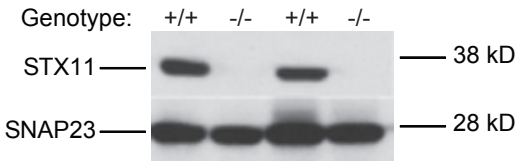

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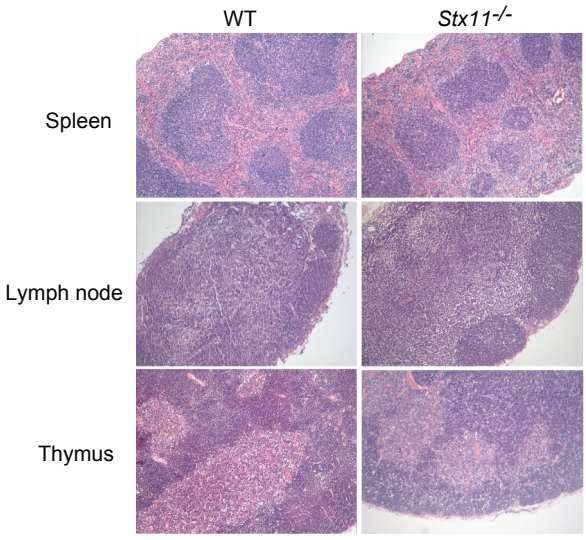
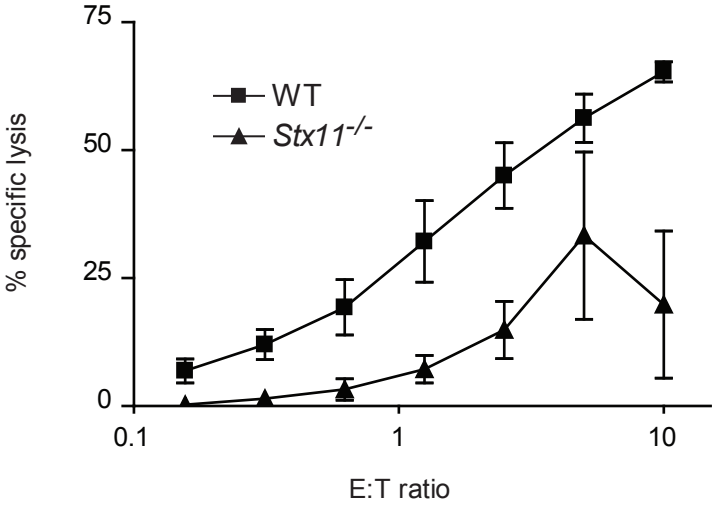
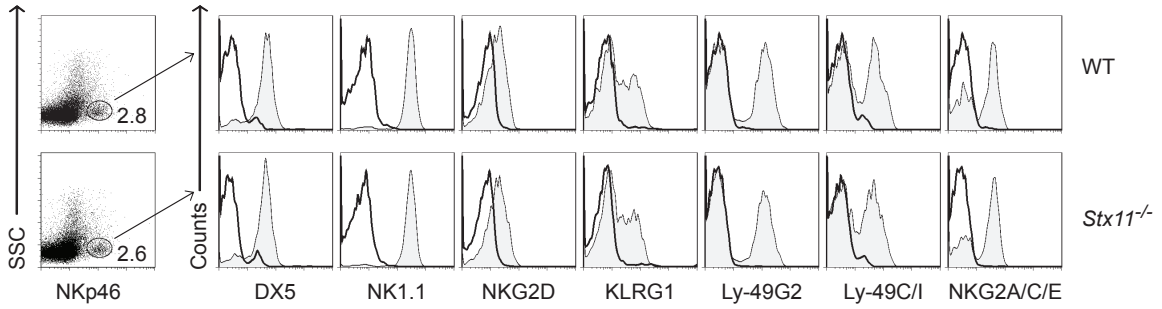


D'Orlando et al., Supplementary Figure 2



D'Orlando et al., Supplementary Figure 3

A



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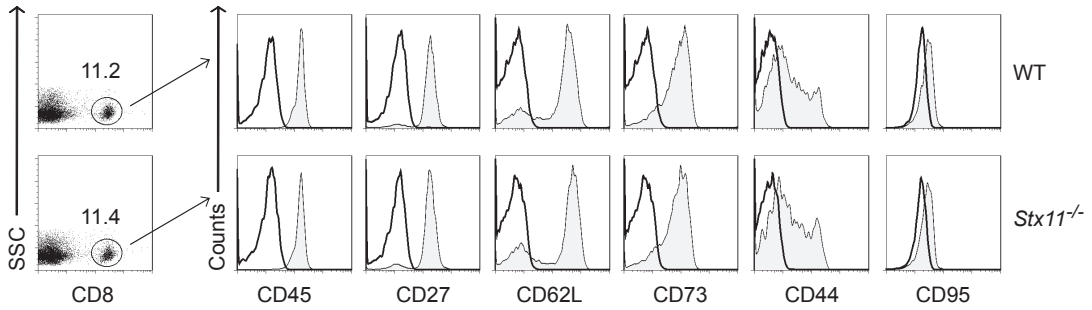

Table S1. Cell counts in primary and secondary lymphatic organs of the immune system.

	WT (x 10^7)	*Stx11* $^{-/-}$ (x 10^7)
Spleen	8.82 ± 0.87 (n=8)	11.01 ± 1.08 (n=8)
Lymph node	3.05 ± 0.90 (n=5)	3.43 ± 0.83 (n=4)
Bone marrow	3.45 ± 0.26 (n=8)	2.94 ± 0.26 (n=8)
Thymus	7.43 ± 0.69 (n=7)	8.15 ± 1.38 (n=7)

Values are expressed as mean ± SEM.

Supplemental Methods

Generation of *Stx11*$^{-/-}$. Genomic DNA from 129/SvJ mice was used to construct the targeting vector. A 4 kb PCR product including intron 2 and a 2 kb PCR product including the 3′ downstream sequence of the *Stx11* gene were placed in the Scrambler V901 vector (Lexington Genetics), flanking a phosphoglycerate kinase (pgk) promoter-driven neomycin resistance cassette. A *pgk* promoter driven diphteria toxin A cassette was added as a negative selection marker. The linearized targeting vector was electroporated into R1 embryonic stem (ES) cells (Nagy Lab). Targeted ES cell clones were verified by Southern blot analysis.

With a 5′ probe of about ~500 bp length, indicated in figure S1A, a smaller HindIII fragment was detected for the allele with the Neo cassette, i.e. the deleted *Stx11* genomic fragment, compared to the WT fragment. Two independent targeted ES cell clones were injected into C57Bl/6 blastocysts and gave highly chimeric mice that were transmitting the *Stx11*$^{-/-}$ allele to the germline. After germline transmission the mutation was confirmed by Southern blotting. Heterozygous offspring mice were crossed to yield homozygous *Stx11*$^{-/-}$ mice. Offspring were routinely genotyped by PCR. The present studies were performed with *Stx11*$^{-/-}$ mice that were backcrossed with C57Bl/6 background for at least 10 generations.

Analysis of ROS formation and phagocytosis

Generation of ROS was determined in a microplate luminometer (LB 96V; Berthold) by measurement of chemiluminescence in the presence of 200 µM lucigenin (9,9'-bis (*N*-methylacridinium nitrate; Roche Applied Science) as described elsewhere [39]. In brief, 2 x 10^5 cells in 200 µl CL-medium (RPMI 1640 buffered with 25 mM HEPES without phenol red (Biochrom), supplemented with 5% FCS) were treated with latex beads (2.5 x 10^7 particles/well,

0.992 µm diameter; Serva), zymosan, or zymosan opsonized with human plasma (each 125 µg/ml; both from Sigma), and chemiluminescence was recorded for 60 min. Individual assay backgrounds were determined in samples of unstimulated cells run in parallel and were subtracted. Data were expressed as relative light units and quantified by integration over the time periods indicated.

For measurement of phagocytosis 5 x 10^5 cells were cultured for 24h in 24 well-plates. Phagocytosis was induced by the addition of 3.4 x 10^7 Fluoresbrite® YG-conjugated monodisperse latex beads (1.92 µm diameter; Polyscience) for 20 min at 37°C. After removal of free particles by aspiration, cells were detached from the plate and washed 4 times in ice cold PBS, supplemented with 0.1% BSA. The percentage of cells which had ingested beads was analyzed by flow cytometry.

β-glucuronidase release

Mouse peripheral blood was collected by heart puncture into 2.7% EDTA in PBS. After centrifugation platelet-rich plasma was removed, erythrocytes were lysed and cells were washed once in HEPES-BSS (10 mM HEPES pH 7.2, 137 mM NaCl, 5 mM D-glucose, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$), and resuspended in the same buffer at 1 x 10^6 cells/ml. Cytochalasin B (Sigma; 5 µg/ml) was added, and cells were prewarmed for 10 min at 37°C before addition of 200 nM PMA, 1 µM Ionomycin, 1 µM A23187, 10 µM fMLP (all from Sigma), or buffer alone. After 30 min incubation at 37°C, degranulation was stopped by rapid cooling on ice. Cells were removed by centrifugation, and duplicates of 50 µl supernatant were transferred in 96-well plates. Release of neutrophil-specific endogenous β-glucuronidase enzymatic activity was assayed by addition of 50 µl reaction buffer (100 mM sodium acetate pH 4, 8 mM 4-nitrophenyl-β-D-glucuronide (Sigma)). The reaction was stopped after 24 to 48h at

37°C by adding 50 µl 400 mM glycine/NaOH pH 10.3. β-glucuronidase release is expressed as percent release compared with the total content detected in supernatants of 0.1% hexadecyltrimethyl ammonium bromide-lysed cells run in parallel. Assay backgrounds determined in samples of unstimulated cells run in parallel were subtracted.

Flow cytometry and intracellular staining. Cells were resuspended in FACS-buffer (2% newborn calf serum, 0.1% NaN_3, 2 mM EDTA in PBS), stained with mAb against CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD45R (RA3-6B2), CD49b/Pan-NK (DX5), Gr-1 (RB6-8C5), IgD (11-26c.2a), IgM (R6-60.2), NK-1.1 (PK136), Ly-49C/I (5E6), NKG2A/C/E (20d5), Ly-49G2 (4D11), CD69 (H1.2F3), CD44 (IM7), CD28 (37.51), CD95 (Jo2), CD25 (7D4), CD62L (MEL-14) (all from BD PharMingen), NKp46 (29A1.4), NKG2D (CX5), KLRG1 (2F1), CD45 (30-F11), CD73 (TY/11.8), CD27 (LG.7F9), CD51 (RMV-7) (all from eBioscience) for 20 min at 4° C. To analyse intracellular content of cytokines, granzymes or perforin WT or *Stx11*$^{-/-}$ CTL recovered after anti-allo stimulation or NK recovered after rhIL-15 stimulation, were re-stimulated *in vitro* with 1 µg/ml phorbol-12-myristate-13-acetate (PMA) plus 1µM Ionomycin (both from Sigma), 1 µg/ml anti-mouse CD3ε and P815 or YAC-1 target cells (E/T ratio 10:1) or left untreated for 3 h. Intracellular FACS staining was performed according to a standard protocol from eBioscience. Briefly, stimulated cells were cultured with 10 µg/ml Brefeldin A (Sigma-Aldrich), washed and surface FACS staining was performed. After fixation and permeabilization CTL and NK cells were incubated with mAb against Granzyme A (3G8.5) (Santa Cruz), Granzyme B (GB12) (Invitrogen), perforin (eBioOMAK-D) (eBioscience), TNF (MP6-XT22) (BD PharMingen) and IFN-γ (37895) (R&D Systems). Data were acquired on a FACSCalibur flow cytometer and analyzed with FlowJo Version 7.6.1 software (TreeStar). Isotype controls were used to set the gates.

Cell isolation

For NK cell isolation by cell sorting, splenocytes from C57Bl/6 mice were labelled with CD49b/DX5/Pan-NK antibody, washed, incubated with magnetic anti-FITC microbeads (Miltenyi Biotec), and enriched on the autoMACS (Miltenyi Biotec) device. Collected cells were surface stained with anti-CD3 or anti-CD8a mAb and $DX5^+CD3^-$ or $CD8^+$ cells were isolated on a FACSARIA cell sorter (routinely ≥92% purity). Otherwise NK cells were enriched using the NK Cell Isolation Kit II (Miltenyi Biotec) on the autoMACS device, instead $CD8^+$ T cells were isolated from spleen by negative selection using magnetic associated cell sorting (MACS). For this, cells were stained with FITC-conjugated mAb against CD4, CD45R/B220, CD49b/DX5/Pan-NK, CD11c, CD11b and F4/80, washed, incubated with magnetic anti-FITC-labeled microbeads and sorted using an autoMACS.

RNA isolation, cDNA synthesis and PCR. For Fig. 1A and 2A total RNA was purified using High Pure RNA Isolation Kit (Roche Applied Science) according to manufacturer's instructions followed by reverse transcription into cDNA using First Strand cDNA Synthesis Kit (Fermentas). PCR was conducted on a thermocycler (Biorad) after mixing the cDNA with High Fidelity PCR Enzyme Mix (Fermentas) and appropriate primers (500 nM each). Primers for mouse *Stx11*, and β2-microglobulin (B2m) genes are as follows (5′ to 3′): *Stx11*, sense: TCA TCC AGG ACA TCC AGG, antisense: ACT CGT ACA TGG CTT GCT G; B2m, sense: CTG ACC GGC CTG TAT GCT AT, antisense: CAG TAT GTT CGG CTT CCC AT. PCR products were separated by 4% agarose (Peqlab) gel electrophoresis in TAE buffer (40 mM Tris pH 8.5, 20 mM acetic acid, 1 mM EDTA). For Fig. 3A total RNA was purified using RNeasy Mini Kit (QIAGEN) according to manufacturer's recommendations followed by reverse transcription into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen). Primers for mouse *Stx11*, and

β-actin genes are as follows (5′ to 3′): *Stx11*, sense: ATG GGG ACG ATG ACT TTG, antisense: GTC GCG CTG TTT CAT CTC; β-actin sense: GTG GGG CGC CCC AGG CAC CA, antisense: CTC CTT AAT GTC ACG CAC GAT TTC. PCR products were separated by 1.5% agarose gel electrophoresis in TAE buffer.

Western blot analysis. Cell pellets were lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$ containing 2% Triton X-100, and 1 mM Na_3VO_4, 10 mM NaF and 1x Complete Protease Inhibitor cocktail (Roche). Proteins were extracted from membrane/organelle fractions using the ProteoExtract® subcellular Proteome Extraction Kit (Calbiochem) following the manufacturer's instructions. The proteins of membrane fractions were separated by SDS-PAGE using 12% polyacrylamide gel and blotted onto nitrocellulose membrane (Biorad). Rabbit anti-human STX11 antibody was a generous gift of Dr. Rytis Prekeris (University of Colorado). Anti-human Syntaxin-BP2 antibody (MUNC18-2) was purchased from R&D Systems. Mouse anti-VTI1B monoclonal antibody and purified mouse anti-TIM23 (clone 32/Tim23) antibody were purchased from BD Transduction Laboratories™. Mouse anti-GAPDH monoclonal antibody was purchased from Santa Cruz. Donkey anti-rabbit IgG-HRP and sheep anti-mouse IgG-HRP were purchased from Amersham Biosciences. Rat anti-mouse perforin purified (clone eBioOMAK-D) antibody was purchased from eBioscience.

ELISA. BMDC or BMM (1 x 10^4 cells/well) supernatant was collected at 24 and 48h after cell stimulation. CTL supernatants were collected at day 5 during the last round of anti-allo stimulation. Cytokines were measured by ELISA using specific antibodies and standard proteins from R&D Systems according to the manufacturer's instructions.

***In vitro* proliferation assay.** $CD8^+$ or $CD4^+$ T cells were isolated from lymph nodes and spleen of OT-I TCR-transgenic mice or OT-II TCR-transgenic mice, respectively. C57BL/6 OT-I mice

transgenic for a $CD8^+$ T cell-restricted TCR recognizing OVA (257-264) peptide and C57BL/6 OT-II mice, whose transgenic CD4-TCRs specifically recognize the OVA (323-339) peptide were provided from Charles River (France) and maintained at the Research Center Borstel animal facilities. BMDC generated from bone marrow of WT and *Stx11*$^{-/-}$ mice were collected at day 8-9 using Accutase (PAA), washed and resuspended at a concentration of 10^7 cells/ml in BMDC medium containing 2 μg/ml OT-I peptide ($OVA^{257-264}$, NeoMPS) for 2-3 h at 37ºC. After that BMDC were washed twice with BMDC medium and plated at a density of 1 x 10^4 cells/well in a 96 well U-bottom plate (Nunc). Alternatively, BMDC were pulsed with 0.1 μM OT-II peptide ($OVA^{323-339}$, present in culture). 1 x 10^5 OT-I $CD8^+$ T cells or OT-II $CD4^+$ T cells were added to the BMDC. Cells were pulsed with 0.5 μCi/well of [^{3}H]thymidine on day 2 (OT-I proliferation) or on day 3 (OT-II proliferation) and then collected 18-24h later. T cell proliferation was evaluated by incorporation of [^{3}H]thymidine from triplicate wells.

LAK cells generation

For the generation of LAK cells splenocytes were plated at 2 x 10^6 cells/ml and cultured in NK medium supplemented with 2000 U/ml rhIL-2. At day 5 NK cells were enriched using the NK Cell Isolation Kit II on the autoMACS device. LAK cells were used at day 6 as effector cells in the *in vitro* killing assay. Before the assay, an aliquot of effector cells was analyzed by flow cytometry to determine the relative percentages of $NKp46^+$ in WT and *Stx11*$^{-/-}$ cell cultures.

Histology. Tissue samples were fixed with 4% formaldehyde and processed for paraffin embedding. Tissue sections were stained with haematoxylin/eosin.