DOCK8 is critical for the survival and function of NKT cells

Supplementary Materials and Methods

BrdU Incorporation

BrdU (Sigma/ B.D.) was given at 0.8mg/ml in drinking water of DOCK8$^{cpm/cpm}$ and WT mice for nine days before staining using BD Pharmingen™ BrdU Flow Kit. All buffers and reagents were included in kit and used in accordance with manufacturer's protocols[1]. Briefly, cells were stained with αGalcer-loaded tetramers before fixation, permeabilization and DNase treatment. Cells were then stained with FITC-conjugated anti-BrdU antibody before data collection on LSRII flow cytometer (BD).

Cytokine Cultures

Thymic NKT cells from DOCK8$^{cpm/cpm}$ and WT mice were cultured for three days in the presence of different cytokines and stained with Live/dead kit (Invitrogen) to test for cell viability. All cytokines were purchased from Peprotech and used at 20ng/ml.

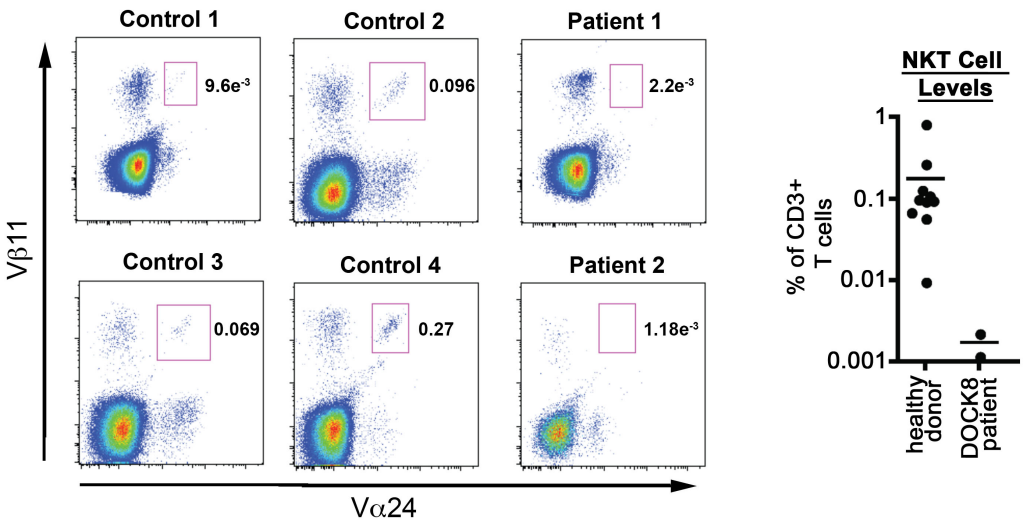
In vivo NKT cell activation

Animals were injected (I.V.) with 1μg αGalCer or PBS and left for 1 hour before harvesting of organs. Lymphocytes from livers and spleens were cultured for a further 4 hours in GolgiPlug (BD Biosciences) before carrying out intracellular cytokine staining as described previously[2].

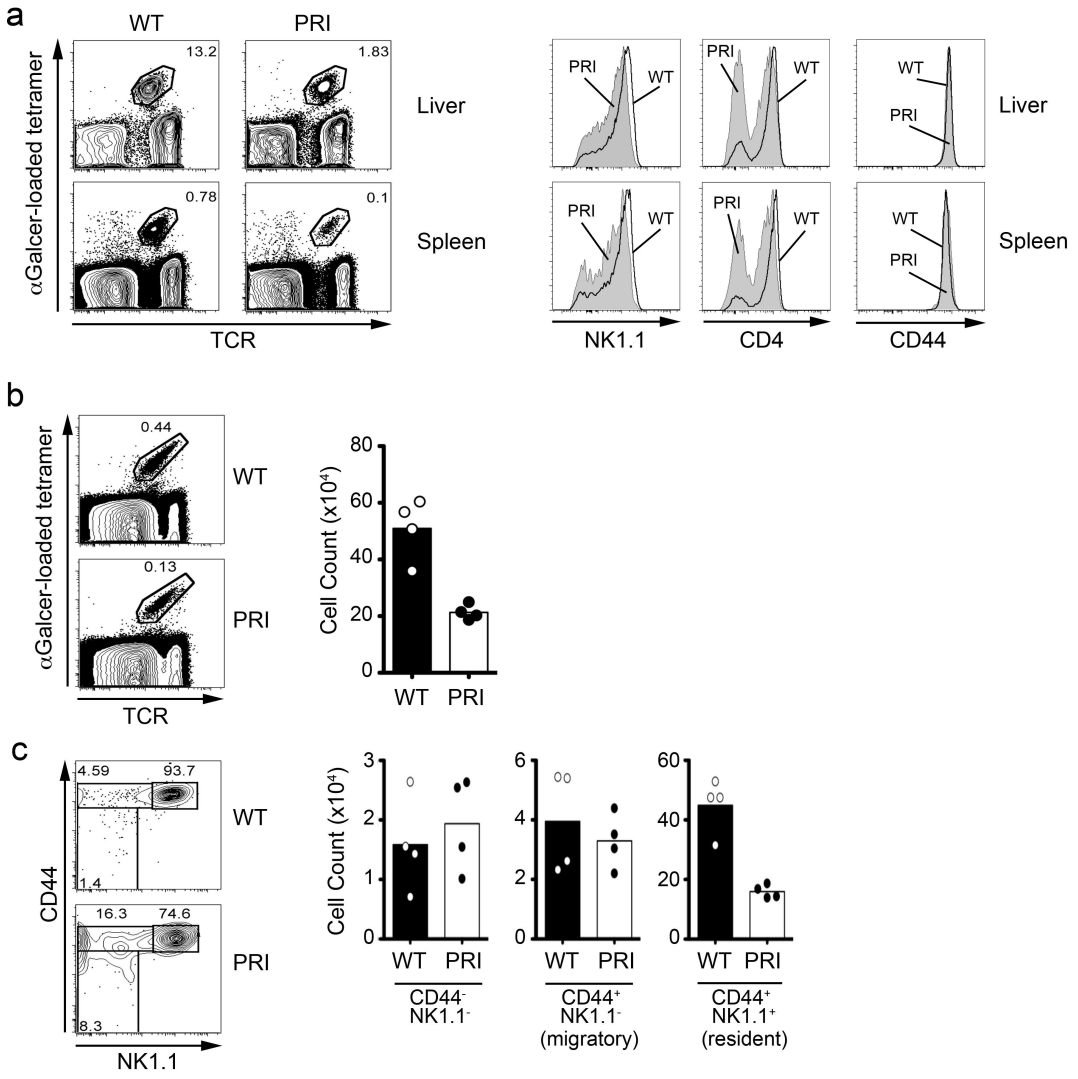
Cell Sorting Experiments

For cell sorting experiments, pure populations of cells (>99% purity) cells were stained with the flow cytometry protocols described above and run on a Beckman Coulter Legacy MoFlo MLS High Speed Cell Sorter at the flow cytometry core facility within the Jenner Institute, Oxford.

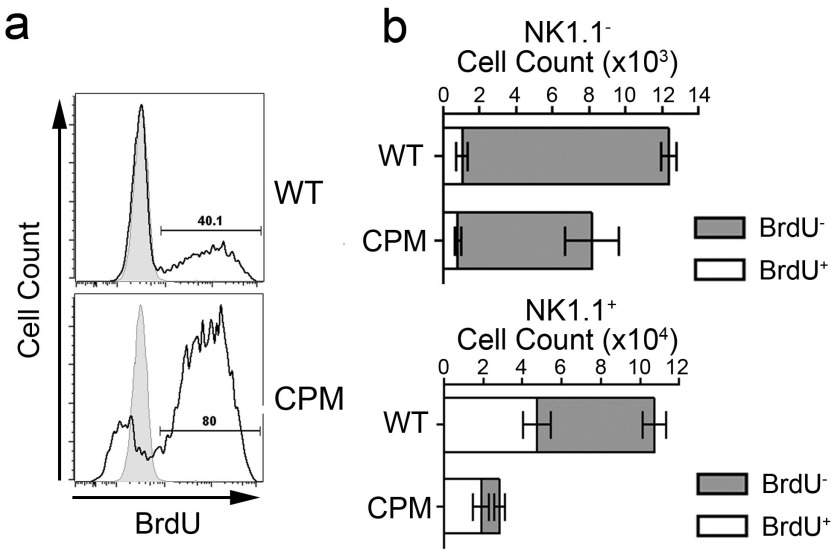
Supplementary Figures



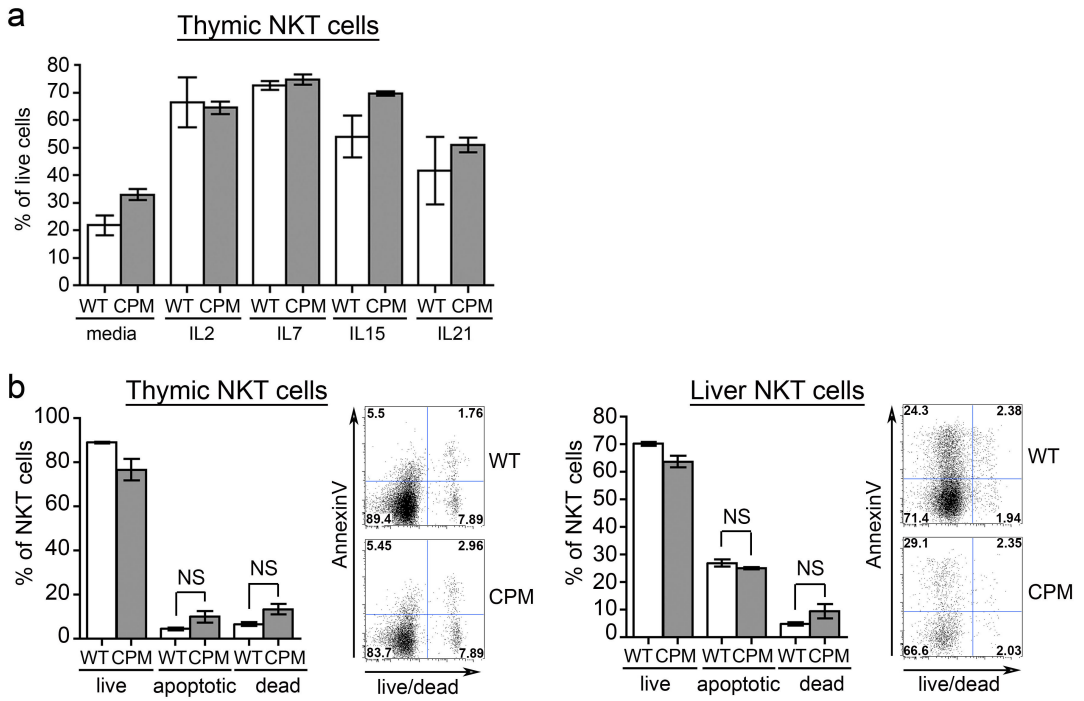
Supplementary Figure 1: DOCK8 is required for maintenance of peripheral NKT cells in humans. Representative FACS plots of PBL from four healthy controls and two DOCK8 deficient patients, depicting levels of NKT cells based on Vα24 and Vβ11 staining. The percentage of NKT cells as a percentage of $CD3^+$ T cells are depicted.



Supplementary Figure 2: Reduction of NKT cells in the liver, spleen and thymus of DOCK8$^{pri/pri}$ mice. (a) Flow cytometry analysis of NKT cells in liver and spleen of WT and DOCK8$^{pri/pri}$ (PRI) mice showing representative staining with αGalCer-loaded tetramer and TCRβ (left panel). Relative expression levels of phenotypic and maturation markers (NK1.1, CD4, CD44) for NKT cells from WT (dark line) and DOCK8$^{pri/pri}$ mice (filled) are depicted (right panel) (b) Representative FACS staining of thymocytes with αGalCer-loaded tetramer and TCRβ (left panel) and absolute numbers of NKT cells (right panel). The data depicts individual mice (circles) arithmetic means (bars). (c) αGalCer-tetramer^{+} cells divided into the three, linear stages of NKT cell development on the basis of CD44 and NK1.1 staining. Data depicts individual mice (circles) with arithmetic means (bars).

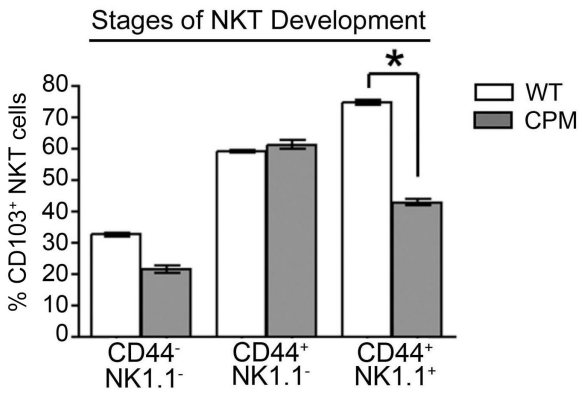


Supplementary Figure 3: Decreased survival and increased turnover of terminally differentiated liver NKT cells in the absence of DOCK8. (a) Histograms showing typical incorporation of BrdU into NKT cells from the livers of WT and DOCK8$^{cpm/cpm}$ (CPM) mice (dark lines) with unlabeled WT controls (filled histograms) (n>4 for each) nine days after addition of BrdU to drinking water. (b) Numbers of BrdU$^+$ and BrdU$^-$ NKT cells were calculated for both NK1.1$^-$ and NK1.1$^+$ subsets in the liver (gated on TCRβ^+ αGalCer-tetramer$^+$). Columns are arithmetic means and error bars SEM. Data are representative of three independent experiments involving 5 biological replicates.



Supplementary Figure 4: Normal Responses to Cytokine Growth Factors and levels of apoptosis in DOCK8$^{cpm/cpm}$ **NKT cells.** (a) Thymic WT and DOCK8$^{cpm/cpm}$ (CPM) NKT cell viability three days after culture in media supplemented cytokine growth factors (20ng/ml), gating from live cells. Bars depict arithmetic means and SEM from 4 biological replicates. Data is representative of 3 separate experiments. (b) Staining of thymic and liver NKT cells for annexinV and live/dead stain to access apoptosis levels. Bars depict arithmetic means and SEM from 3 biological replicates. Data is representative of 2 separate experiments.

a



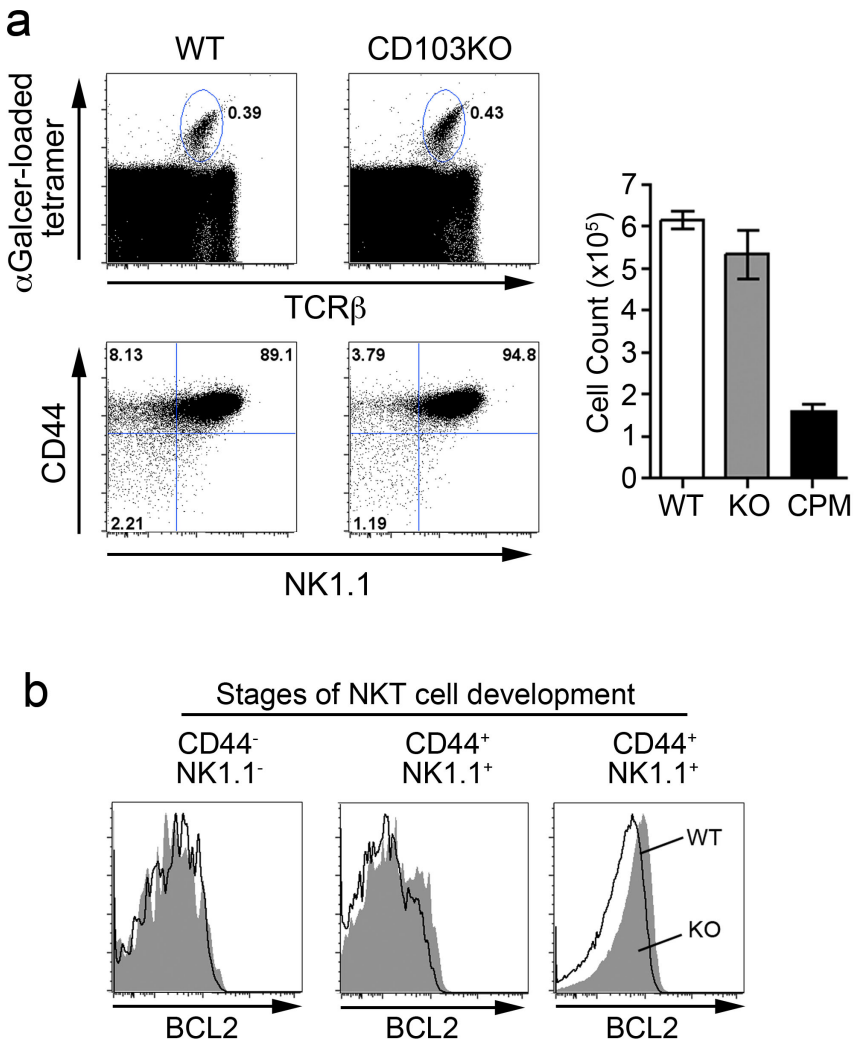
b

	Bcl2 Mean Expression in DOCK8$^{cpm/cpm}$ Thymic NKT cells									
Stage of Development	WT1	WT2	WT3	WT4	Mean (+/-SEM)	CPM1	CPM2	CPM3	CPM4	Mean (+/-SEM)
CD44- NK1.1- (Stage1)	418	268	314	277	319 (+/- 69)	262	218	258	212	238 (+/- 26)
CD44+ NK1.1- (Stage2)	265	150	183	77	169 (+/- 78)	70	101	119	123	103 (+/- 24)
CD44+ NK1.1+ (Stage3)	1175	1061	961	856	1013 (+/- 137)	503	521	554	556	534 (+/- 26)

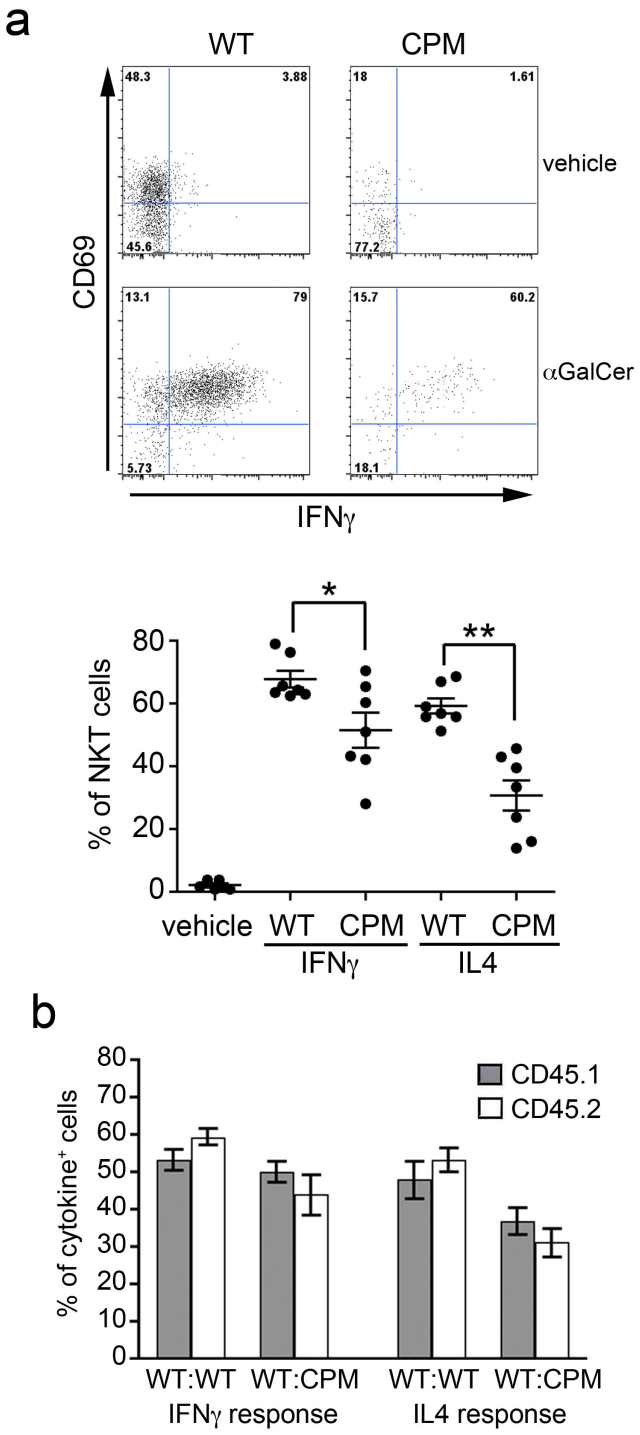
	ICOS Mean Expression in DOCK8$^{cpm/cpm}$ Thymic NKT cells									
Stage of Development	WT1	WT2	WT3	WT4	Mean (+/- SEM)	CPM1	CPM2	CPM3	CPM4	Mean (+/- SEM)
CD44- NK1.1- (Stage1)	10830	16511	16111	18000	15363 (+/- 3129)	9085	6877	6002	7712	7419 (+/- 1312)
CD44+ NK1.1- (Stage2)	17563	23349	23771	27731	23104 (+/- 4188)	15874	14092	13094	13930	14248 (+/- 1169)
CD44+ NK1.1+ (Stage3)	2974	3982	4179	4366	3875 (+/- 621)	4062	3976	3219	3966	3806 (+/- 394)

	Tbet Mean Expression in DOCK8$^{cpm/cpm}$ Thymic NKT cells									
Stage of Development	WT1	WT2	WT3	WT4	Mean (+/-SEM)	CPM1	CPM2	CPM3	CPM4	Mean (+/-SEM)
CD44- NK1.1- (Stage1)	143	77	128	101	112 (+/- 29)	82	91	74	99	87 (+/-11)
CD44+ NK1.1- (Stage2)	446	449	514	449	465 (+/- 33)	317	287	352	323	320 (+/- 27)
CD44+ NK1.1+ (Stage3)	1241	1355	1124	1218	1235 (+/- 95)	1300	1313	1377	1392	1346 (+/- 46)

Supplementary Figure 5: DOCK8 results in loss of CD103^{+} NKT cells and expression of the survival factor Bcl-2. a) FACS analysis of thymic NKT development in WT and DOCK8$^{cpm/cpm}$ (CPM) showing proportions of CD103^{+} cells. Bars depict arithmetic means and SEM. Data is representative of 3 separate experiments. Statistical significance was tested by unpaired t-test, *$p<0.05$. b) Tables illustrating mean expression levels of factors associated with NKT cell development including Bcl-2, ICOS and Tbet. Mean expression based on 4 replicates is shown with SEM. Data are representative of 3 independent experiments.



Supplementary Figure 6: CD103 knockout mice show no alterations in thymic NKT cell development. a) Flow cytometric analysis of thymic NKT cells development in WT and CD103KO (KO) mice showing representative staining of thymocytes with αGalCer-loaded tetramer and TCRβ. αGalCer-tetramer^{+} NKT cells were further analyzed for CD44 and NK1.1 expression. Numbers of mature, NK1.1^{+} NKT cells were calculated in WT, DOCK8$^{cpm/cpm}$ (CPM) and CD103KO (KO) mice. Bars show arithmetic means and SEM. b) Bcl2 expression profile at all stages of NKT cell development comparing WT (dark line) and CD103KO (filled). Data are representative of 2 independent experiments.



Supplementary Figure 7: The impaired activation of splenic DOCK8$^{cpm/cpm}$ NKT cells is restored in mixed bone marrow chimeras. (a) Mice were injected with αGalCer with responses quantified using CD69 gating, IFNγ and IL4 staining by ICS after 1 hr (upper panel). Graph (lower panel) shows the response for individual mice (circles) as a percentage of NKT cells with arithmetic means and SEM. Statistical significance was tested by unpaired t-test, *p<0.05, **p<0.01. (b) The percentage of

DOCK8 deficient and WT NKT cells secreting IFNγ and IL4 in mixed chimeras 1hr after intravenous αGalCer injection. Irradiated mice were reconstituted with 80% CD45.2 WT or DOCK8$^{cpm/cpm}$ (CPM) BM mixed with 20% CD45.1 WT BM and injected with αGalCer. I.V. Bars represent the arithmetic means with SEM of responses generated in 5 individual animals.

References

1. Lambe, T. *et al.* DOCK8 is essential for T-cell survival and the maintenance of CD8+ T-cell memory. *Eur. J. Immunol.* **41**, 3423–3435 (2011).
2. Schmieg, J., Yang, G., Franck, R. W., van Rooijen, N. & Tsuji, M. Glycolipid presentation to natural killer T cells differs in an organ-dependent fashion. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1127–1132 (2005).