Supplementary Files

Regulation of NK cell development, maturation, and anti-tumor responses by the nuclear receptor NR2F6

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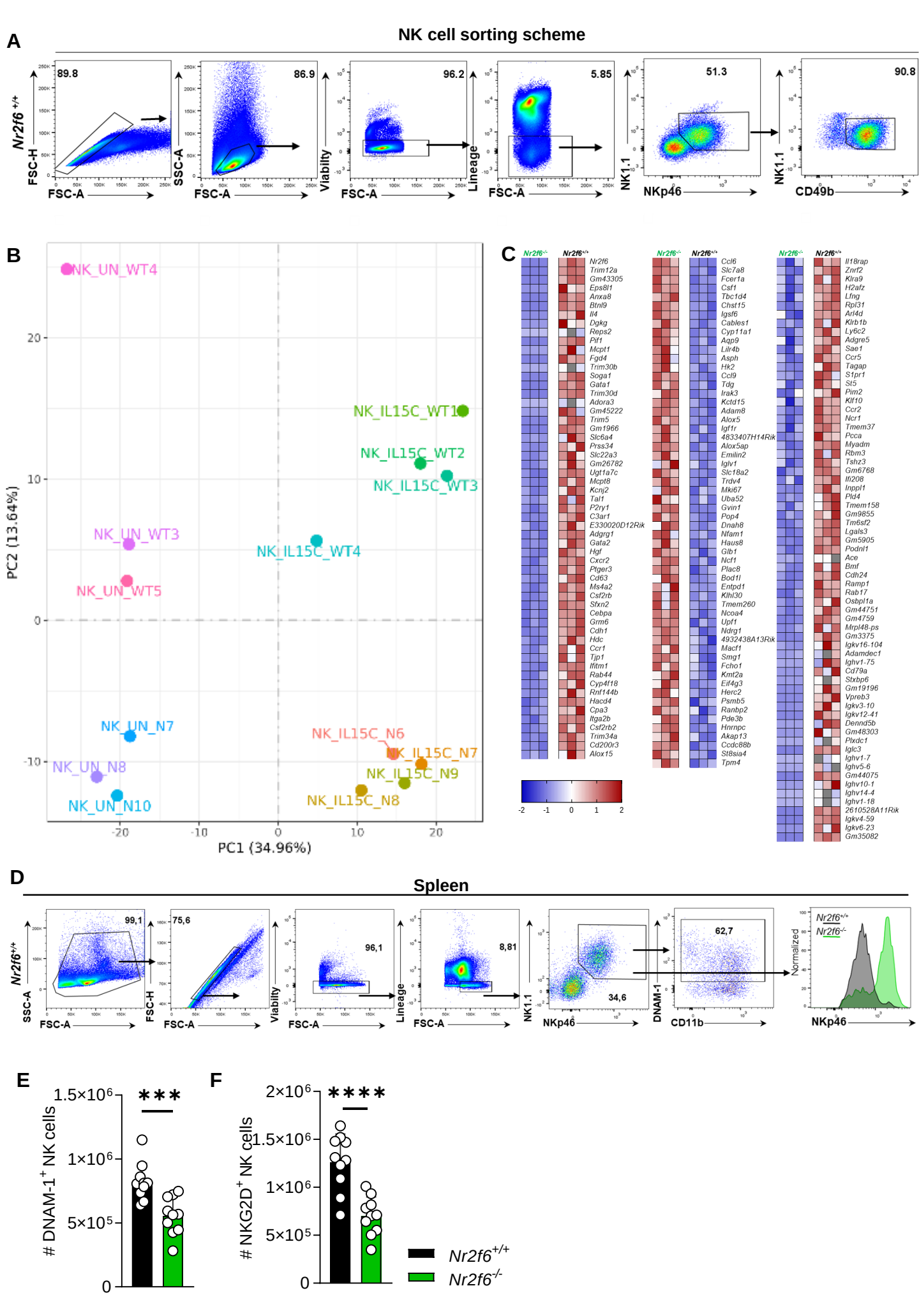

A
NK cell sorting scheme
Nr2f6 +/+
89.8
FSC-H
FSC-A
86.9
SSC-A
FSC-A
96.2
Viability
FSC-A
5.85
Lineage
FSC-A
51.3
NK1.1
NKp46
90.8
NK1.1
CD49b
B
NK_UN_WT4
NK_IL15C_WT1
NK_IL15C_WT2
NK_IL15C_WT3
NK_UN_WT3
NK_IL15C_WT4
NK_UN_WT5
NK_UN_N7
NK_IL15C_N6
NK_IL15C_N7
NK_UN_N8
NK_IL15C_N9
NK_UN_N10
NK_IL15C_N8
PC2 (13.64%)
PC1 (34.96%)
C
Nr2f6-/-
Nr2f6+/+
Nr2f6
Trim12a
Gm43305
Eps8l1
Anxa8
Btnl9
Il4
Dgkg
Reps2
Pif1
Mcpt1
Fgd4
Trim30b
Soga1
Gata1
Trim30d
Adora3
Gm45222
Trim5
Gm1966
Slc6a4
Prss34
Slc22a3
Gm26782
Ugt1a7c
Mcpt8
Kcnj2
Tal1
P2ry1
C3ar1
E330020D12Rik
Adgrg1
Gata2
Hgf
Cxcr2
Ptger3
Cd63
Ms4a2
Csf2rb
Sfxn2
Cebpa
Grm6
Cdh1
Hdc
Ccr1
Tjp1
Ifitm1
Rab44
Cyp4f18
Rnf144b
Hacd4
Cpa3
Itga2b
Csf2rb2
Trim34a
Cd200r3
Alox15
Ccl6
Slc7a8
Fcer1a
Csf1
Tbc1d4
Chst15
Igsf6
Cables1
Cyp11a1
Aqp9
Lilr4b
Asph
Hk2
Ccl9
Tdg
Irak3
Kctd15
Adam8
Alox5
Igf1r
4833407H14Rik
Alox5ap
Emilin2
Iglv1
Slc18a2
Trdv4
Mki67
Uba52
Gvin1
Pop4
Dnah8
Nfam1
Haus8
Glb1
Ncf1
Plac8
Bod1l
Entpd1
Klhl30
Tmem260
Ncoa4
Upf1
Ndrg1
4932438A13Rik
Macf1
Smg1
Fcho1
Kmt2a
Eif4g3
Herc2
Psmb5
Ranbp2
Pde3b
Hnrnpc
Akap13
Ccdc88b
St8sia4
Tpm4
Il18rap
Znrf2
Klra9
H2afz
Lfng
Rpl31
Arl4d
Klrb1b
Ly6c2
Adgre5
Sae1
Ccr5
Tagap
S1pr1
St5
Pim2
Klf10
Ccr2
Ncr1
Tmem37
Pcca
Myadm
Rbm3
Tshz3
Gm6768
Ifi208
Inppl1
Pld4
Tmem158
Gm9855
Tm6sf2
Lgals3
Gm5905
Podnl1
Ace
Bmf
Cdh24
Ramp1
Rab17
Osbpl1a
Gm44751
Gm4759
Mrpl48-ps
Gm3375
Igkv16-104
Adamdec1
Ighv1-75
Cd79a
Stxbp6
Gm19196
Vpreb3
Igkv3-10
Igkv12-41
Dennd5b
Gm48303
Plxdc1
Iglc3
Ighv1-7
Ighv5-6
Gm44075
Ighv10-1
Ighv14-4
Ighv1-18
2610528A11Rik
Igkv4-59
Igkv6-23
Gm35082
-2
-1
0
1
2
D
Spleen
Nr2f6+/+
99,1
SSC-A
FSC-A
75,6
FSC-H
FSC-A
96,1
Viability
FSC-A
8,81
Lineage
FSC-A
NK1.1
NKp46
34,6
62,7
DNAM-1
CD11b
Nr2f6+/+
Nr2f6-/-
Normalized
NKp46
E
DNAM-1+ NK cells
1.5×10^6
1×10^6
5×10^5
0
***
F
NKG2D+ NK cells
2×10^6
1.5×10^6
1×10^6
5×10^5
0
****
Nr2f6+/+
Nr2f6-/-

Supplementary Figure 1: **Characterization of *Nr2f6*-deficient NK cells via RNA-Seq analysis.**

(A) Gating strategy for splenic NK cell fluorescence-activated cell sorting from wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice. Single events were selected, debris was excluded, and only viable cells were included from total splenocytes. Lin^{+} cells (CD3^{+}CD19^{+}) were excluded and NK cells were identified by NK1.1, NKp46 and CD49b expression.

(B) Principal component analysis (PCA) of sorted splenic NK cells **(A)** from healthy wild-type (*Nr2f6*$^{+/+}$) and *Nr2f6*-deficient (*Nr2f6*$^{-/-}$), or IL-15C treated wild-type (*Nr2f6*$^{+/+}$) and *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) (UN: n = 3, IL-15C: n = 4) mice.

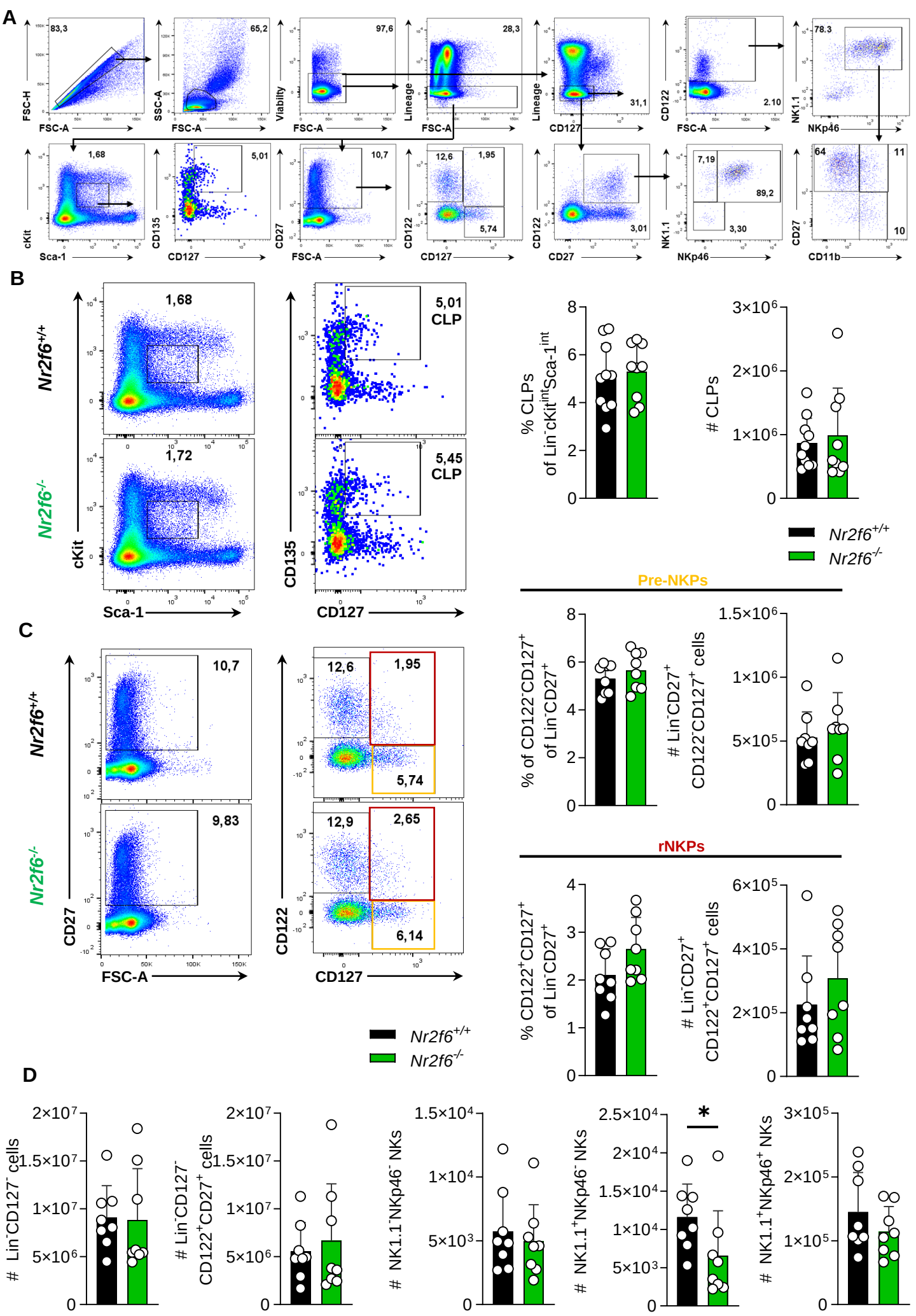
(C) Heatmap of differentially expressed genes (DEG) of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) splenic NK cells. All genes were z-score normalized, and DEGs were defined by DESeq2 (adjusted p-value (padj) < 0.05) (n = 3 per genotype).

(D) Gating strategy for splenic and blood NK cells from wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice. Debris was excluded, single events were selected and only viable cells were included from total splenocytes or blood lymphocytes. Lin^{+} cells (CD3^{+}CD19^{+}) were excluded and NK cells were identified by expression of NK1.1 and NKp46. NK cells were further characterized by e.g., expression of NKp46, DNAM-1 or NKG2D.

(E) Quantification of total cell counts of DNAM-1^{+} splenic NK cells (CD3^{-}CD19^{-}NK1.1^{+}NKp46^{+}) in wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice.

(F) Quantification of total cell counts of NKG2D^{+} splenic NK cells (CD3^{-}CD19^{-}NK1.1^{+}NKp46^{+}) in wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice.

(A-C) RNA sequencing and all downstream analyses were performed on splenic NK cells from *n*=3 per genotype. (E, F) Representative data is shown as pooled experiments of at least three independent experiments n =10. Each dot represents the data of an individual mouse. Results are shown as mean ± SD. The normality of data was evaluated by the Shapiro-Wilk test. An asterisk indicates statistically significant differences between genotypes calculated using Student's *t*-test. A *p*-value < 0.05 was considered statistically significant. ***$p < 0.001$ ****$p < 0.0001$.


A
83,3
FSC-H
FSC-A
65,2
SSC-A
FSC-A
97,6
Viability
FSC-A
28,3
Lineage
FSC-A
Lineage
CD127
31,1
CD122
FSC-A
2.10
78.3
NK1.1
NKp46
1,68
cKit
Sca-1
5,01
CD135
CD127
10,7
CD27
FSC-A
12,6
1,95
5,74
CD122
CD127
CD122
CD27
3,01
7,19
89,2
3,30
NK1.1
NKp46
64
11
10
CD27
CD11b
B
Nr2f6+/+
Nr2f6-/-
1,68
1,72
cKit
Sca-1
5,01
CLP
5,45
CLP
CD135
CD127
% CLPs
of Lin-cKitintSca-1int
CLPs
Nr2f6+/+
Nr2f6-/-
Pre-NKPs
C
Nr2f6+/+
Nr2f6-/-
10,7
9,83
CD27
FSC-A
12,6
1,95
5,74
12,9
2,65
6,14
CD122
CD127
% of CD122-CD127+
of Lin-CD27+
Lin-CD27+
CD122-CD127+ cells
rNKPs
% CD122+CD127+
of Lin-CD27+
Lin-CD27+
CD122+CD127+ cells
Nr2f6+/+
Nr2f6-/-
D
Lin-CD127- cells
Lin-CD127-
CD122+CD27+ cells
NK1.1-NKp46- NKs
NK1.1+NKp46- NKs
*
NK1.1+NKp46+ NKs

Supplementary Figure 2: ***Nr2f6*-deficient mice have normal NK cell development in the bone marrow.**

(A) Gating strategy from singlet, cells, live, lineage$^-$, CLP (Lin$^-$CD117^{int}Sca1^{int}CD135$^+$CD127$^+$), different NK progenitor (Lin$^-$CD27$^+$CD122$^-$CD127$^+$; Lin$^-$CD27$^+$CD122$^+$CD127$^+$), immature (Lin$^-$CD127$^-$CD122$^+$CD27$^+$NK1.1$^-$NKp46$^-$; Lin$^-$CD127$^-$CD122$^+$CD27$^+$NK1.1$^+$NKp46$^-$) and mature NK (Lin$^-$CD127$^-$CD122$^+$NK1.1$^+$NKp46$^+$CD27$^+$CD11b$^-$; Lin$^-$CD127$^-$CD122$^+$NK1.1$^+$NKp46$^+$CD27$^+$CD11b$^+$; Lin$^-$CD127$^-$CD122$^+$NK1.1$^+$NKp46$^+$CD27$^-$CD11b$^+$) populations in the bone marrow from femur and tibia of healthy wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice.

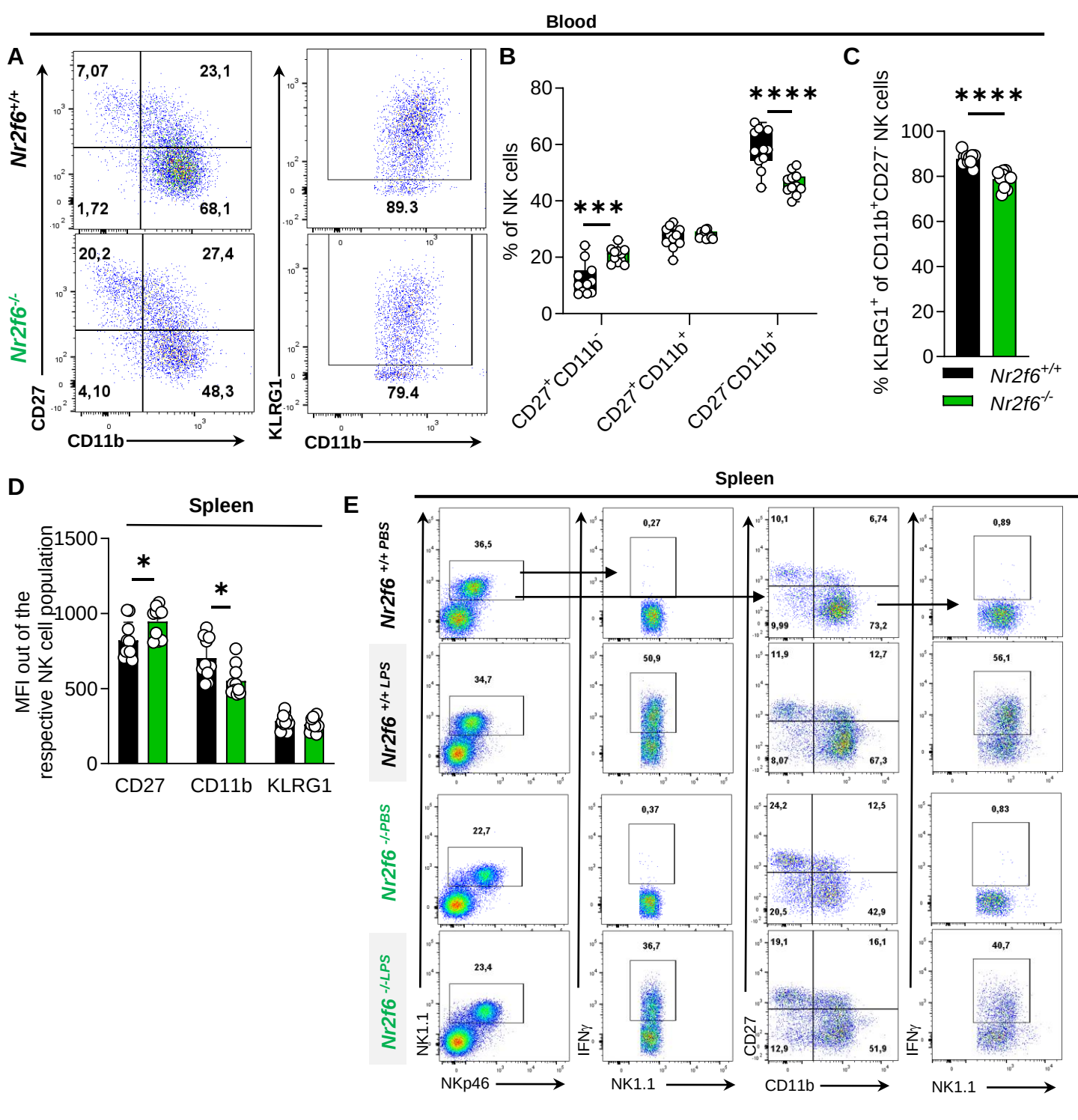
(B) Representative dot plots, quantification of frequency of parent and total cell counts of CLP (CD135$^+$CD127$^+$) of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) bone marrow-derived Lin$^-$CD117^{int}Sca1^{int} cells.

(C) Representative dot plots, quantification of the frequency of parent and total cell counts of CD122$^-$CD127$^+$ pre-NK progenitors and CD122$^+$CD127$^+$ refined NK progenitors (rNKPs) out of wild-type (*Nr2f6*$^{+/+}$) *or Nr2f6*-deficient (*Nr2f6*$^{-/-}$) bone marrow-derived Lin$^-$CD27$^+$ cells.

(D) Quantification of total cell counts of Lin$^-$CD127$^-$ cells, Lin$^-$CD127$^-$CD122$^+$CD27$^+$ NK-progenitors, Lin$^-$CD127$^-$CD122$^+$CD27$^+$ stage A-C NK cells (NK1.1$^-$NKp46$^-$, NK1.1$^+$NKp46$^-$, NK1.1$^+$NKp46$^+$) of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) bone marrow derived cells.

(B) Representative data is shown as pooled experiments of at least three independent experiments n =10 (*Nr2f6*$^{+/+}$) and n=9 (*Nr2f6*$^{-/-}$)

(C, D) Representative data is shown as pooled experiments of at least three independent experiments n = 8. Each dot represents the data of an individual mouse. Results are shown as mean ± SD. An asterisk indicates statistically significant differences between genotypes calculated using Student's t-test or Mann-Whitney *U* test. A p-value < 0.05 was considered statistically significant. *p < 0.05


Blood
A
Nr2f6+/+
Nr2f6-/-
7,07
23,1
1,72
68,1
20,2
27,4
4,10
48,3
CD27
CD11b
89.3
79.4
KLRG1
CD11b
B
% of NK cells
***
****
CD27+CD11b-
CD27+CD11b+
CD27-CD11b+
C
% KLRG1+ of CD11b+CD27- NK cells
****
Nr2f6+/+
Nr2f6-/-
D
Spleen
MFI out of the respective NK cell population
*
*
CD27
CD11b
KLRG1
E
Spleen
Nr2f6 +/+ PBS
Nr2f6 +/+ LPS
Nr2f6 -/-PBS
Nr2f6 -/-LPS
36,5
0,27
10,1
6,74
9,99
73,2
0,89
34,7
50,9
11,9
12,7
8,07
67,3
56,1
22,7
0,37
24,2
12,5
20,5
42,9
0,83
23,4
36,7
19,1
16,1
12,9
51,9
40,7
NK1.1
NKp46
IFNγ
NK1.1
CD27
CD11b
IFNγ
NK1.1

Supplementary Figure 3: **Terminal NK cell maturation block in the blood of *Nr2f6*-deficient mice.**

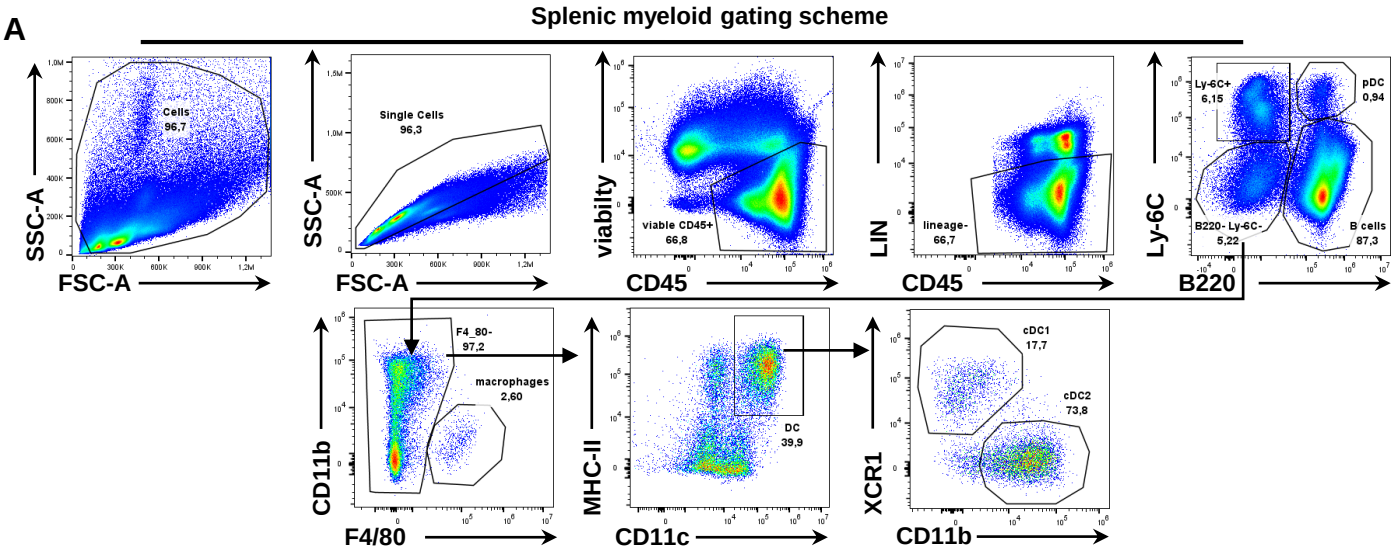
(A) Representative dot-plots of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) NK1.1^{+}NKp46^{+}, CD27^{-}CD11b^{-}, CD27^{+}CD11b^{-}, CD27^{+}CD11b^{+}, mature CD27^{-}CD11b^{+} and terminal mature CD11b^{+}KLRG1^{+} NK cell populations in the blood.

(B) Quantification of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) NK1.1^{+}NKp46^{+}, CD27^{-}CD11b^{-}, CD27^{+}CD11b^{-}, CD27^{+}CD11b^{+}, mature CD27^{-}CD11b^{+} and **(C)** terminal mature CD11b^{+}KLRG1^{+} blood derived NK cell frequencies.

(D) Quantification of the MFI of CD27, CD11b and KLRG1 in wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) splenic NK cells (CD3^{-}CD19^{-}NK1.1^{+}NKp46^{+}).

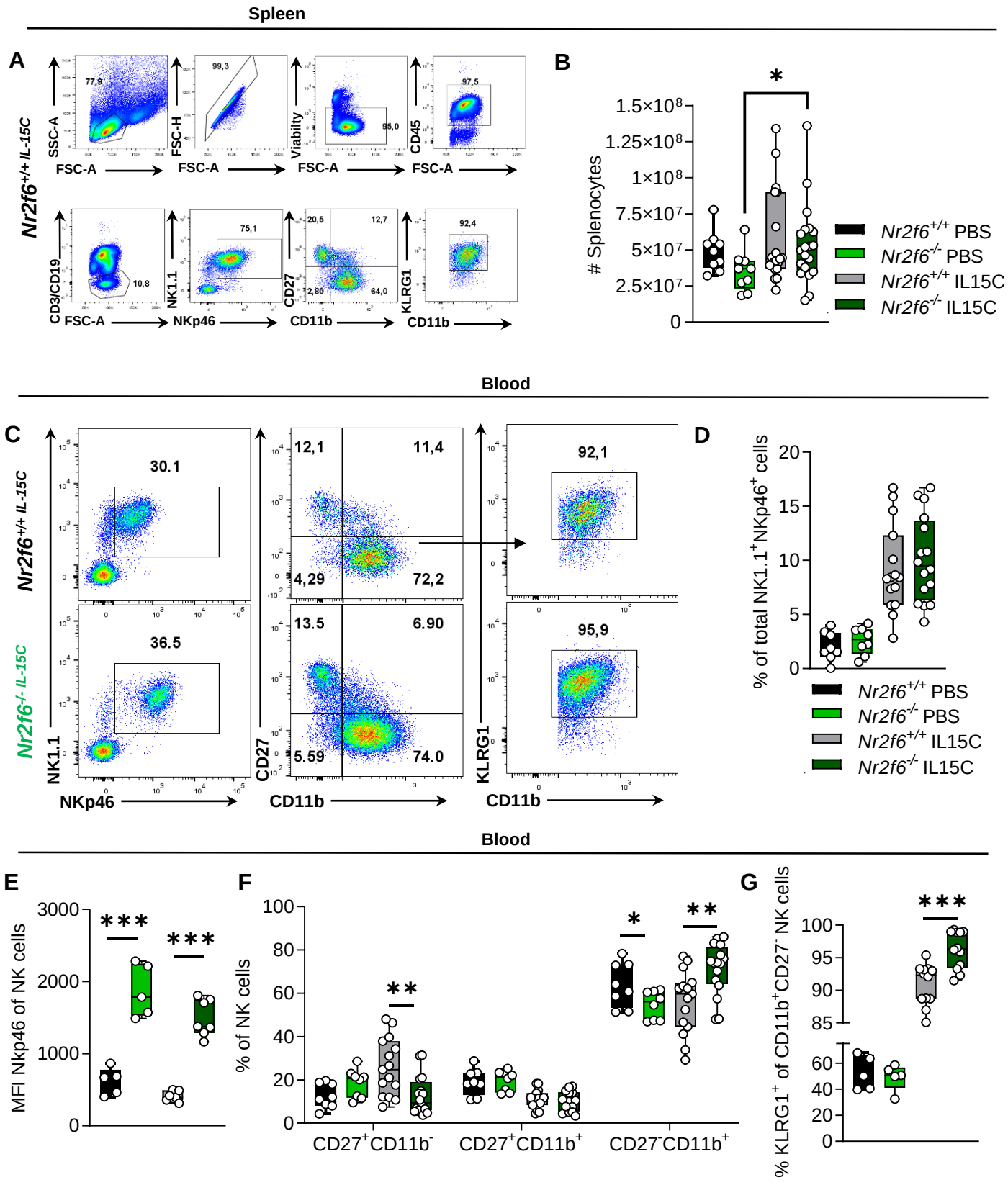
(E) Representative dot-plots of splenic NK cells (CD45^{+}CD3^{-}NK1.1^{+}NKp46^{+}) and IFNγ-producing NK cells in wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice after PBS or LPS injection.

(B, C) Representative data is shown as pooled experiments of at least two independent experiments $n = 11$. (D) Representative data is shown as pooled experiments of at least two independent experiments $n = 10$. Each dot represents the data of an individual mouse. Results are shown as mean ± SD. An asterisk indicates statistically significant differences between genotypes calculated using Student's t test. A p-value < 0.05 was considered statistically significant. ***$p < 0.001$; ****$p < 0.0001$.



Supplementary Figure 4:

(A) Gating strategy of splenic derived myeloid cell populations. Single events were selected, debris was excluded, and only viable cells were included from total splenocytes.


Spleen
A
Nr2f6+/+ IL-15C
SSC-A
FSC-A
77,9
FSC-H
99,3
Viability
95,0
CD45
97,5
CD3/CD19
10,8
NK1.1
75,1
CD27
20,5
12,7
2,80
64,0
KLRG1
92,4
NKp46
CD11b
B
Splenocytes
1.5×10^8
1.25×10^8
1×10^8
7.5×10^7
5×10^7
2.5×10^7
0
*
Nr2f6+/+ PBS
Nr2f6-/- PBS
Nr2f6+/+ IL15C
Nr2f6-/- IL15C
Blood
C
Nr2f6+/+ IL-15C
Nr2f6-/- IL-15C
30.1
36.5
12,1
11,4
4,29
72,2
13.5
6.90
5.59
74.0
92,1
95,9
NK1.1
NKp46
CD27
CD11b
KLRG1
D
% of total NK1.1+NKp46+ cells
20
15
10
5
0
Nr2f6+/+ PBS
Nr2f6-/- PBS
Nr2f6+/+ IL15C
Nr2f6-/- IL15C
Blood
E
MFI Nkp46 of NK cells
3000
2000
1000
0
***
***
F
% of NK cells
100
80
60
40
20
0
**
*
**
CD27+CD11b-
CD27+CD11b+
CD27-CD11b+
G
% KLRG1+ of CD11b+CD27- NK cells
100
95
90
85
60
40
20
0
***

Supplementary Figure 5: IL-15C treatment of *Nr2f6*-deficient mice rescues NK maturation and effector responses

(A) Gating strategy of NK cell maturation following IL-15C treatment in the spleen or blood of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice.

(B) Quantification of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) splenocyte numbers after PBS or 3 x IL-15/IL-15Rα-complex treatment on day 9.

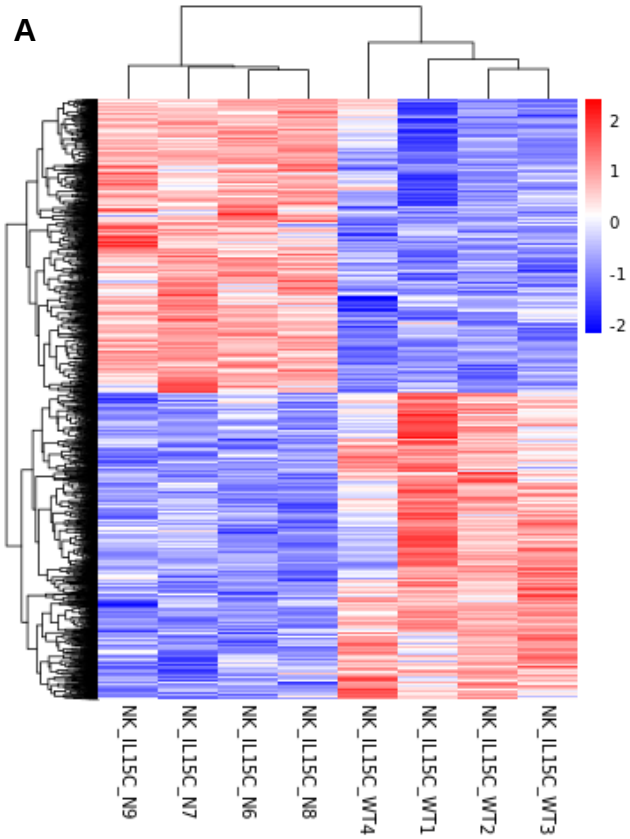
(C) Representative dot-plots of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) NK1.1^{+}NKp46^{+}, CD27^{-}CD11b^{-}, CD27^{+}CD11b^{-}, CD27^{+}CD11b^{+}, mature CD27^{-}CD11b^{+} and terminal mature CD11b^{+}KLRG1^{+} NK cell populations in the blood following 3 x IL-15/IL-15Rα-complex or PBS treatment (out of CD45^{+}CD3^{-}CD19^{-}) on day 9.

(D) Quantification of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) NK1.1^{+}NKp46^{+}, blood derived NK cell frequencies following 3 x IL-15/IL-15Rα-complex or PBS treatment on day 9.

(E) Quantification of the MFI of NKp46 out of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) NK1.1^{+}NKp46^{+} NK cells in the blood either PBS or IL-15C treated.

(F) Quantification of blood derived CD27^{+}CD11b^{-}, CD27^{+}CD11b^{+} , mature CD27^{-}CD11b^{+} and **(G)** terminal mature CD11b^{+}KLRG1^{+} NK cell frequencies of wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) following 3 x IL-15/IL-15Rα-complex or PBS treatment (out of CD45^{+}CD3^{-}CD19^{-}) on day 9.

(B) Representative data are shown as pooled experiments of five independent experiments $n = 9$ (PBS treated wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice) and n=19 (IL15C treated wild-type (*Nr2f6*$^{+/+}$) mice) or n=20 (IL-15C treated *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice). (D, F, G) Representative data are shown as pooled experiments of four independent experiments $n = 8$ (PBS treated wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice) and n=15 (IL15C treated wild-type (*Nr2f6*$^{+/+}$) mice) or n=16 (IL-15C treated *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice). (E) The representative data shown are from two pooled experiments out of five replicative experiments, with n =2-7 per group and experiment. $n = 5$ (PBS treated wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice) and n=7 (IL15C treated wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice). Each dot represents the data of an individual mouse. Results are shown as mean ± SD. An asterisk indicates statistically significant differences between genotypes calculated using Student's t-test, or Mann-Whitney *U* test. A p-value < 0.05 was considered statistically significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



Supplementary Figure 6: Characterization of *Nr2f6*-deficient NK cells following IL-15C treatment via RNA-Seq analysis.

(A) Heatmap of differentially expressed genes (DEG) in splenic NK cells from IL-15C treated wild-type (*Nr2f6*$^{+/+}$) or *Nr2f6*-deficient (*Nr2f6*$^{-/-}$) mice. All genes were z-score normalized, and DEGs were defined by DESeq2 (adjusted p-value (padj) < 0.05) (n = 4 per genotype).

Table 1: Antibody list for flow cytometry and sorting

AB	Fluorochrome	Clone	Company	Order Number
B220	Biotin	RA3-6B2	BioLegend	103204
CCR5	PE	HM-CCR5	BioLegend	107005
CD117	APC-Cy7	2B8	BioLegend	105825
CD11b	BV510	M1/70	BioLegend	101245
CD11b	PerCP-Cy5.5	M1/70	BioLegend	101229
CD11b	BV605	M1/70	BioLegend	101257
CD11c	Biotin	N418	BioLegend	117304
CD11c	BV510	N418	BioLegend	117353
CD122	APC-Cy7	TM-b1	BioLegend	123221
CD127 (IL-7Ra)	PE	SB/199	BioLegend	121111
CD127 (IL-7Ra)	PE-Cy7	A7R34	BioLegend	135014
CD135 (Flk2, Flt3)	APC	A2F10	BioLegend	135310
CD19	Biotin	6D5	BioLegend	115503
CD19	Pacific Blue	1D3	BioLegend	115523
CD19	FITC	1D3	BioLegend	152403
CD226 (DNAM-1)	PE-Cy7	10E5	BioLegend	128811
CD27	Pacific Blue	LG.3A10	BioLegend	124217
CD27	FITC	LG3AC	eBiosciences	11-0271-82
CD3	Pacific Blue	17A2	BioLegend	100214
CD3	PerCP-Cy5.5	2C11	BioLegend	100327
CD3e	Biotin	145-2C11	BioLegend	100304
CD45	V500	30-F11	BD	561487
CD45	AF700	30-F11	BioLegend	103127
CD49b	PB	DX5	BD	563063
F4/80	PE/Dazzle 594	BM8	BioLegend	123145
GR1 (Ly6G Ly6C)	Biotin	RB6-8C5	BioLegend	108404
IL15 RA	AF647/APC	6B4C88	BioLegend	153505
KLRG1	PE-Cy7	2F1/KLRG1	BioLegend	138415
Langerin	PE	4C7	BioLegend	144203
Ly-6C	BV421	HK1.4	BioLegend	128032
MHC-II (I-A/I-E)	PE-Cy7	M5/114	BioLegend	107629
NK1.1	APC	PK136	BioLegend	108709
NK1.1	PerCP	PK136	BioLegend	108725
NK1.1	PerCP-Cy5.5	PK136	BioLegend	108727
Nkp46 (CD335)	APC	29A1.4	BioLegend	137607
Nkp46 (CD335)	BV605	29A1.5	BioLegend	137619
Nkp46 (CD335)	PE	29A1.4	BioLegend	137603
Sca-1	PE	D7	BioLegend	108107
Ter119	Biotin	Ter-119	BioLegend	116204
XCR1	BV650	ZET	BioLegend	148220
Fixable Viability Stain	575V		BD	565694
Fixable Viability Stain	780		BD	565388
Granzyme B	PE	NGZB	eBioscience	12-8898-80
IFNγ	PE-Cy7	XMG1.2	Biolegend	505825
Ki-67	PE	16A8	Biolegend	652404
Perforin	APC	S16009A	Biolegend	154303
TNFα	BV510	MP6-XT22	Biolegend	560659

Supplementary Figure 7: Table 1: Antibody list for flow cytometry and sorting