Immunity, Volume *57*

Supplemental information

Circulating NK cells establish tissue residency upon acute infection of skin and mediate accelerated effector responses to secondary infection

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Figure S1

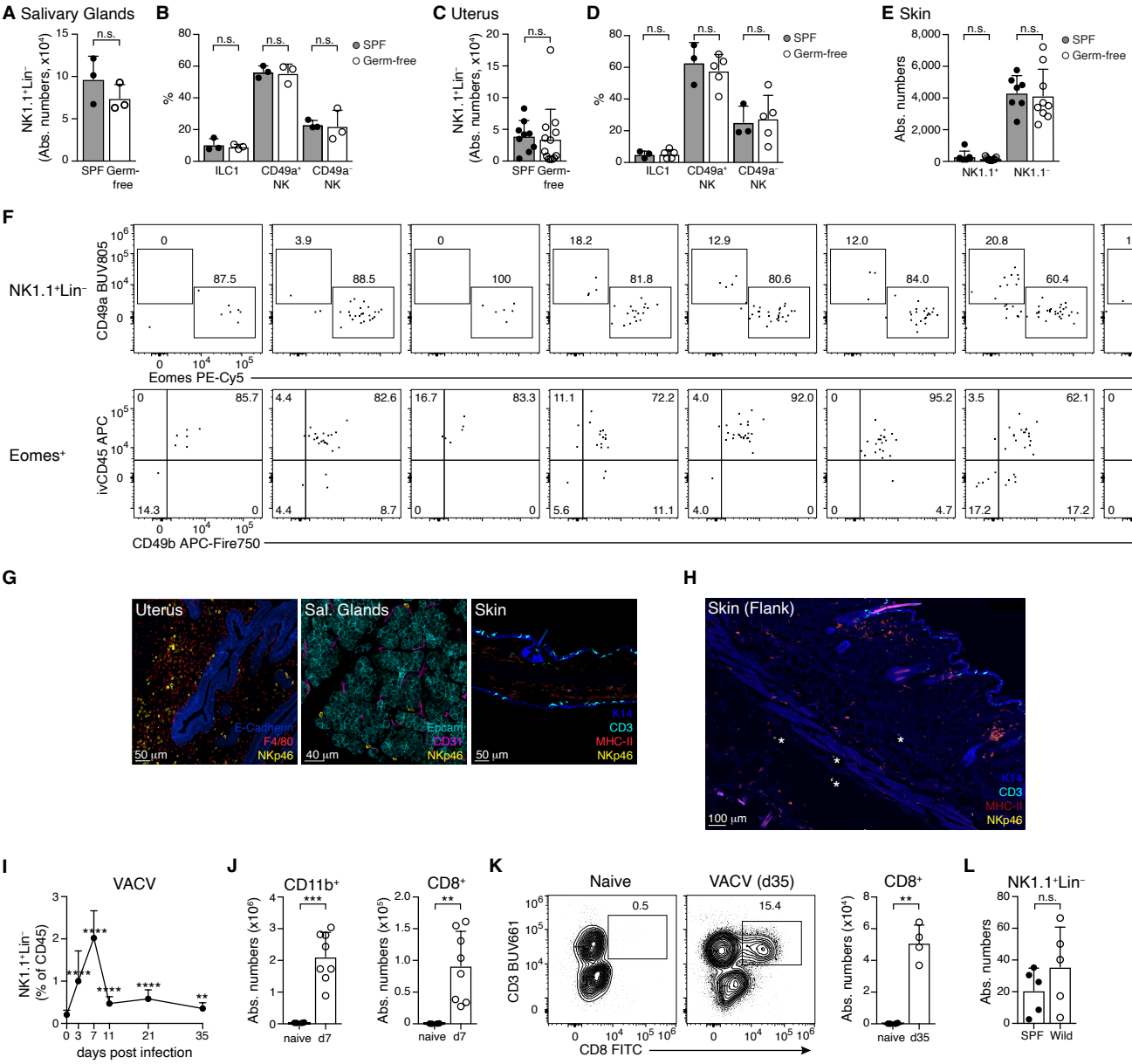
A Salivary Glands
B
C Uterus
D
E Skin
SPF
Germ-free
n.s.
NK1.1⁺Lin⁻ (Abs. numbers, x10⁴)
%
Abs. numbers
ILC1
CD49a⁺ NK
CD49a⁻ NK
NK1.1⁺
NK1.1⁻
F
NK1.1⁺Lin⁻
Eomes⁺
CD49a BUV805
Eomes PE-Cy5
ivCD45 APC
CD49b APC-Fire750
0
87.5
3.9
88.5
100
18.2
81.8
12.9
80.6
12.0
84.0
20.8
60.4
10.5
78.9
85.7
14.3
4.4
82.6
8.7
16.7
83.3
11.1
72.2
5.6
11.1
4.0
92.0
95.2
4.7
3.5
62.1
17.2
93.3
6.7
G
Uterus
E-Cadherin
F4/80
NKp46
50 μm
Sal. Glands
Epcam
CD31
40 μm
Skin
K14
CD3
MHC-II
H
Skin (Flank)
100 μm
I
VACV
NK1.1⁺Lin⁻ (% of CD45)
days post infection
J
CD11b⁺
CD8⁺
naive
d7
K
Naive
VACV (d35)
CD3 BUV661
CD8 FITC
d35
L
NK1.1⁺Lin⁻
SPF
Wild

Figure S1: **Immune populations in naïve and VACV-infected skin. Related to Figure 1.** (A-E) Numbers of $Lin^{-}NK1.1^{+}$ cells (A, C) or $Lin^{-}CD90^{+}CD127^{+}Il18ra^{+}$ ILC subsets (E) and frequency of $Eomes^{-}$ ILC1 and $Eomes^{+}$ NK subsets out of $Lin^{-}NK1.1^{+}$ cells (B and D) in indicated tissues of mice housed in SPF or germfree conditions. (F) Representative FACS plots show $Lin^{-}NK1.1^{+}$ (top row) and $Eomes^{+}$ NK cells (bottom row) in naïve ears. Individual ears are shown. (G, H) Confocal immunofluorescence images of skin cryosections from indicated organs of naïve mice. (I) Frequency of $Lin^{-}NK1.1^{+}$ cells in ear skin of mice infected with VACV at the indicated times post infection. (J, K) Numbers and representative gating of $CD11b^{+}$ cells and $CD8^{+}$ T cells in naïve versus VACV-infected ear skin at indicated times post infection. (L) Absolute numbers of $Lin^{-}NK1.1^{+}$ cells in the ear skin of naïve mice housed in SPF or wildling conditions. Data are representative of three independent experiments with n=3 mice (A, B, D, G, H) or n=4-8 ears (F, J-L) per group. Data in (C, E, I) were pooled from two independent experiments with n=9-13 mice (C) or n=7-23 ears (E, I) per group. Error bars indicate mean ± SD. p values were calculated by unpaired Student's t test. **p<0.01, ***p<0.001, ****p<0.0001, n.s. not significant.

Figure S2

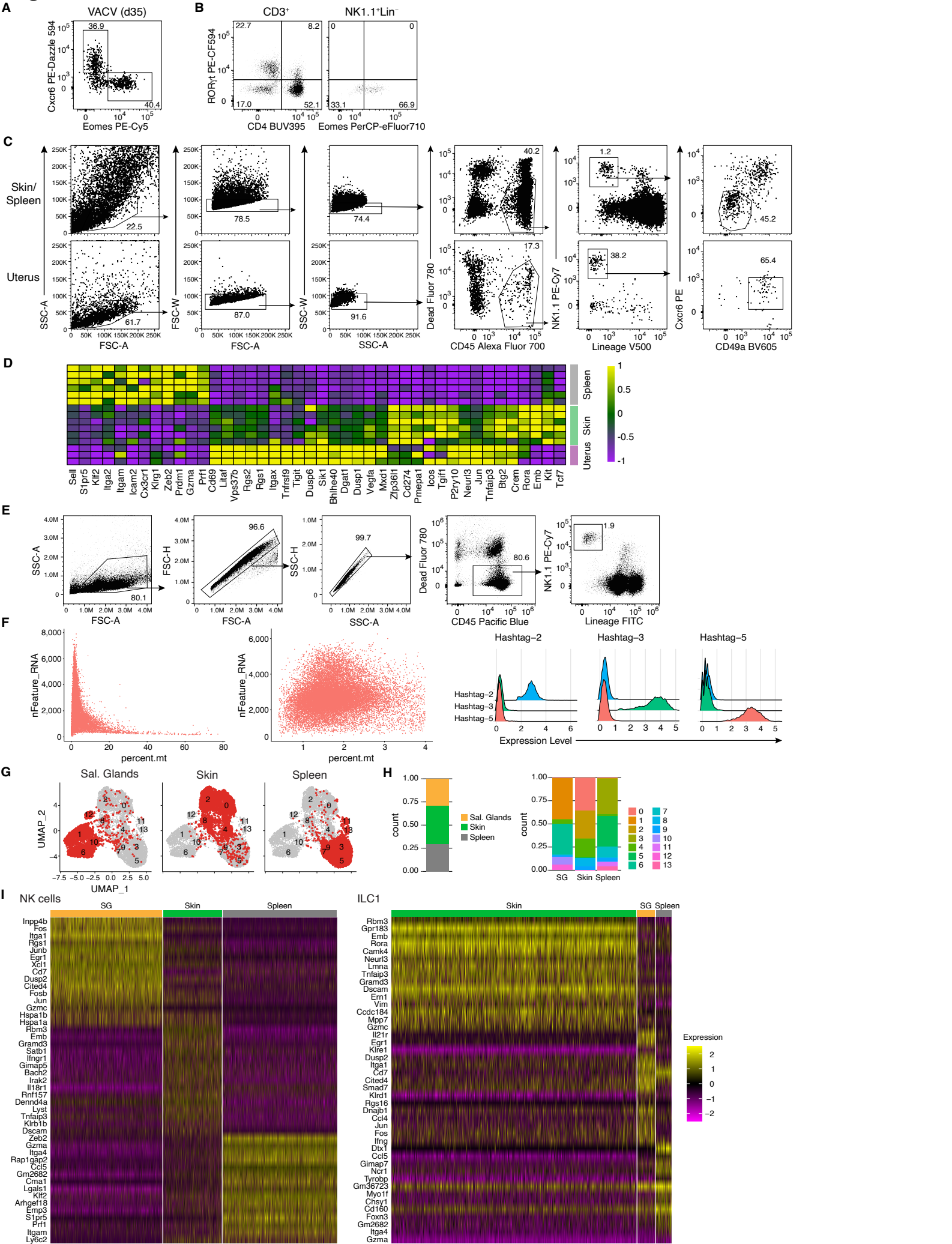
A
VACV (d35)
Cxcr6 PE-Dazzle 594
Eomes PE-Cy5
36.9
40.4
B
CD3+
NK1.1+Lin−
RORγt PE-CF594
CD4 BUV395
Eomes PerCP-eFluor710
22.7
8.2
17.0
52.1
33.1
66.9
C
Skin/ Spleen
Uterus
SSC-A
FSC-A
FSC-W
SSC-W
Dead Fluor 780
CD45 Alexa Fluor 700
NK1.1 PE-Cy7
Lineage V500
Cxcr6 PE
CD49a BV605
22.5
78.5
74.4
40.2
1.2
45.2
61.7
87.0
91.6
17.3
38.2
65.4
D
Spleen
Skin
Uterus
Sell
S1pr5
Klf2
Itga2
Itgam
Icam2
Cx3cr1
Klrg1
Zeb2
Prdm1
Gzma
Prf1
Cd69
Litaf
Vps37b
Rgs2
Rgs1
Itgax
Tnfrsf9
Tigit
Dusp6
Sik1
Bhlhe40
Dgat1
Dusp1
Vegfa
Mxd1
Zfp36l1
Cd274
Pmepa1
Icos
Tgif1
P2ry10
Neurl3
Jun
Tnfaip3
Btg2
Crem
Rora
Emb
Kit
Tcf7
E
SSC-A
FSC-A
FSC-H
SSC-H
Dead Fluor 780
CD45 Pacific Blue
NK1.1 PE-Cy7
Lineage FITC
80.1
96.6
99.7
80.6
1.9
F
nFeature_RNA
percent.mt
Hashtag−2
Hashtag−3
Hashtag−5
Expression Level
G
Sal. Glands
Skin
Spleen
UMAP_2
UMAP_1
H
count
Sal. Glands
Skin
Spleen
SG
Skin
Spleen
I
NK cells
SG
Skin
Spleen
Inpp4b
Fos
Itga1
Rgs1
Junb
Egr1
Xcl1
Cd7
Dusp2
Cited4
Fosb
Jun
Gzmc
Hspa1b
Hspa1a
Rbm3
Emb
Gramd3
Satb1
Ifngr1
Gimap5
Bach2
Irak2
Il18r1
Rnf157
Dennd4a
Lyst
Tnfaip3
Klrb1b
Dscam
Zeb2
Gzma
Itga4
Rap1gap2
Ccl5
Gm2682
Cma1
Lgals1
Klf2
Arhgef18
Emp3
S1pr5
Prf1
Itgam
Ly6c2
ILC1
Skin
SG
Spleen
Rbm3
Gpr183
Emb
Rora
Camk4
Neurl3
Lmna
Tnfaip3
Gramd3
Dscam
Ern1
Vim
Ccdc184
Mpp7
Gzmc
Il21r
Egr1
Klre1
Dusp2
Itga1
Cd7
Cited4
Smad7
Klrd1
Rgs16
Dnajb1
Ccl4
Jun
Fos
Ifng
Dtx1
Ccl5
Gimap7
Ncr1
Tyrobp
Gm36723
Myo1f
Chsy1
Cd160
Foxn3
Gm2682
Itga4
Gzma
Expression

Figure S2: mRNA sequencing reveals unique and tissue-specific NK and ILC1 gene signatures. Related to Figure 2.

(A, B) Representative FACS plots of $Lin^{-}NK1.1^{+}$ (A, B right) and $CD3^{+}$ T cells (B left) in ear skin of mice infected with VACV (A) or *S. aureus* (B). (C, E) Full gating strategy for NK cells sorted for bulk (C, D) and scRNA-seq analysis (E-I). (D) Heat map depicting z-score of significantly DEG in the indicated tissues. (F-I) scRNA-seq of hashtagged skin, spleen and SG $Lin^{-}NK1.1^{+}$ cells sorted from VACV-infected mice on d25 pi. (F) General QC, filtering of low-quality cells (feature counts > 1000 and percentage of genes mapped to mitochondrial genome < 8) and doublets, and demultiplexing of CD45 and Hashtag (HT) antibodies to identify tissue of origin. Single cell transcriptome visualization using a UMAP color-coded by hashtag (tissue origin) (G) and bar graph show the proportion of cells per tissue and cluster (H). (I) Heat map of the top 15 most DEG across NK cells (clusters 1, 3, 5, 6, 7, 9, 10, 13, left) and ILC1 (0, 2, 11, 12) in the indicated tissues. Data in (A, B) are representative of three independent experiments with n=4 ears per group.

Figure S3

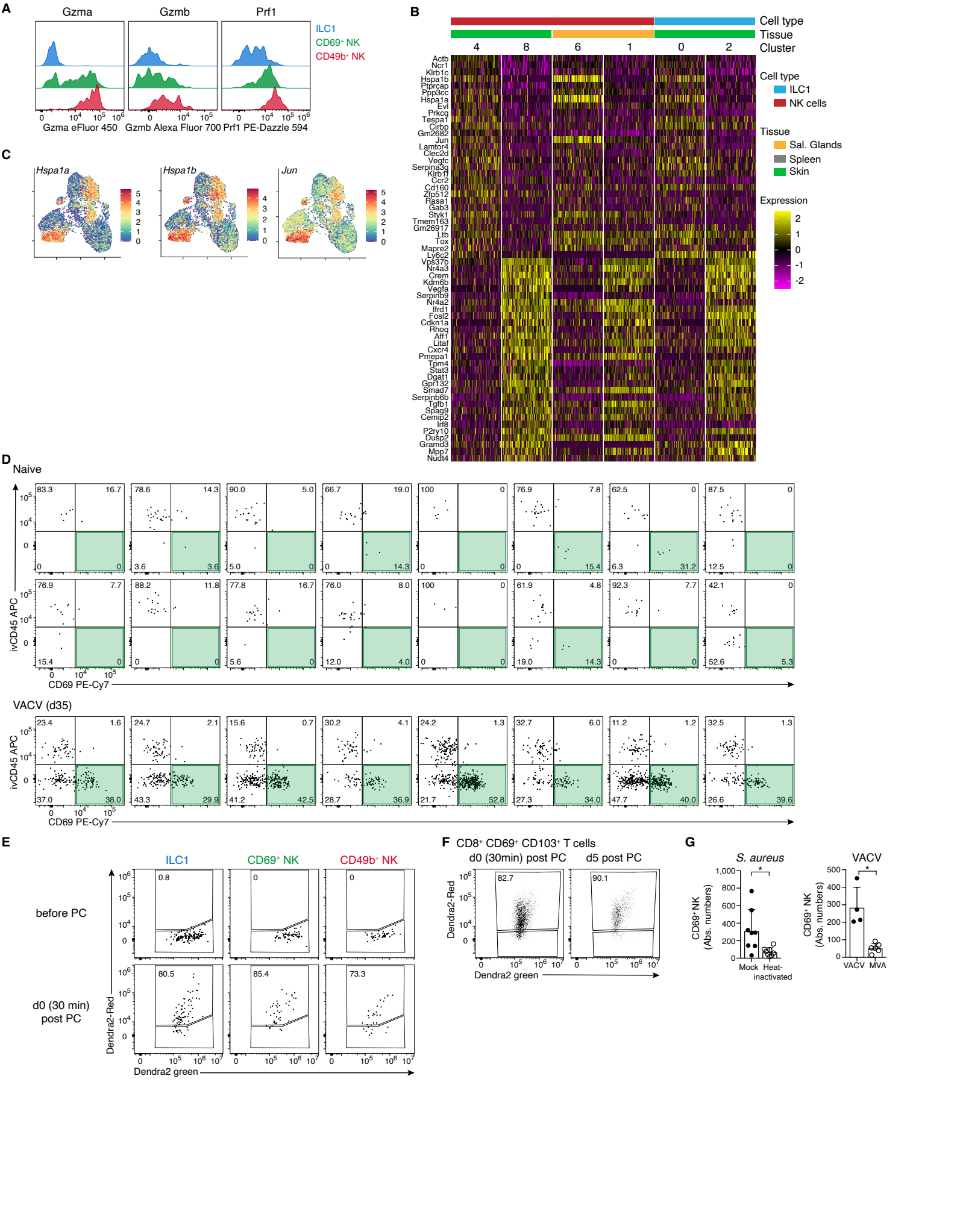
A
Gzma
Gzmb
Prf1
ILC1
CD69+ NK
CD49b+ NK
Gzma eFluor 450
Gzmb Alexa Fluor 700
Prf1 PE-Dazzle 594
B
Cell type
Tissue
Cluster
4 8 6 1 0 2
Actb Ncr1 Klrb1c Hspa1b Ptprcap Ppp3cc Hspa1a Evl Prkcq Tespa1 Cirbp Gm2682 Jun Lamtor4 Clec2d Vegfc Serpina3g Klrb1f Ccr2 Cd160 Zfp512 Rasa1 Gab3 Styk1 Tmem163 Gm26917 Ltb Tox Mapre2 Ly6c2 Vps37b Nr4a3 Crem Kdm6b Vegfa Serpinb9 Nr4a2 Ifrd1 Fosl2 Cdkn1a Rhoq Aff1 Litaf Cxcr4 Pmepa1 Tpm4 Stat3 Dgat1 Gpr132 Smad7 Serpinb6b Tgfb1 Spag9 Cemip2 Irf8 P2ry10 Dusp2 Gramd3 Mpg7 Nudt4
Cell type
ILC1
NK cells
Tissue
Sal. Glands
Spleen
Skin
Expression
C
Hspa1a
Hspa1b
Jun
D
Naive
VACV (d35)
ivCD45 APC
CD69 PE-Cy7
E
ILC1
CD69+ NK
CD49b+ NK
before PC
d0 (30 min) post PC
Dendra2-Red
Dendra2 green
F
CD8+ CD69+ CD103+ T cells
d0 (30min) post PC
d5 post PC
Dendra2-Red
Dendra2 green
G
S. aureus
CD69+ NK (Abs. numbers)
Mock
Heat-inactivated
VACV
CD69+ NK (Abs. numbers)
VACV
MVA

Figure S3: Skin NK cells from naïve and infected mice. Related to Figure 3.

(A) Representative histograms show marker protein expression by indicated skin NK subsets 4 weeks post VACV infection. (B) Heat map showing gene expression of top 30 DEG between cluster 4 and 8 for all clusters. (C) UMAP visualization of selected gene expression, correlating with subclustering of trNK (cl4+8, cl1+6) and ILC1 (cl0+2). (D) FACS plots show frequency of $Eomes^+$ NK subsets from individual naïve (top) and VACV-infected (bottom) ear skins after intravascular labelling with anti-CD45 (ivCD45). (E, F) Representative FACS plots show $Dendra2^{Red+}$ fraction before and 30 mins (d0) or 5 days post photoconversion (PC) among indicated subtypes of $Lin^-NK1.1^+$ cells (E), or $CD8^+CD69^+CD103^+$ tissue-resident T cells (F). (G) Absolute numbers of ear skin $CD69^+$ NK cells 4 weeks post *S. aureus*, heat-inactivated *S. aureus*, VACV-wt or non-replicating VACV (MVA) infection. Data are representative of three (A, D, G) or two (B, C, E, F) independent experiments with n=4-16 ears per group. Error bars indicate mean ± SD. p values were calculated by unpaired Student's t test. *$p<0.05$.

Figure S4

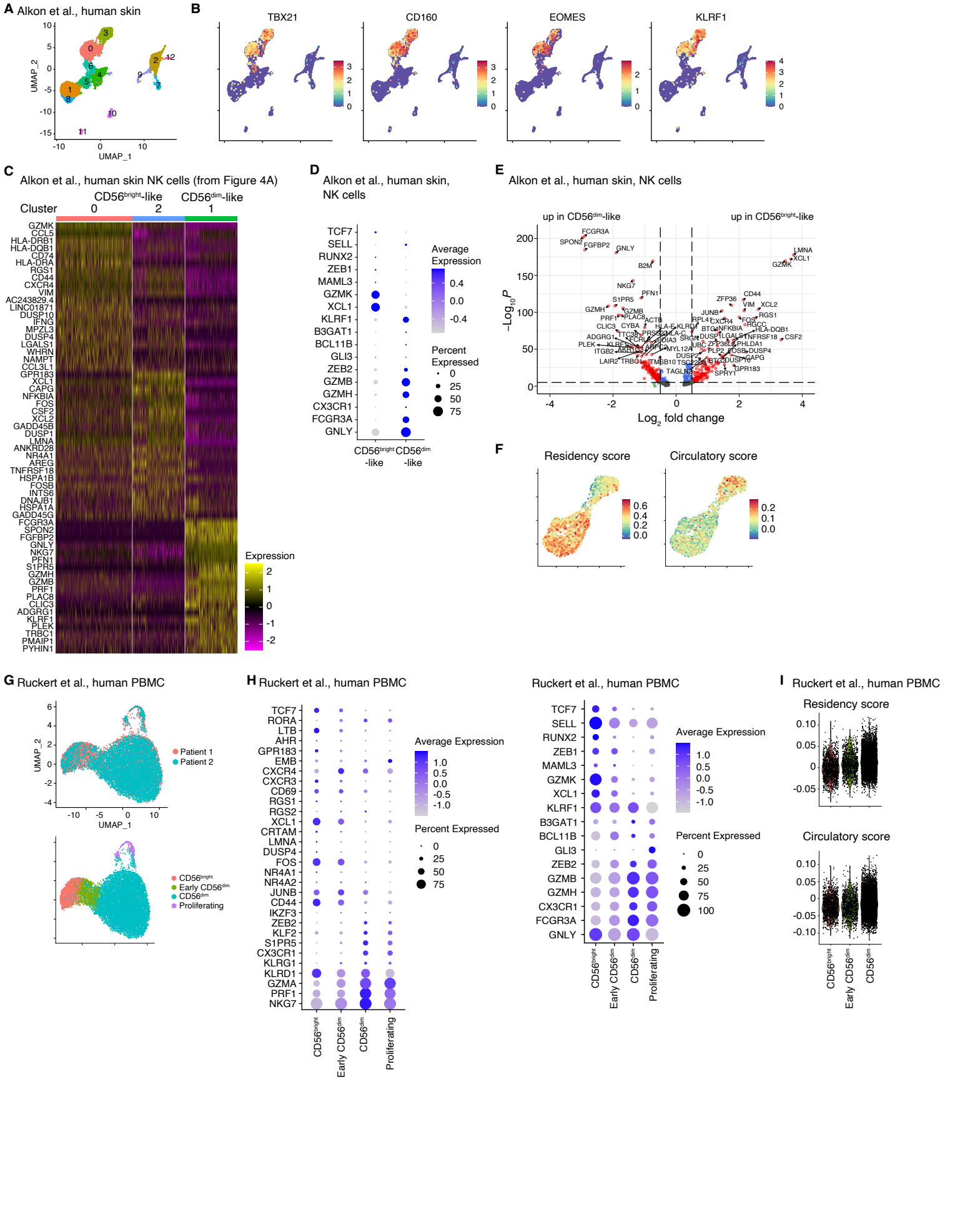
A Alkon et al., human skin
UMAP_2
UMAP_1
B
TBX21
CD160
EOMES
KLRF1
C
Alkon et al., human skin NK cells (from Figure 4A)
CD56bright-like
CD56dim-like
Cluster 0 2 1
Expression
D
Alkon et al., human skin, NK cells
Average Expression
Percent Expressed
CD56bright -like
CD56dim -like
E
Alkon et al., human skin, NK cells
up in CD56dim-like
up in CD56bright-like
−Log10P
Log2 fold change
F
Residency score
Circulatory score
G Ruckert et al., human PBMC
Patient 1
Patient 2
CD56bright
Early CD56dim
CD56dim
Proliferating
H Ruckert et al., human PBMC
Average Expression
Percent Expressed
I Ruckert et al., human PBMC
Residency score
Circulatory score

Figure S4: Gene expression analysis of human NK cells from skin and peripheral blood. Related to Figure 4.

Analysis of single-cell transcriptome of healthy human skin data generated by Alkon et al.[1] (A-F), and human PBMC data generated by Ruckert et al.[2] (G-I). (A and B) UMAP visualization delineating NK clusters, identified based on expression of TBX21, EOMES, CD160 and KLRF1. (C-E) Analysis of human NK cells re-clustered from cluster 0 and 3 as in Figure 4. Heatmap (C) and volcano plot (E) show DEG across indicated NK clusters. (D, H) Bubble plot representation of z-score of selected marker genes of indicated NK clusters. (F) UMAP of a score based on genes associated to tissue-resident memory of T cells. (G) UMAP visualization based on origin or on identified clusters. (I) Violin plot visualization of residency and circulating scores, based on genes associated to tissue-resident memory of T cells as in Figure 4E, in the indicated subsets.

Figure S5

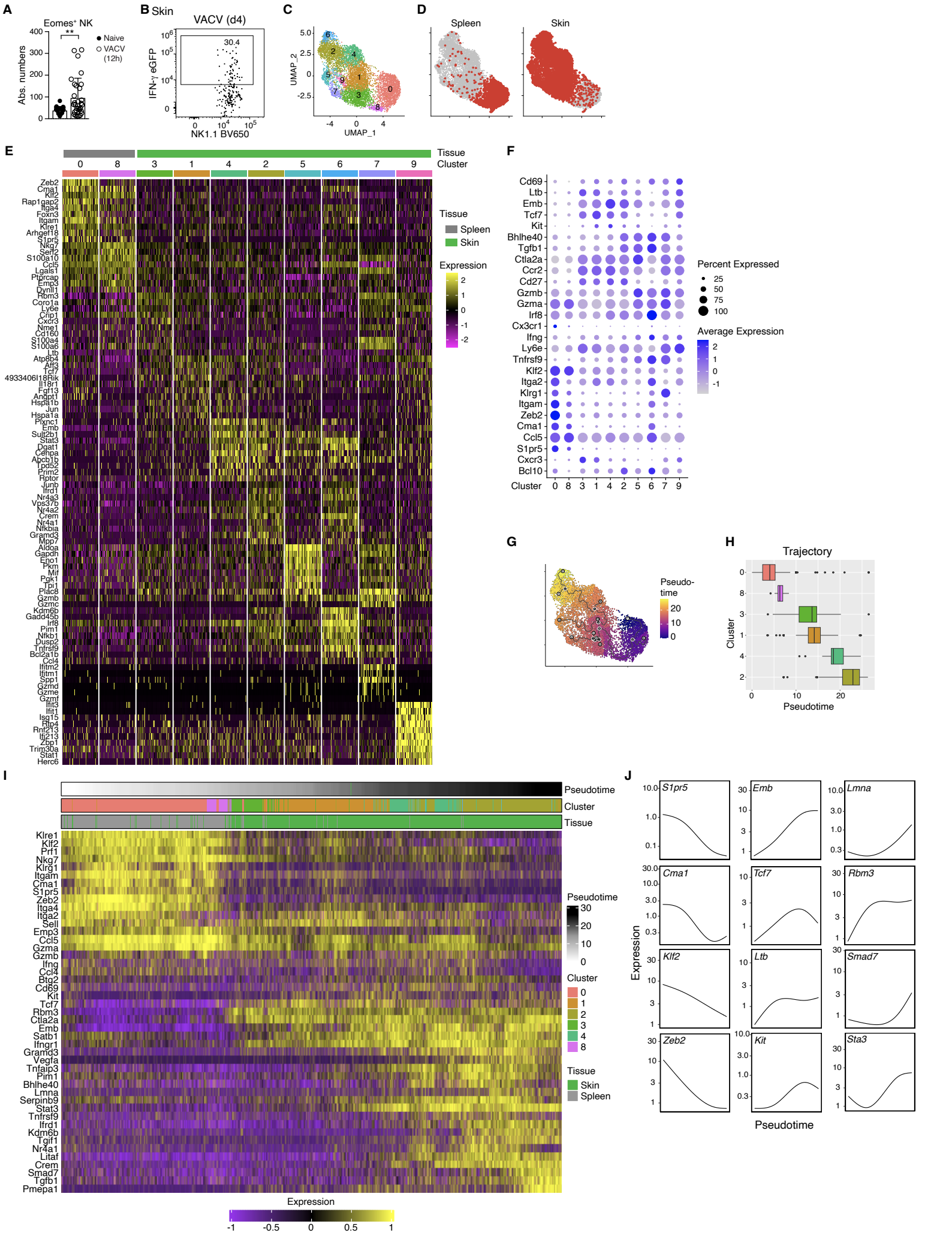
A
Eomes+ NK
Abs. numbers
Naive
VACV (12h)
B Skin
VACV (d4)
30.4
IFN-γ eGFP
NK1.1 BV650
C
UMAP_2
UMAP_1
D
Spleen
Skin
E
Tissue
Cluster
Spleen
Skin
Expression
F
Percent Expressed
Average Expression
Cluster
G
Pseudo-time
H
Trajectory
Cluster
Pseudotime
I
Pseudotime
Cluster
Tissue
Expression
J
S1pr5
Emb
Lmna
Cma1
Tcf7
Rbm3
Klf2
Ltb
Smad7
Zeb2
Kit
Sta3
Expression
Pseudotime

Figure S5: Rapid recruitment and activation of NK cells VACV infected skin. Related to Figure 5.

(A) Absolute numbers of Eomes^{+} NK cells from naïve ear skins or 12h post VACV-wt infection. (B) Representative FACS plot showing IFN-γ(eGFP)-expressing NK cells in ear skin on d4 post VACV infection. (C-F) Single cell mRNA-Seq analysis of spleen and skin NK cells sorted on d8 post VACV infection. (C, D) UMAP visualisation of clusters (C) and tissue origin (D). (E) Heatmap showing top 10 most DEG across clusters. (F) Bubble plot representation of z-score of selected marker genes between NK cell clusters. (G, H) Pseudotime and trajectory analysis using Monocle3. (I, J) Expression of selected genes in NK cells along pseudotime. Data in (A) are pooled from three independent experiments with n=18 or n=30 ears per group. Data in (B) are representative of two independent experiments with n=4 ears.

Figure S6

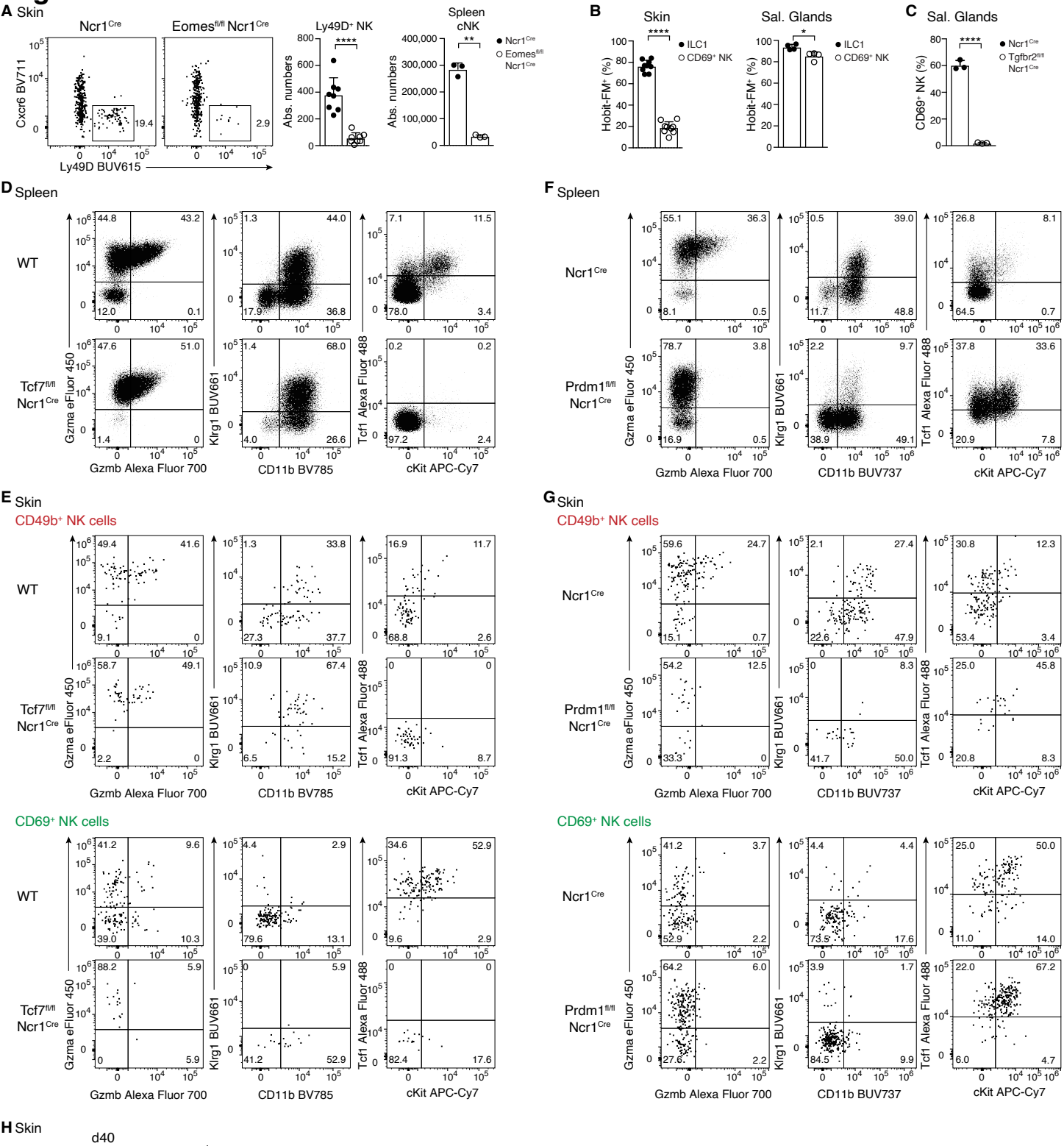
A Skin
Ncr1Cre
Eomesfl/fl Ncr1Cre
Cxcr6 BV711
Ly49D BUV615
19.4
2.9
Ly49D+ NK
Abs. numbers
****
Spleen
cNK
**
Ncr1Cre
Eomesfl/fl Ncr1Cre
B
Skin
Sal. Glands
Hobit-FM+ (%)
****
*
ILC1
CD69+ NK
C
Sal. Glands
CD69+ NK (%)
****
Ncr1Cre
Tgfbr2fl/fl Ncr1Cre
D Spleen
WT
Tcf7fl/fl Ncr1Cre
Gzma eFluor 450
Gzmb Alexa Fluor 700
Klrg1 BUV661
CD11b BV785
Tcf1 Alexa Fluor 488
cKit APC-Cy7
F Spleen
Ncr1Cre
Prdm1fl/fl Ncr1Cre
CD11b BUV737
E Skin
CD49b+ NK cells
CD69+ NK cells
G Skin
CD49b+ NK cells
CD69+ NK cells
H Skin
d40
Eomes Fatemap
Eomes PE-Cy5
% Eomes+ (of FM+)

Figure S6: Eomes, Tcf1 and Blimp1 regulate the differentiation of circulating and skin-resident NK cells. Related to Figure 6.

Analysis of NK cell subsets (A-G) and ILC1 (B) in the indicated organs of $Eomes^{fl/fl}$ $Ncr1^{cre}$ (A), Hobit-FM (B), $Tgfbr2^{fl/fl}$ $Ncr1^{cre}$ (C), $Tcf7^{fl/fl}$ $Ncr1^{cre}$ (D, E) and $Prdm1^{fl/fl}$ $Ncr1^{cre}$ (F, G) and indicated control mice on d30 post VACV infection. Spleen (A) and SG (B, C) were analyzed from naïve mice. (A) Alternative gating strategy for ear skin NK cells in absence of Eomes expression as $Lin^{-}NK1.1^{+}Ly49D^{+}$ cells. Spleen NK cells were gated as $Lin^{-}NK1.1^{+}CD49a^{-}CD127^{-}$ cells. (H) Representative FACS plot showing $Lin^{-}NK1.1^{+}$ of Eomes-FM mice on d40 post infection. Bar graphs shows frequency of $Eomes^{+}$ cells within $Lin^{-}NK1.1^{+}$ FM^{+} cells. Data are representative of two (A, B, D, E, H) or three (C, F, G) independent experiments with n=3-4 mice (A-D, F, H) or n=8-9 ears (A, B, E, G) per group. Error bars indicate mean ± SD. p values were calculated by unpaired Student's t test (A-C). *p < 0.05, ****p< 0.0001.

Figure S7

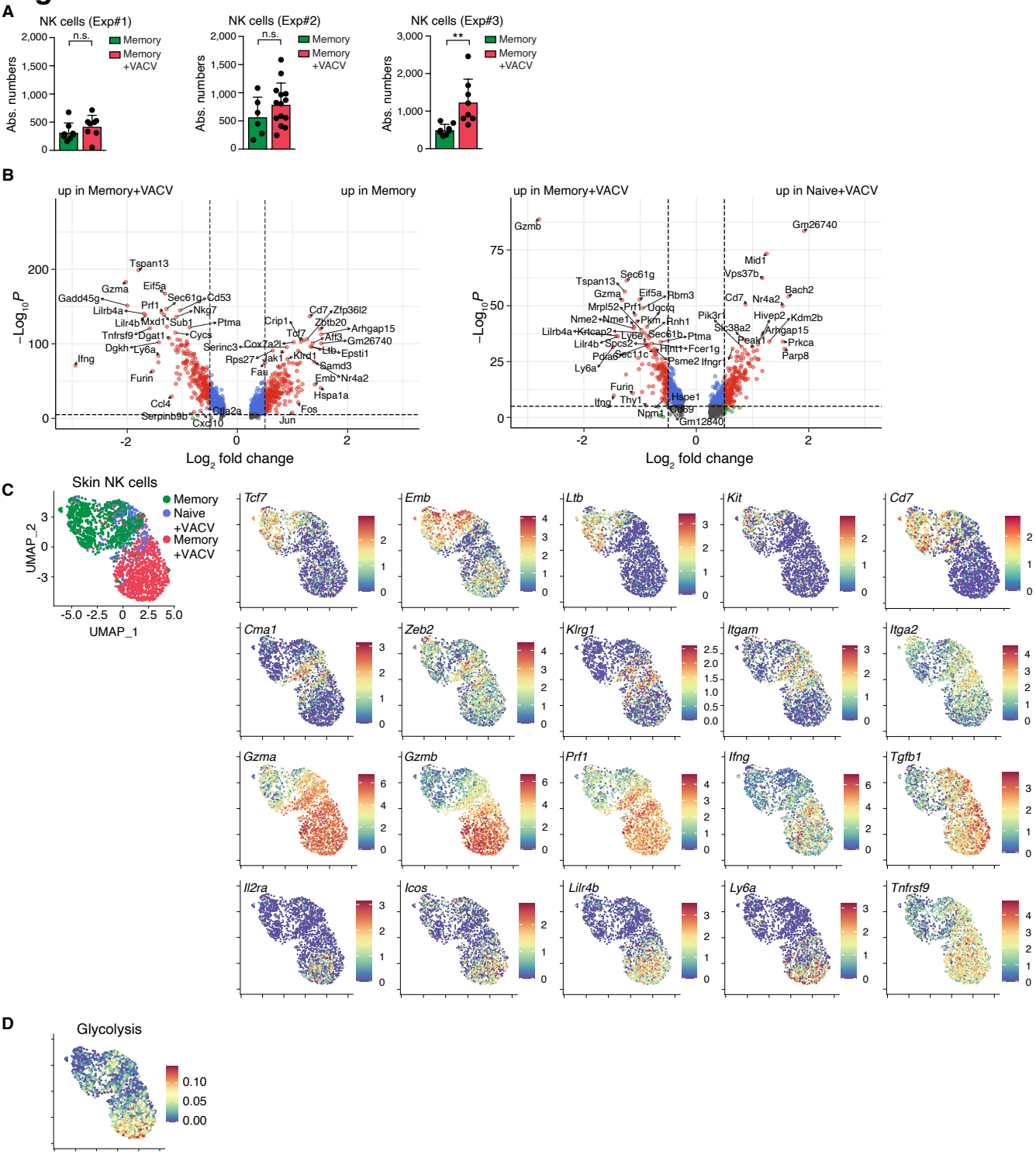
A
NK cells (Exp#1)
NK cells (Exp#2)
NK cells (Exp#3)
Abs. numbers
n.s.
**
Memory
Memory +VACV
B
up in Memory+VACV
up in Memory
up in Naive+VACV
−Log10 P
Log2 fold change
C
Skin NK cells
Memory
Naive +VACV
Memory +VACV
UMAP_1
UMAP_2
Tcf7
Emb
Ltb
Kit
Cd7
Cma1
Zeb2
Klrg1
Itgam
Itga2
Gzma
Gzmb
Prf1
Ifng
Tgfb1
Il2ra
Icos
Lilr4b
Ly6a
Tnfrsf9
D
Glycolysis

Figure S7: Skin-resident NK cells rapidly reprogram into effector cells during secondary infection. Related to Figure 7.

Analysis of ear skin NK cells as in Figure 7A. (A) Absolute numbers of Eomes$^+$ NK cells. Three independent experiments with n=6-14 ears per group are shown. (B) Volcano plot showing DEG. (C, D) Single cell mRNA-Seq analysis as indicated in Figure 7G. UMAP visualization of selected marker gene expression (C) or glycolytic score (D). Error bars indicate mean ± SD. p values were calculated by unpaired Student's t test. **p<0.01, n.s. not significant.