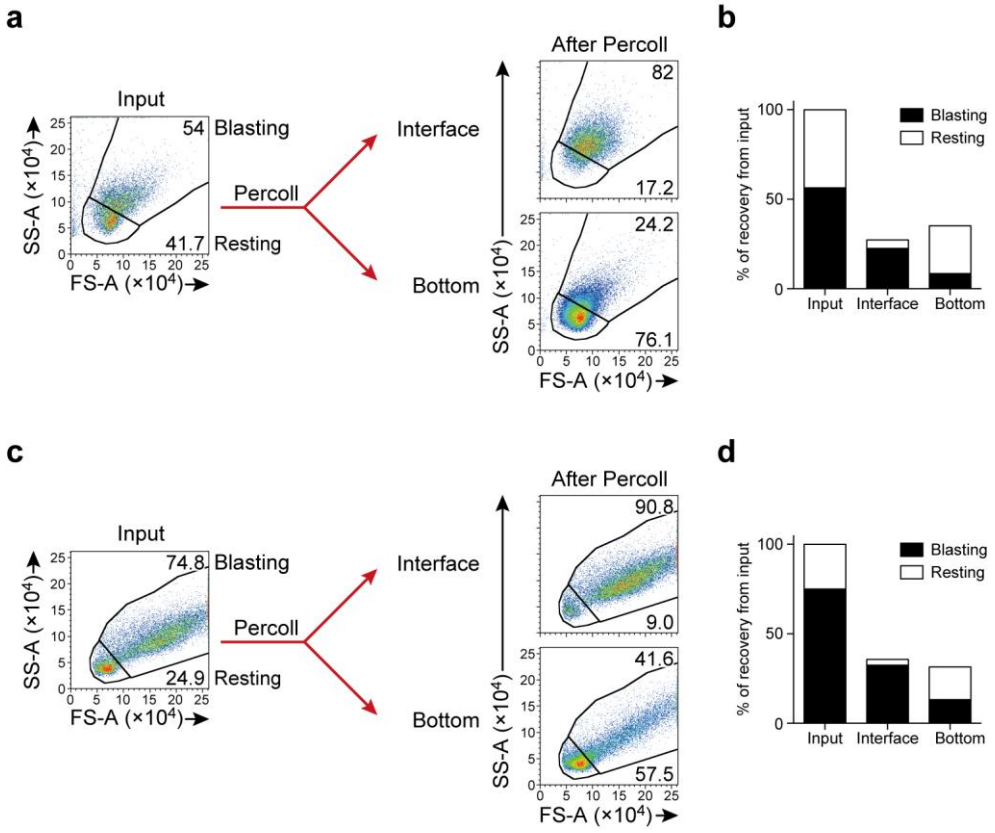
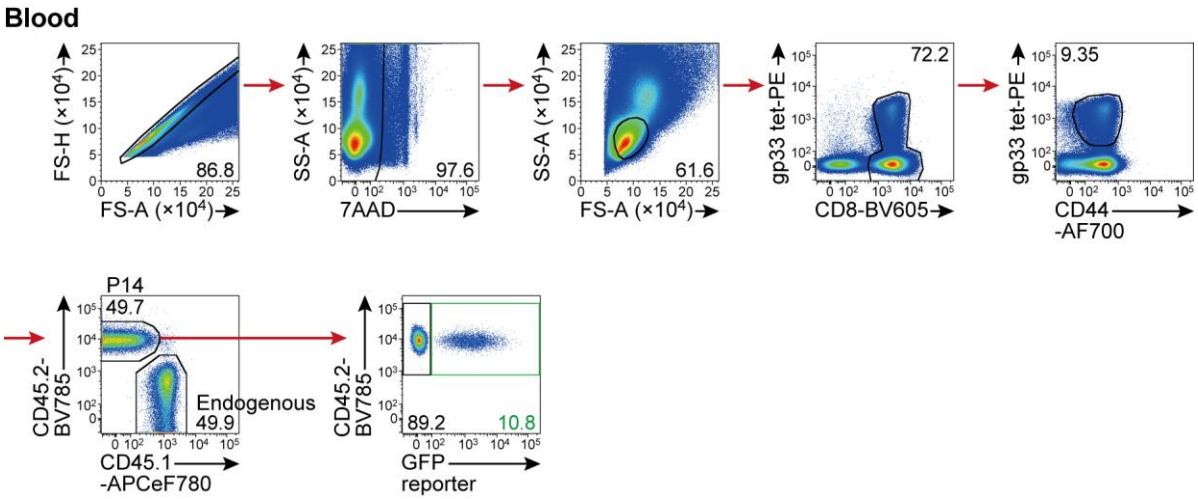


Supplementary Figure 1

Additional two examples of activated CD8$^+$ T cell enrichment with Percoll centrifugation.

(Related to Figure 2b,c)

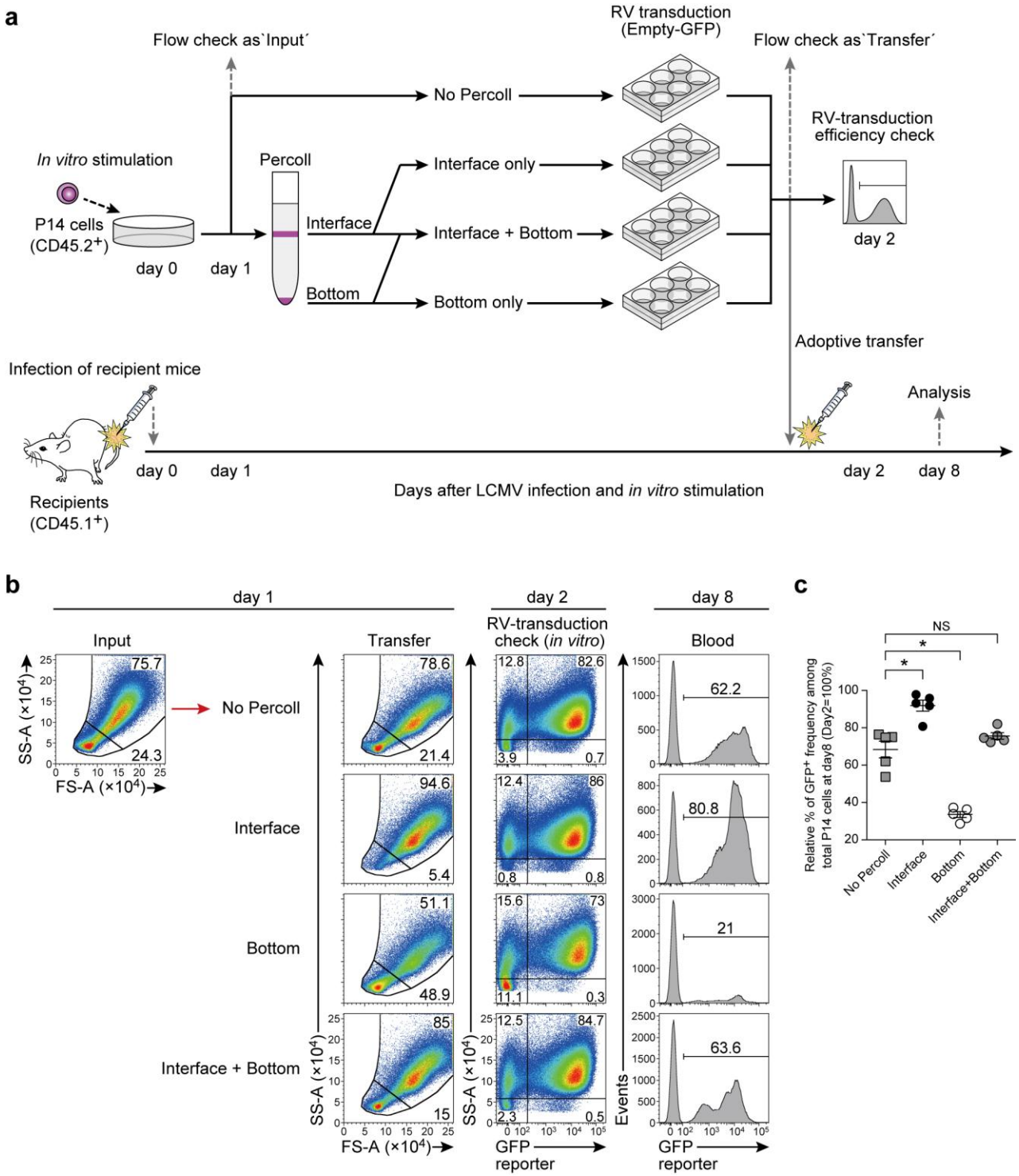
As shown in **Fig.1** and **2b,c**, *in vitro* activated CD8$^+$ T cells were enriched at day 1 using 30% and 60% Percoll centrifugation (**a** and **b**: repeat 1 at 18 hrs after stimulation, **c** and **d**: repeat 2 at 25hrs after stimulation). (**a** and **c**) Flow plots that are gated on the 7AAD negative CD8 positive population show enrichment step from input (left) to shortly after Percoll separation at day 1 (right). (**b** and **d**) Graph showing recovery of small ('resting') and large ('blast') size cells in each interface and bottom after Percoll centrifugation. Note that the recovery of large-sized cells (black bar) was 40% (18 hrs-stimulated; **Supplementary Fig. 1b**), 60% (22 hrs; **Fig. 2c**), and 43.4% (25 hrs; **Supplementary Fig. 1d**).



Supplementary Figure 2

Staining and gating strategy to detect LCMV gp33-specific CD8$^+$ T cells *in vivo* used in this study.

At the indicated time points in each Figure legend, peripheral blood or spleens were harvested and single cell suspensions were prepared using ACK lysis buffer, followed by staining with anti-CD8, CD44, CD45.1, CD45.2 antibodies, and D^{b}gp33 tetramer. In some experiments, to detect only transferred P14 cells, TCR Vα2 was used instead of D^{b}gp33 tetramer. Antigen-specific CD8$^+$ T cells are identified by first gating on singlet cells (FS-A and FS-H), and then on live cells (7AAD negative), and lymphocytes (FS-A and SS-A). Antigen–specific CD8$^+$ T cells were identified by successively gating on CD8, D^{b}gp33 tetramer (or TCR Vα2) and CD44 positive populations. Transferred P14 cells (and endogenous gp33-specific CD8$^+$ T cells in case of D^{b}gp33 tetramer staining) were further subgated on the basis of CD45.1 and CD45.2 congenic marker expression. RV-reporter expression was analyzed on the final P14 cell gate. In selected experiments, differentiation and function of effector and memory P14 cells were evaluated by additional surface marker staining such as CD127 and KLRG1 and standard intracellular cytokine staining after 5 hrs restimulation with cognate peptide. Representative blood sample used in Figure 2d as no Percoll condition is shown.

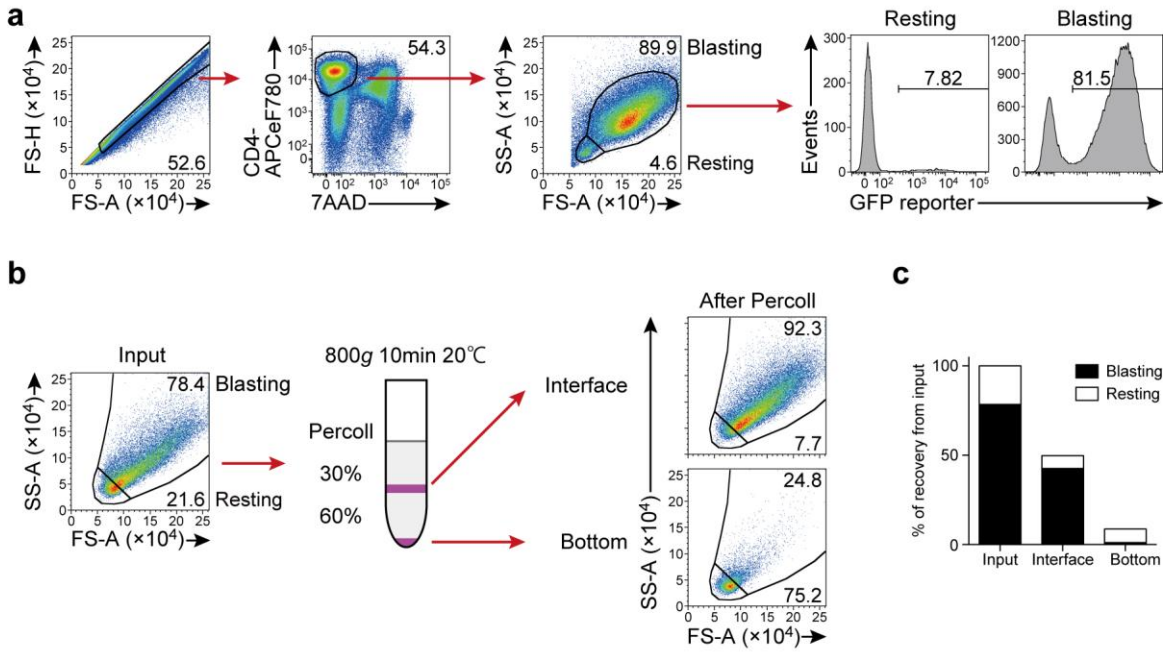

a
Flow check as`Input´
RV transduction
(Empty-GFP)
Flow check as`Transfer´
No Percoll
RV-transduction
efficiency check
In vitro stimulation
Percoll
Interface only
P14 cells
(CD45.2+)
Interface
Interface + Bottom
day 2
day 0
day 1
Bottom
Bottom only
Adoptive transfer
Infection of recipient mice
Analysis
day 0
day 1
day 2
day 8
Days after LCMV infection and in vitro stimulation
Recipients
(CD45.1+)
b
day 1
day 2
day 8
Input
Transfer
RV-transduction
check (in vitro)
Blood
75.7
24.3
No Percoll
78.6
21.4
12.8
82.6
3.9
0.7
62.2
Interface
94.6
5.4
12.4
86
0.8
0.8
80.8
Bottom
51.1
48.9
15.6
73
11.1
0.3
21
Interface + Bottom
85
15
12.5
84.7
2.3
0.5
63.6
SS-A (×10^4)
FS-A (×10^4)
GFP
reporter
Events
c
NS
*
*
Relative % of GFP+ frequency among
total P14 cells at day8 (Day2=100%)
No Percoll
Interface
Bottom
Interface+Bottom

Supplementary Figure 3

Small-sized cell frequency at adoptive transfer inversely correlates with RV-transduction efficiency *in vivo*.

(Related to Figure 2a-g)

(**a**) Experimental design. Donor P14 cells and recipient mice were stimulated *in vitro* and infected with LCMV Arm at day 0, respectively. At day 1, activated cells were harvested from the flask and analyzed immediately by flow cytometry to determine the ratio of small-sized cells to large-sized cells in 'Input' population. An aliquot of activated cells was set aside for a no Percoll control prior to Percoll centrifugation. After Percoll, interface (blasting cells) and bottom (resting cells) cells were separately collected, and a re-mixture of the interface and bottom cells was made. Then the equal numbers of the 4 populations ($3x10^6$ cells per well) were transduced with empty-GFP RV. After 4 hrs, aliquots of the 4 populations were checked by flow cytometry ('transfer' sample), and $1x10^5$ cells of each population were adoptively transferred to infected mice. RV-transduction efficiency and engraftment of RV-transduced cells were analyzed at day 2 and 8, respectively. (**b**) Flow plots gated on live P14 cells show the composition of blasting and resting cells in input (left) and 4 populations at transfer (right-left) at day 1, and RV transduction efficiency at day 2 (right-middle), and frequency of RV-transduced cells at day 8 (right-right). (**c**) Graph showing 'Retention rate' of RV-transduced cells among total P14 cells from day 2 (*in vitro*) to day 8 (blood). Bars show mean ± s.e.m. *p<0.005 (two-tailed t test); NS p>0.05. All data in this figure are from one experiment (n=5 per group). All animal experiments used in this Figure were in accordance with the Institute Animal Care and Use Guidelines for the University of Pennsylvania.


a
FS-H (×10⁴)
FS-A (×10⁴)
52.6
CD4-APCeF780
7AAD
54.3
SS-A (×10⁴)
FS-A (×10⁴)
89.9
Blasting
4.6
Resting
Resting
Blasting
Events
7.82
81.5
GFP reporter
b
Input
SS-A (×10⁴)
FS-A (×10⁴)
78.4
Blasting
21.6
Resting
800g 10min 20℃
Percoll
30%
60%
Interface
Bottom
After Percoll
92.3
7.7
24.8
75.2
c
% of recovery from input
Blasting
Resting
Input
Interface
Bottom
d
Timing of RV transduction after in vitro stimulation
0h (No RV)
24h
48h
72h
96h
0
75.6
81.2
41.1
22.3
Events
GFP reporter

Supplementary Figure 4

Activated CD4$^+$ T cells can be enriched by Percoll density centrifugation.

(Related to Figure 2a-c and 5a)

Polyclonal CD4$^+$ T cells were prepared from C57/Bl6 spleens using EasySep Mouse CD4$^+$ T Cell Isolation Kit (STEMCELLS) and stimulated *in vitro* with anti-CD3ε (final 1 µg/mL), anti-CD28 (final 0.5 µg/mL) antibodies and rIL-2 (final 100 U/mL) as shown in **Fig.1**. CD4$^+$ T cells were spin-transduced with empty-GFP RV using Polybrene (final 4 µg/mL) and tissue culture treated plates at day 1 (**a**) or indicated timing (**d**) after stimulation without enrichment of blasting cells, and analyzed for GFP expression one day after RV-transduction. Some of CD4$^+$ T cells were processed by density centrifugation with 30% and 60% Percoll layers at day 1 (**b, c**). Data are representative of two independent experiments (one technical replicate per experiment). (**a**) Small sized 'resting' CD4$^+$ T cells are mostly RV negative. (**b**) Density centrifugation enriches activated 'blasting' CD4$^+$ T cells at day 1. Flow plots that are gated on 7-AAD negative CD4 positive population show enrichment step from input (left) to shortly after Percoll separation at day 1 (right). (**c**) Graph showing recovery of small ('resting') and large ('blast') size CD4$^+$ cells in each interface and bottom after Percoll centrifugation. (**d**) RV transduction efficiency to CD4$^+$ cells peaks at 48 hours after *in vitro* stimulation.

Supplementary Table 1 List of antibodies used in this study.

Antibody	Clone	Fluorochrome	Source	Dilution
$CD3_{\varepsilon}$	145-2C11	Purified	Biolegend	1:1000
CD4	RM4-5	APC-EFluor780	eBioscience	1:200
CD8α	53-6.7	Brilliant Violet 605	Biolegend	1:200
CD8α	53-6.7	APC-eFluor780	eBioscience	1:200
CD28	37.51	Purified	Biolegend	1:2000
CD44	IM7	Alexa Fluor 700	Biolegend	1:400
CD44	IM7	APC-eFluor780	eBioscience	1:400
CD45.1	A20	APC-R700	BD Biosciences	1:200
CD45.1	A20	APC-eFluor780	eBioscience	1:200
CD45.2	104	Brilliant Violet 785	Biolegend	1:50
Thy1.1 (CD90.1)	OX-7	Brilliant Violet 605	Biolegend	1:200
CD127 (IL-7Rα)	A7R34	Brilliant Violet 421	Biolegend	1:200
hNGFR (CD271)	ME20.4	PerCP-Cy5.5	Biolegend	1:800
IFNγ	XMG1.2	APC	eBioscience	1:200
IL-2	JES6-5H4	PE	Biolegend	1:200
KLRG1	2F1	PerCP-eFluor 710	eBioscience	1:200
TCR Vα2	B20.1	PerCP-Cy5.5	Biolegend	1:200
TCR Vα2	B20.1	PerCP-eFluor 710	eBioscience	1:200
TNFα	MP6-XT22	PE-Cyanine7	eBioscience	1:200
gp33 tetramer	(n/a)	PE	NIH tetramer core	1:500