Supplemental Information

The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis

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Figure S1. Generation of composite FACS plots & CX3CR1 is preferentially induced on anti-viral CD8\(^+\) T\(_{\text{Eff}}\). Related to Figure 1. (A) To accurately represent a group average in FACS plots, all FACS dot plots and histograms within this manuscript, unless specified otherwise, are composites of all mice within a group. Composite plots were generated by randomly selecting equal numbers of each cell population of interest from each mouse within a group. These cells were pooled into a single file (‘concatenation’ in FlowJo) and plotted. (B-D) Data are from the same experiment(s) as in Figure 1A,B. Blood-derived CD3\(^+\)CD8\(^+\) and CD3\(^+\)CD4\(^+\) T cells from Cx3cr1\(^{-/-}\) mice were analyzed before (day 0) and 8 days after infection with LCMV. (B) CX3CR1-GFP expression was measured on polyclonal CD4\(^+\) and CD8\(^+\) T cells. (C) Composite FACS histograms of CX3CR1-GFP expression on resting CD8\(^+\) T\(_N\) (day 0) and on LCMV specific T\(_{\text{Eff}}\) assessed after gating on CD3\(^+\)CD8\(^+\) T cells that were stained with gp33-MHC-I dextramer (Dex\(^+\)) (day 8). (D) CX3CR1-GFP expression on T\(_N\) and LCMV specific T\(_{\text{Eff}}\). Left: Symbols represent individual mice, horizontal bars represent group mean. Right: Geometric mean fluorescence intensity (GeoMFI) of CX3CR1-GFP fluorescence on day 0 T\(_N\) and Dex\(^+\) T\(_{\text{Eff}}\) on day 8. (E) Composite FACS histograms of
CX3CR1-GFP expression on resting CD4$^+$ T$_N$ (day 0) and activated (CD44$^{\text{high}}$) CD4$^+$ T cells on day 8 after LCMV infection. (F) CX3CR1-GFP expression on CD4$^+$ T$_{Eff}$. **Left:** Symbols represent individual mice, horizontal bars represent group mean. **Right:** Geometric mean fluorescence intensity (GeoMFI) of CX3CR1-GFP fluorescence on CD4$^+$ T$_N$ and activated CD44$^{\text{high}}$ CD4$^+$ T cells. $n = 3$ experiments (3-5 mice each). Total mice: 12 (d0) and 13 (d8). Bars represent group mean ± SD. Composite FACS histograms were compiled from 5 mice (d8) or 4 mice (d0) and are representative of three independent experiments.
Figure S2. CX3CR1 induction on CD8+ T cells requires cognate antigen recognition and occurs by a variety of infections. Relates to Figure 1. (A,B) Naïve OT-I Cx3cr1+/gfp CD45.1+ CD45.2+ and P14 Cx3cr1+/gfp CD45.1+ CD45.2+ T cell receptor transgenic (TCR-tg) T cells were co-transferred into C57BL/6 followed by LCMV or LCMV-ova infection. (A) Composite FACS plot of 4 mice, gated on TCR-tg T cells. Representative of n = 2 experiments (4 mice each). (B) Symbols represent individual mice. LCMV: n = 2 experiments (4 mice each; 8 mice total). LCMV-ova: 1 experiment, 2 mice total. *** p<0.001 by one-way ANOVA with Tukey’s multiple comparisons test. The percentage of CX3CR1 expressers among P14 is lower after LCMV-ova infection than LCMV infection due to the competition between the OT-I and P14 responding to the LCMV-ova infection. This competition occurred because high numbers of OT-I needed to be transferred (10^6 OT-I vs 10^4 p14) to allow their recovery after LCMV infection. (C-E) Naïve OT-I or P14 TCR-tg Cx3cr1+/gfp CD45.1+ T cells were transferred into
C57BL/6 followed by LCMV, LCMV-ova, vesicular stomatitis virus (VSV)-ova or *Listeria monocytogenes* (LM)-ova infection. Data are from blood-derived OT-I of 5 mice / group (infected) and 2 mice / group ( naïve), and are representative of two experiments per infection model. (C) Left: Composite FACS plot of 5 mice / group (after infection) and 2 mice / group ( naïve), gated on TCR-tg T cells. Middle: Symbols represent individual mice. Right: Depicted is group mean ± SD. (D-E) Kinetics of CX3CR1-GFP expression on TCR-tg T cells. (D) Left: Composite flow cytometry plot of 5 mice / group (after infection) and 2 mice / group ( naïve), gated on TCR-tg T cells. Right: Depicted is group mean ± SD. (E) Timecourse of T cell differentiation marker expression. Depicted is group mean ± SD.
Figure S3. CX3CR1 is a differentiation marker for CD8\(^+\) T\(_{\text{eff}}\). Relates to Figure 1 and 2. Naive CD45.1\(^+\) OT-I x Cx3cr1\(^{+/-}\) T cells were transferred into C57BL/6 hosts followed by LCMV-ova or VSV-ova infection. Up-regulation of CD44 and the 1B11 isoform of CD43, and down-regulation of CD62L are indicative of antigen-experience. (A,C & E) Composite FACS plots of 5 infected and 2 mice (c, e) and 3 naïve mice (a) / group / day, gated on OT-I cells. Data are representative of two experiments of 5 mice each. (B & D) Depicted is group mean \(\pm\) SD. Data are pooled from (B), two experiments (CD43 one experiment) and (D) three experiments (CD127 two experiments) of 5 mice each. * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\) by repeated measures one-way ANOVA with Tukey’s multiple comparisons test. (C-E) Same experiment(s) as in Figure 2A&B. (F) Composite flow cytometry plots of 5 LCMV-ova infected mice. Dot plots: gated on OT-I cells. Histograms: gated on CX3CR1-GFP (top row) or fractalkine-Ig (bottom row) negative, intermediate or high OT-I cells.
Figure S4. CX3CR1⁺, CX3CR1int and CX3CR1hi T Eff are functionally distinct and also present in human blood. Relates to Figure 2-4. Naïve OT-I CX3CR1-GFP CD45.1⁺ T cells were transferred into C57BL/6 followed by LCMV-ova or VSV-ova infection. Data are from spleen-derived OT-I, 10 days after infection. (A) Composite FACS plots of 5 mice / group, gated on OT-I cells. Representative of two experiments. (B) The amount of cytokine produced by the cytokine producers is depicted relative to the mean of the CX3CR1 plots. Data were pooled from two experiments of 4-5 mice each (9 mice total). Same experiments as in Figure 2A. * p<0.05, ** p<0.01, *** p<0.001 by repeated measures one-way ANOVA with Tukey’s multiple comparisons test. (C) CX3CR1 antibody staining on CD3⁺CD8⁺CD45RO⁺ T cells from 4 anonymous human donors. (D, E) Mathematical modeling of CX3CR1⁺ TMem and CX3CR1int TMem population sizes between days 55-128 (D) and days 128-350 (E) after infection, with and without CX3CR1int→CX3CR1 conversion.
Figure S5. Differential cytokine production and cytotoxicity by CX3CR1\(^{-}\), CX3CR1\(^{int}\) and CX3CR1\(^{hi}\) T\(_{Mem}\). 

**Relates to Figure 4.** Naïve OT-I Cx3cr1\(^{+/+}\) CD45.1\(^{+}\) T cells were transferred into C57BL/6 followed by LCMV-ova or VSV-ova infection and effector function of the resulting T\(_{Mem}\) was measured at 5+ weeks after infection. (A) Composite FACS plots of OT-I T\(_{Mem}\) (4 mice / group). (B) Frequencies of individual cytokine producing cells, (C) amount of produced cytokine, relative to the mean of the neg population, and (D) frequency of single, double and triple cytokine producers were assessed among splenic OT-I T\(_{Mem}\), \( n = 3 \) experiments (3-4 mice each). Total mice: 11 LCMV-ova, 12 VSV-ova. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) by repeated measures (B,C) one-way or (D) two-way ANOVA with (B,C) Tukey’s or (D) Bonferroni multiple comparisons test. (E) Sorted OT-I T\(_{Mem}\) subsets were pooled from spleen, blood and LNs of 5 mice 39 days after VSV-ova infection and incubated with a 1:1 mixture of differentially labelled SIINFEKL-loaded B cells (targets) and control B cells for 63h. (F) Sorted p14 T\(_{Mem}\) subsets were incubated with a 1:1 mixture of gp33-loaded splenocytes (targets) and un-loaded control splenocytes. (E-F) Depicted is group mean + SD (3-5 wells / group). Total wells: 4 (E, CX3CR1\(^{lo}\)), 5 (E, CX3CR1\(^{int}\), CX3CR1\(^{hi}\)), 3
Figure S6. Phenotypic and transcriptional characteristics of CX3CR1<sup>-</sup>, CX3CR1<sup>int</sup> and CX3CR1<sup>hi</sup> T<sub>Mem</sub>. Relates to Figure 4. Naïve OT-I C<sup>x<sup>3</sup>cr1<sup>-/-</sup></sup> CD45.1<sup>+</sup> T cells were transferred into C57BL/6 followed by LCMV-ova or VSV-ova infection. The phenotype of the OT-I T<sub>Mem</sub> subsets was assessed >5 weeks after infection. (A) Top
+ bottom left: Composite flow FACS plots of 4 mice (infected) and 2 naïve mice/ Bottom right: n = 2 experiments (3-5 mice each; 8 mice total). (B) n = 2 experiments (3-5 mice each). LCMV-ova: 7 mice total. VSV-ova: 8 mice total. c, n = 1 experiment (4 mice). (B-C) Depicted is group mean ± SD. (D) Naïve OT-I CD45.1+ T-cells were transferred into C57BL/6 followed by LCMV-ova infection. Eomes and Tbet expression was analyzed on naïve and TMem subsets (identified by differential CX3CR1 antibody staining) >9 weeks after infection. OT-I cells are derived from spleen and LN. n = 2 experiments with 1-4 mice (neg/int/hi) or 2 mice (naïve) per experiment. Total mice: 4 (naïve), 6 (neg/int/hi Tbet stain), 4 (neg/int/hi Eomes stain). * p<0.05, ** p<0.01, *** p<0.001 by repeated measures (B-C) or ordinary (D) one-way ANOVA with Tukey’s multiple comparisons test.
Figure S7. CX3CR1 expression levels on T$_{\text{Mem}}$ do not identify the same populations as identified by CD43{1B11}/CD27, and CD62L expression by CX3CR1$^+$, CX3CR1$^{\text{int}}$ and CX3CR1$^{\text{hi}}$ T$_{\text{Mem}}$. Relates to Figure 4. (A-D) Phenotype of adoptively transferred OT-I or gp33 Dextramer$^+$ (endogenous) T$_{\text{Mem}}$ >6 weeks after viral infection. (A) Left & middle: Composite FACS plots from one representative experiment showing peripheral blood OT-I, CX3CR1-GFP$^+$ T cells from 5 LCMV-ova infected mice. Right: Pooled data from 2 experiments (4-5 mice each; 9 mice total). (B-C) Left & middle: Composite FACS plots from one experiment showing peripheral blood gp33 Dextramer$^+$ CD44$^+$ CD8$^+$ CX3CR1$^{\text{int}}$/gfp$^+$ T cells from 6 (B) and 5 (C) LCMV infected mice. Right: Group mean ± SD. Total mice: 6 (B) and 5 (C). D, Left & middle: Composite FACS plots from one experiment showing peripheral
blood OT-I, $Cx3cr1^{+/gfp}$ T cells from 3 VSV-ova infected mice. Right: Group mean + SD (3 mice). (E,F) Naïve OT-I $Cx3cr1^{+/gfp}$ CD45.1$^+$ T cells were transferred into C57BL/6 followed by LCMV-ova or VSV-ova infection. (E) Composite FACS plots of 4 mice (infected) and 2 naïve mice. (F) $n = 2$ experiments of 4 mice (Exp 1) and 3 mice (Exp 2). Depicted is group mean + SD. * p<0.05, ** p<0.01, *** p<0.001 by repeated measures one-way ANOVA with Tukey’s multiple comparisons test. (G) $Cx3cr1^{+/gfp}$ mice were infected with LCMV. Composite FACS plots of 4 mice from one of the two experiments shown in Figure 4E, gated on gp33-specific CD8$^+$ T cells. (H) Sort gates of the experiment show in Figure 4F.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice
Mice of both genders and of ages 6-20 weeks were used. In adoptive transfer experiments, donor and recipient mice were gender matched. Within each experiment, mice of different groups were age matched. All mice were housed, bred and crossed in a specific pathogen-free barrier facility of Harvard Medical School. Experiments using TCR-tg T cells were performed either with Rag1+/+ and Rag1−/− TCR-tg donor T cells, the latter to rule out any potential effects of TCRs with other than the transgenic specificity. Whenever experiments were performed with Rag1+/+ and Rag1−/− TCR-tg donor T cells, the results were comparable. Therefore, it is not specified which experiment was performed with Rag1+/+ and which with Rag1−/− TCR-tg cells, and data from both types of experiments may be shown pooled if the experimental setup was otherwise the same.

T cell isolation from murine tissues
10⁶ Va2 CD8 T cells were transferred into naïve C57BL/6 recipient mice. If experiments required flow cytometric sorting of T cells, cells were transferred into naïve C57BL/6 recipient mice. If experiments required flow cytometric sorting of T cells, 1-5x10⁶ naïve Va2 CD8 T cells were transferred. Figure 3A: number of transferred T Eff: 2x10⁵ (Exp.1) and 1.6x10⁵ (Exp. 2). Small intestines were depleted of Peyer’s Patches and first incubated in HBSS / 2.5 mM EDTA for 20 min. to isolate IEL. The remaining tissue was digested for 20 min. in HBSS / 2.5% FCS / 25µg/ml DNaseI (Roche) / 500µg/ml Collagenase D (Roche) to isolate LPL. IEL and LPL were further purified on a Percoll (GE Healthcare) density gradient.

Prior to FACS sorting or when harvesting sorted T cell subsets, T cells were enriched by MACS using the CD8α+ T cell isolation kit (Miltenyi Biotec) for endogenous T cells and CD45.1-biotin or CD45.2-biotin (BioLegend) staining followed by anti-biotin MicroBeads (Miltenyi Biotec) for adoptively transferred TCR-tg cells.

In Fig. 3A (Exp 1+2), the number of recovered OT-I memory cells is expressed relative to the mean number recovered from the transferred CX3CR1hi cells of the respective experiment.

Parabiosis
Pairs of mice were matched by gender and body weight, and anesthetized i.p. with Ketamine (100mg/kg), 10 mg/kg Xylazine (10mg/kg), and Acepromazine (3mg/kg). Hair was removed from the opposing lateral flanks of both mice by shaving and depilatory cream. A longitudinal skin incision was made from the olecranon area to the knee joint on each mouse of the parabiotic pair, and the subcutaneous fascia was bluntly dissected to create ~0.5 cm skin flap. Animals were positioned side-by-side and the right olecranon and right knee joint of one animal was sutured to the corresponding left olecranon and left knee joint of the other. The dorsal and ventral skin flaps were then attached by continuous 6-0 suture at the olecranon and knee regions, and staples along the lateral abdominal aspect of the mice.

Thoracic duct lymph collection
Mice were administered ~0.5ml of olive oil by gavage approximately 1hr prior to surgery to better visualize lymphatic vessels and the cisterna chyli. Mice were then anesthetized by IP injection of 100 mg/kg Ketamine HCl, 10 mg/kg Xylazine and 3 mg/kg Acepromazine. After surgical site preparation, the mice receive a heparinized polyethylene catheter in the left jugular vein for administration of fluids (Ringer’s lactate, 1U/ml Heparin) prior to and throughout the thoracic duct cannulation procedure. Next, an incision is made through the left abdominal wall exposing the intestines, spleen, left kidney, and adrenal gland, which are gently mobilized and held by hemostat clamps to expose the thoracic duct and cisterna chyli. The thoracic duct is then punctured and a heparinized cannula is inserted and fixed in place by adding a drop of tissue glue. Thoracic duct lymph was collected for 1-3h and assayed by flow cytometry. If thoracic duct cannulation was performed on one parabiotic partner, the parabiotic mice were surgically separated immediately prior to performing the thoracic duct cannulation procedure.

Flow cytometry
The following antibody clones were used: CD3e (17A2 & 145-2C11), CD8a (53-6.7), CD8b (yts 156.7.7), CD4 (GK1.5 & RM4-5), CD45.1 (A20), CD45.2 (104), CD27 (LG.3A10), CXCR3 (CXCR3-173), CX3CR1 (SA011F11), IL-2 (JES6-5H4), TNFa (MP6-XT22), IFNg (XMG1.2), KLRG1 (2F1), CD62L (MEL14), CD44 (IM7), CD11b (M1/70), Tbet (O4-46), Eomes (Dan1Imag), CD43 (1B11), CD69 (H1.2F3), CD103 (M290), CD45 (30-F11), Ter-119 (Ter-119), Ki67 (B56). All MAb were purchased from BioLegend or BD Biosciences. Double-sorted TMem were >98% pure. When congenic CX3CR1hi and CX3CR1int TMem were co-transferred (Figure 3F), half of the recipients received CD45.1hiCD45.2 + TMem and CD45.1hiCD45.2int TMem, and the other half received CD45.1hiCD45.2 int TMem and CD45.1CD45.2int TMem. To average out any
potential effects the congeneric markers may have on T<sub>men</sub> fates. Sort purity in Figure 3D (Exp1/Exp2): CX3CR1<sup>+</sup>: 100% / 99.9%, CX3CR1<sup>int</sup>: 98.3% / 99.8%, CX3CR1<sup>hi</sup>: 100% / 100%. Sort purity in Figure 3F (Exp1/Exp2): CD45.1<sup>+</sup>CD45.2<sup>+</sup> CX3CR1<sup>+</sup>: 98.9% / 99.7%, CD45.1<sup>+</sup>CD45.2<sup>-</sup> CX3CR1<sup>+</sup>: 99.9% / 99.7%, CD45.1<sup>+</sup>CD45.2<sup>-</sup> CX3CR1<sup>int</sup>: 99.6% / 99.5%, CD45.1<sup>+</sup>CD45.2<sup>-</sup> CX3CR1<sup>hi</sup>: 99.2% / 99.4%.

Cells were sorted with a FACSaria and analyzed with a FACSconto, LSR II (all BD Biosciences), or CytoFlex (Beckman Coulter).

Infections
Tri-segmented LCMV-ova was generated as follows: The OVA open reading frame (ORF) was amplified from cDNA using the PCR Expternder System (5 Prime) and primers OVA-BbStart (TGAAGACAGCAAGATGGCTCCATCGGTGCAGCA) and OVA-Bbstop (TGAAGACAGCTCTAAAAGGGAACACATCTGCCA) containing the Bbs1 restriction site. After Bbs1 digestion, the PCR product was cloned into plasmid pol-I S-GPC/Bbsl (Emonet SF, 2009) to generate plasmid pol-I S-GPC/OVA. The fluorescent protein Katushka ORF was amplified from cDNA using the PCR Expternder System (5 Prime) and primers Katushka-BsStart (CGTCTCCAAGGATGGTGGGTAGATAGCGT) and Katushka_BsStop (CGTCTCTTCTTCAGTGCACCATCCTTAGCCA) containing BsmBI restriction sites. After BsmBI digestion the PCR product was cloned into the BsmBI site of plasmid pol-I S-BsmBI/NP (Emonet SF, 2009) to generate plasmid pol-I S-Katushka/NP. For subsequent virus rescue, subconfluent BHK-21 cells (2x10<sup>6</sup> cells/M6 well) were transfected for 5 h using 2.5 µl of Lipoctectamine 2000 (Invitrogen) per µl of plasmid DNA. The plasmid cocktail included: pC-NP (0.8 µg) and pC-L (1 µg), together with plasmids pol-I L (1.4 µg), pol-I S-Katushka/NP (0.8 µg) and pol-I S-GPC/OVA (0.8 µg) that directed intracellular synthesis, via RNA pol-I, of the viral L and S genome RNA species. After 5 h transfection, cells were washed once with DMEM containing 2 % FBS and fresh (3 ml/M6 well) DMEM containing 10 % FBS added to cells and incubated for 72 h prior collecting the tissue culture supernatant for virus titration.

Transwell chemotaxis, killing and homing assay
Chemotaxis assay: 10<sup>6</sup> spleen-derived, MACS-enriched CD8<sup>+</sup> T cells (CD8α<sup>+</sup> T cell isolation kit; Miltenyi Biotec) were loaded in the upper chamber of 5µm pore size Transwell plates. The lower chamber contained medium without or with indicated concentrations of recombinant murine CCL19. The absolute number of CX3CR1<sup>+</sup>, CX3CR1<sup>int</sup> and CX3CR1<sup>hi</sup> OT-I T<sub>mem</sub> that had migrated into the chemokine-containing lower chamber after 3 hours at 37°C was quantifyed and expressed relative to the corresponding populations’ input numbers (% migrated) or numbers within the no chemokine-containing lower well (chemotactic index).

To assess the abilzity of CX3CR1<sup>+</sup>, CX3CR1<sup>int</sup> and CX3CR1<sup>hi</sup> OT-I T<sub>mem</sub> to specifically lyse peptide-loaded target cells, 10,000 sorted CX3CR1<sup>+</sup>, CX3CR1<sup>int</sup> and CX3CR1<sup>hi</sup> OT-I T<sub>mem</sub> were co-incubated with differentially marked SIINFEKL-loaded target B cells or splenocytes and equal numbers of differentially marked non-loaded control B cells or splenocytes. 3 days later, the relative abundance of control targets vs SIINFEKL-loaded targets was assessed, corrected for their relative input ratio (theoretically 1:1). B cells were isolated from spleens by MACS enrichment for CD43<sup>+</sup> cells (CD43 MicroBeads, Miltenyi).

To determine the short-term homing efficiency of T<sub>mem</sub> CD8<sup>+</sup> T cells were enriched by negative selection from mice that had received naïve OT-I and LCMV-ova >9 weeks before. Enriched CD8<sup>+</sup> T cells containing CX3CR1<sup>+</sup>, CX3CR1<sup>int</sup> and CX3CR1<sup>hi</sup> OT-I T<sub>mem</sub> were transferred i.v. into naïve mice. 2h later, OT-I T<sub>mem</sub> were quantified in peripheral LN (axillary, brachial, inguinal, cervical), mesenteric LN, spleen and blood.

Statistics
In adoptive transfer experiments, T cells were harvested from multiple mice, then pooled to even out any potential differences between mice, and the resulting cells were then sorted into indicated subsets. When the sorted subsets were transferred into recipients, no specific method of randomization was used to determine which recipient received which subset, as all recipients were considered equal. In FACs experiments, no calculations of subset percentages were performed on fewer than 20 cells (pre-established exclusion criteria). The investigator was not blinded during data analysis. When one variable was compared in more than two groups, one-way ANOVA was used, with Tukey’s multiple comparisons test to compare the means of all groups. When two variables were compared in more than two groups, two-way ANOVA was used, with Bonferroni’s multiple comparisons test to compare the means of all groups.
**Mathematical modeling**

The rate of conversion from CX3CR1\textsuperscript{int} to CX3CR1\textsuperscript{T_Mem}, and the related population dynamics of CX3CR1\textsuperscript{−}, CX3CR1\textsuperscript{int} and CX3CR1\textsuperscript{hi} T\_Mem were estimated as follows:

The change in the size $N$ of each T\_Mem population over time $t$ is a function of the growth rate $G$, the death rate $D$, and the rate of conversion $C$ from CX3CR1\textsuperscript{int} to CX3CR1\textsuperscript{−} T\_Mem, and can be expressed by the following differential equations:

For CX3CR1\textsuperscript{hi}:

$$ \frac{dN_{hi}}{dt} = (G_{hi} - D)N_{hi} $$ \ (I)

For CX3CR1\textsuperscript{int}:

$$ \frac{dN_{int}}{dt} = (G_{int} - D - C)N_{int} $$ \ (II)

For CX3CR1\textsuperscript{neg}:

$$ \frac{dN_{neg}}{dt} = (G_{neg} - D)N_{neg} + C N_{int} $$ \ (III)

$G$ was experimentally determined to be different for all three T\_Mem subsets (Figure 3E): $G_{int} = 1.8 \times G_{neg}$ and $G_{int} = 11.8 \times G_{hi}$. $D$ was assumed to be constant for all T\_Mem subsets, which may not be accurate, but we currently have no means to experimentally test this in vivo.

Solving the above equations, followed by their simplification leads to:

$$\frac{1}{11.8} G_{int} - D = \frac{1}{t} \ln \frac{N_{hi}}{N_{hi}^0}$$ \ (I')

$$G_{int} - D - C = \frac{1}{t} \ln \frac{N_{int}}{N_{int}^0}$$ \ (II')

$$N_{neg} = \frac{C N_{int}^0}{4 G_{int} - C} \left[ \frac{N_{int}}{N_{int}^0} - e^{(E G_{int} - D)t} \right] + N_{neg}^0 e^{(E G_{int} - D)t}$$ \ (III')

The population sizes $N_{hi}$, $N_{int}$ and $N_{neg}$ are taken from the experimental data in Figure 3C. Due to the sparseness of experimental data on T\_Mem subset sizes beyond day 55, we chose to estimate the parameters for the two intervals separately. The three unknowns ($G_{int}$, $D$, and $C$) for the time intervals day 55-128 and day 128-350 were solved using Mathematica’s `NSolve` function. The table below shows the averages over all mice.

<table>
<thead>
<tr>
<th>Interval</th>
<th>$D$</th>
<th>$C$</th>
<th>$G_{int}$</th>
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<tbody>
<tr>
<td>day 55 to day 128</td>
<td>1.557%</td>
<td>0.478%</td>
<td>1.461%</td>
</tr>
<tr>
<td>day 128 to day 350</td>
<td>0.753%</td>
<td>0.788%</td>
<td>1.302%</td>
</tr>
</tbody>
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By setting $C$ to zero, we determined what the size of the CX3CR1\textsuperscript{neg} population would have been without contributions from the CX3CR1\textsuperscript{int} subset.