

Supplementary Materials for

**Memory CD8<sup>+</sup> T cells mediate early pathogen-specific protection via localized delivery of chemokines and IFN $\gamma$  to clusters of monocytes**

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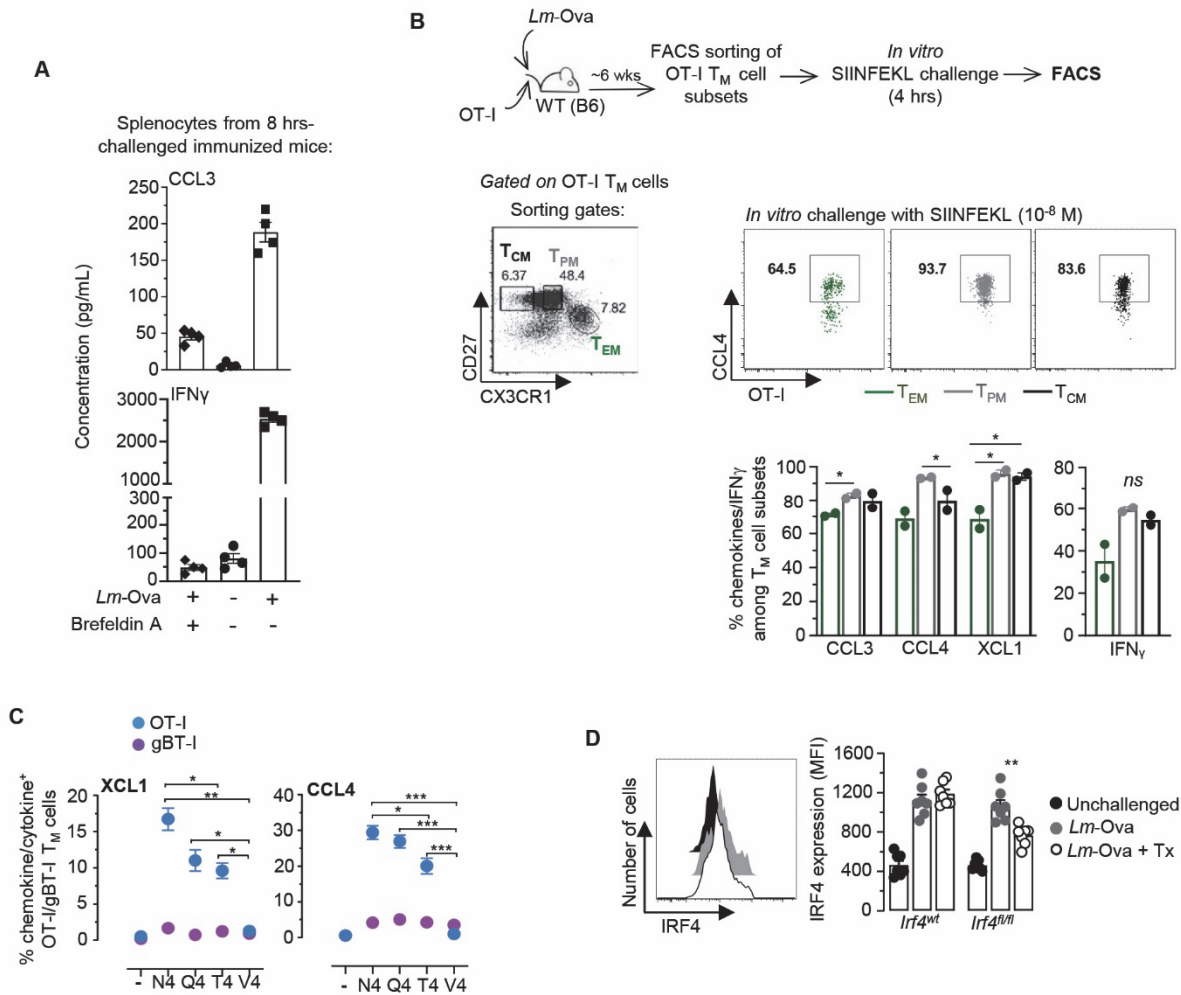
**The PDF file includes:**

Figs. S1 to S8  
Legends for tables S1 to S3  
Legends for movies S1 to S3

**Other Supplementary Material for this manuscript includes the following:**

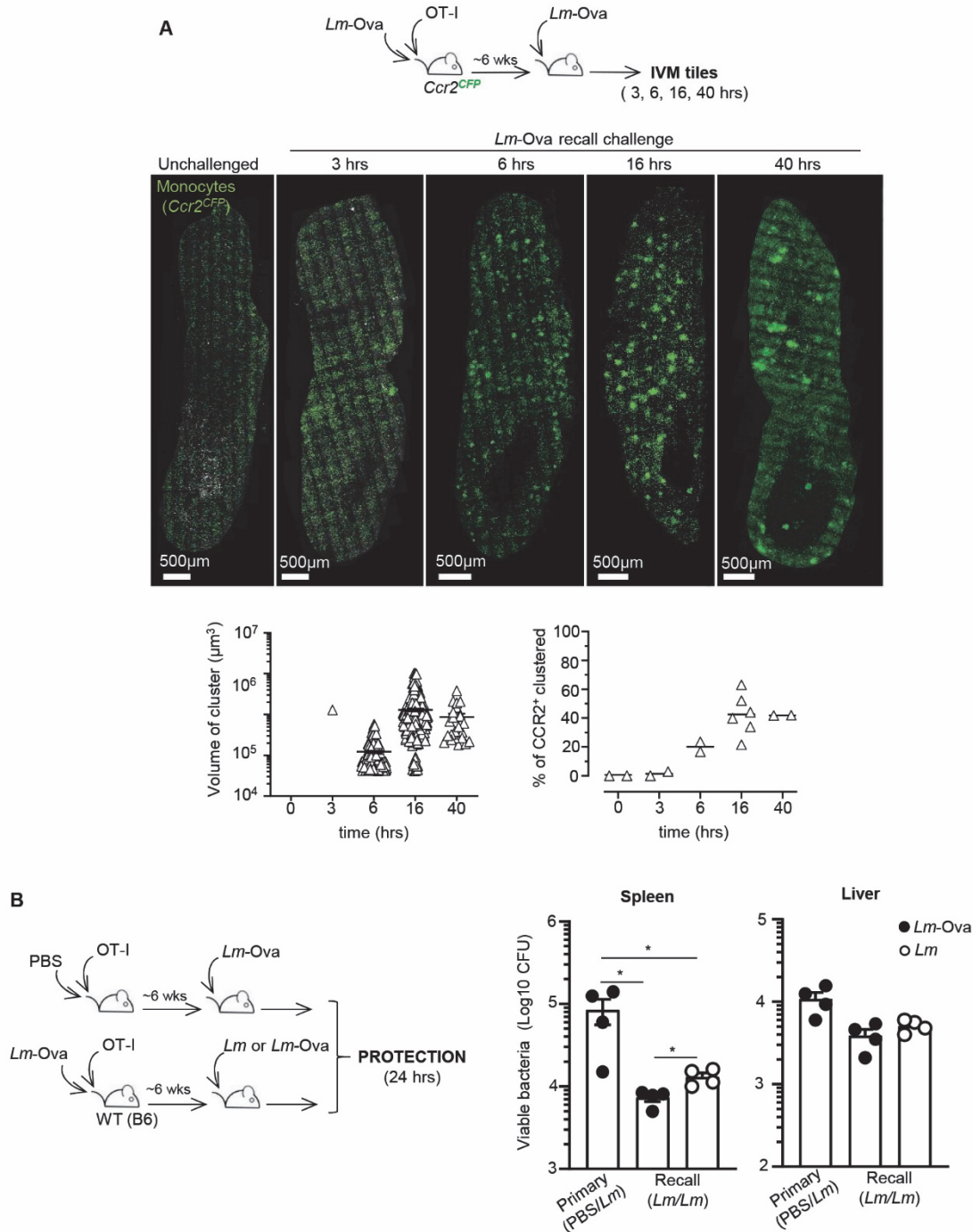
Tables S1 to S3  
Movies S1 to S3

## Supplemental Figure Legends:



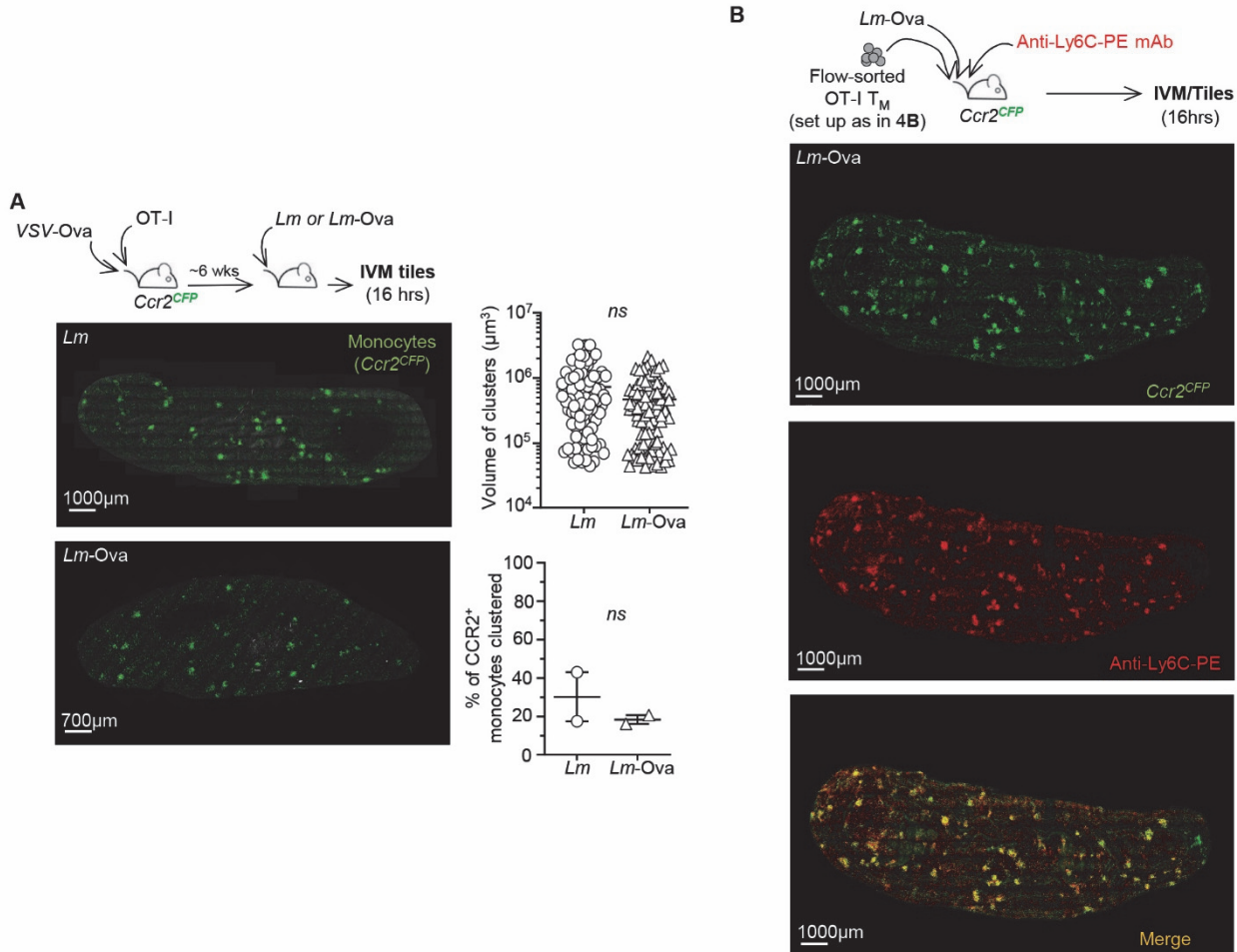
**Figure S1, related to Figures 2 and 3.** (A) Spleen cells were isolated from *Lm-Ova*-immunized mice ( $10^4$ ) that were rechallenged 6 wks later with  $10^6$  *Lm-Ova*. At 8 hrs post challenge, spleen cells were incubated or not with Golgi plug/stop, before collecting supernatants 4 hrs later. CCL3 and IFN $\gamma$  were next quantified by ELISA. (B) Subsets of OT-I T<sub>M</sub> cells were flow-sorted from the spleens of mice transferred with naïve OT-I cells and immunized with  $10^4$  *Lm-Ova* 6 wks before (schematic), based on CX3CR1 and CD27 cell surface marker expression (T<sub>EM</sub>: CX3CR1<sup>hi</sup>CD27<sup>low</sup>, T<sub>PM</sub>: CX3CR1<sup>int</sup>CD27<sup>hi</sup>, T<sub>CM</sub>: CX3CR1<sup>low</sup>CD27<sup>hi</sup>). Sorted (gates are shown) OT-I T<sub>M</sub> subsets were next stimulated for 4 hrs with the SIINFEKL peptide ( $10^{-8}$ M) *in vitro* and stained for cell-surface CD8, CXCR3, KLRG1 and intracellular CCL3, CCL4, XCL1 and IFN $\gamma$ . Representative dot plots with summary bar graphs (each symbol is 1 mouse) show the proportion of chemokines<sup>+</sup> or cytokines<sup>+</sup> cells among CD8<sup>+</sup> T<sub>M</sub> subsets with indicated p-value. (C) Mice grafted with OT-I Td<sup>+</sup> and CD45.1<sup>+/+</sup> gBT-I cells were immunized with  $10^4$  *Lm-Ova*-gB, and ~6 wks later challenged or not for 16 hrs with  $10^6$  *Lm-Ova* N4 or *Lm* expressing 3 different *Ova* APLs, namely *Lm-Ova* Q4, *Lm-Ova* T4 or *Lm-Ova* V4. Spleen cell suspensions were next incubated with Golgi Plug/Stop and stained for cell-surface CD8, CD3, CD45.1 and indicated intracellular XCL1 or CCL4 chemokines. Graphs represent the proportion of OT-I or gBT-I T<sub>M</sub> cells expressing indicated intracellular markers after challenge with *Lm*-expressing N4, Q4, T4 or V4. (D) *Rosa26<sup>CreERT2</sup>Irf4<sup>fllox/fllox</sup>Cd45.2<sup>+/+</sup>* and *Irf4<sup>wt</sup>Cd45.1<sup>+/-</sup>* OT-I cells were co-transferred to *Cd45.1<sup>+/+</sup>* WT recipient mice and immunized with  $10^4$  *Lm-Ova* the next day. Six wks later, mice received Tx (1mg/day) or vehicle i.p. every day for 5 days before secondary challenge infection

with  $10^6$  *Lm-Ova*. At 16 hrs, IRF4 expression was determined as described above in *Irf4<sup>fllox/fllox</sup>* versus *Irf4<sup>wt</sup>* OT-I T<sub>M</sub> cells in the different experimental conditions. Representative FACS dot plots and histograms staining are shown. Panels pool the result of 2 independent replicate experiments with n=6 (C) and 7 (D) mice. P-values are indicated.

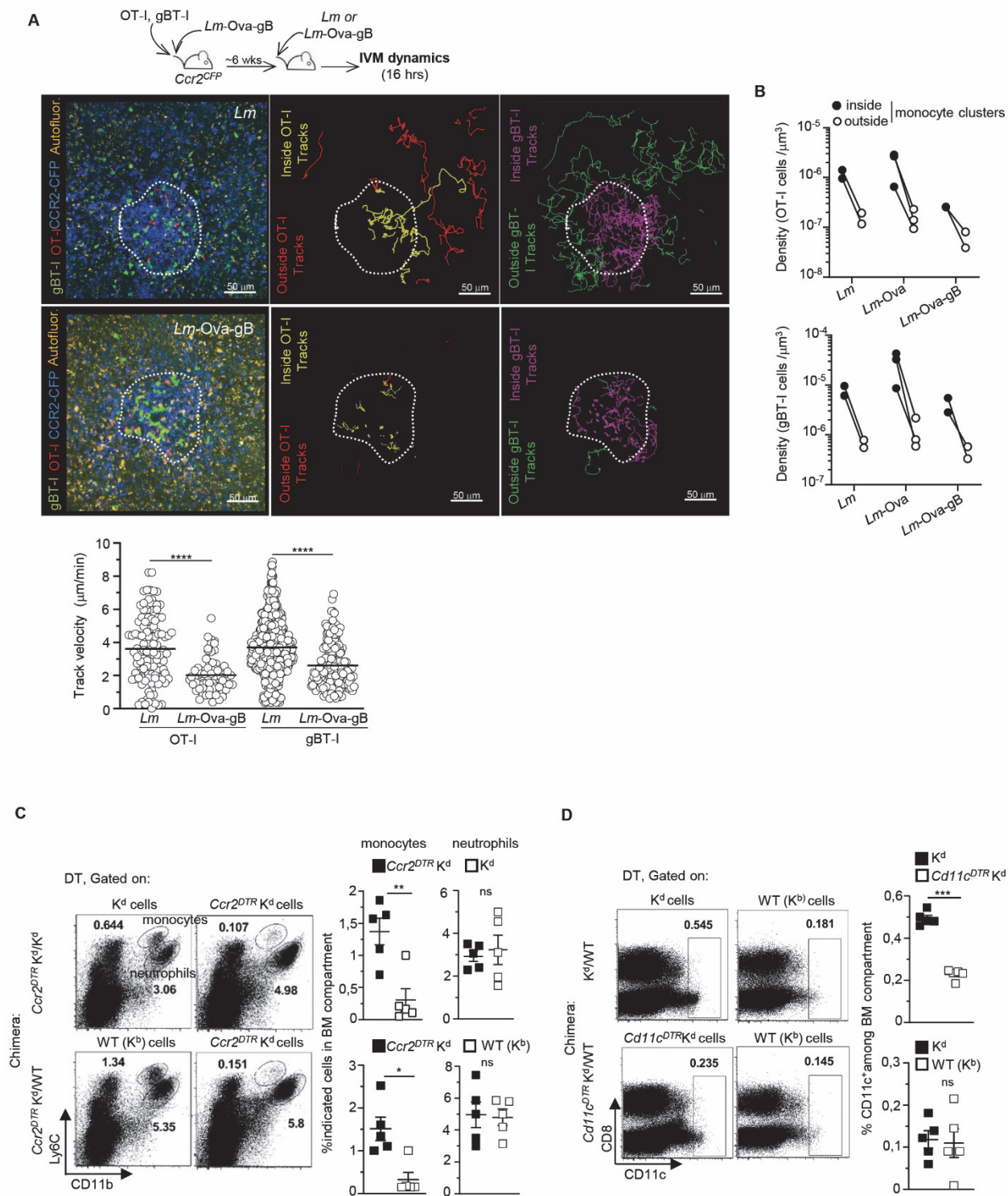


**Figure S2, related to Figure 4.** (A) *Ccr2<sup>CFP</sup>* mice grafted with OT-I cells were immunized with  $10^4$  *Lm-Ova* and 6 wks later challenged or not with  $10^6$  *Lm-Ova* for 3, 6, 16 and 40 hrs. *Ccr2<sup>CFP</sup>* monocytes are in green and representative mouse spleen tile reconstructions are shown. Graphs shows the volume of individual clusters and the average proportion of *CCR2<sup>+</sup>Ly6C<sup>+</sup>* monocytes clustered in a pool of 2 independent experiments (n=2-5 mice). (B) Age- and sex-matched WT B6

mice were transferred with OT-I cells and injected with PBS or immunized with  $10^4$  *Lm*-Ova. 6 wks later, PBS-injected mice were challenged with  $10^6$  *Lm*-Ova (“Primary”), and *Lm*-Ova-immunized mice were challenged with  $10^6$  *Lm* or *Lm*-Ova (“Recall”). Bacterial titers in spleens and livers were determined 24 hrs post challenge. Bar graphs represent 1 of 2 representative experiments with each symbol corresponding to an individual mouse (n=4 mice).

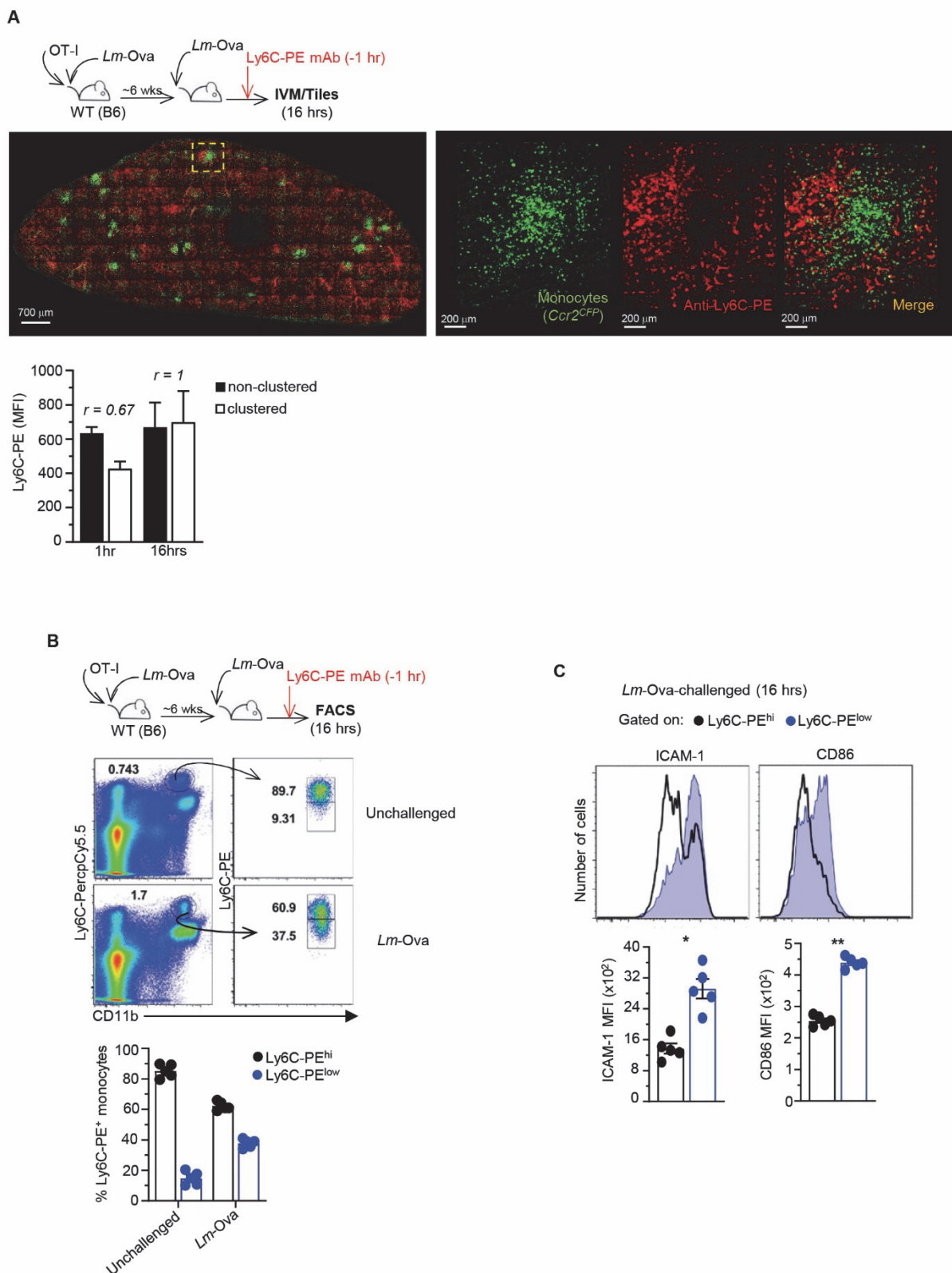






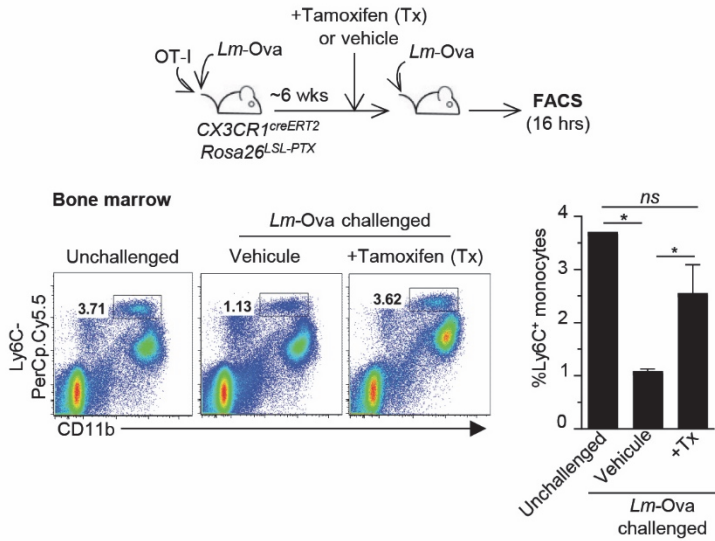
**Figure S4, related to Figure 5.** *Ccr2<sup>CFP</sup>* mice were co-transferred with naïve OT-I Td<sup>+</sup> and gBT-I GFP<sup>+</sup> cells and immunized with 10<sup>4</sup> *Lm*-Ova-gB. Six wks later, mice were challenged with 10<sup>6</sup> *Lm* or *Lm*-Ova-gB and IVM images in the spleen of live mice were recorded 16 hrs later. (A) Representative IVM image (right) of OT-I (red) and gBT-I (green) T<sub>M</sub> cells localized in a cluster (delimited area by white dashed line) of CCR2<sup>CFP</sup> monocytes (blue) are shown with autofluorescence (yellow). OT-I T<sub>M</sub> (outside, red and inside, yellow) and gBT-I T<sub>M</sub> cell tracks (outside, green and inside, purple) inside and outside the cluster of CCR2<sup>CFP</sup> monocytes are also shown (center and left images). Graphs represent the speeds of OT-I and gBT-I T<sub>M</sub> cells in the

clusters. (B) Graphs represent the density of OT-I or gBT-I T<sub>M</sub> cells inside and outside of monocyte clusters after 10<sup>6</sup> *Lm*, *Lm-Ova* or *Lm-Ova-gB* challenges. (C, D) Efficiency of DT-mediated depletion in indicated groups and compartments of mixed BM chimeras. Representative FACS dot plots in a pool of 2 experiments with p-value are shown (n=5 mice).



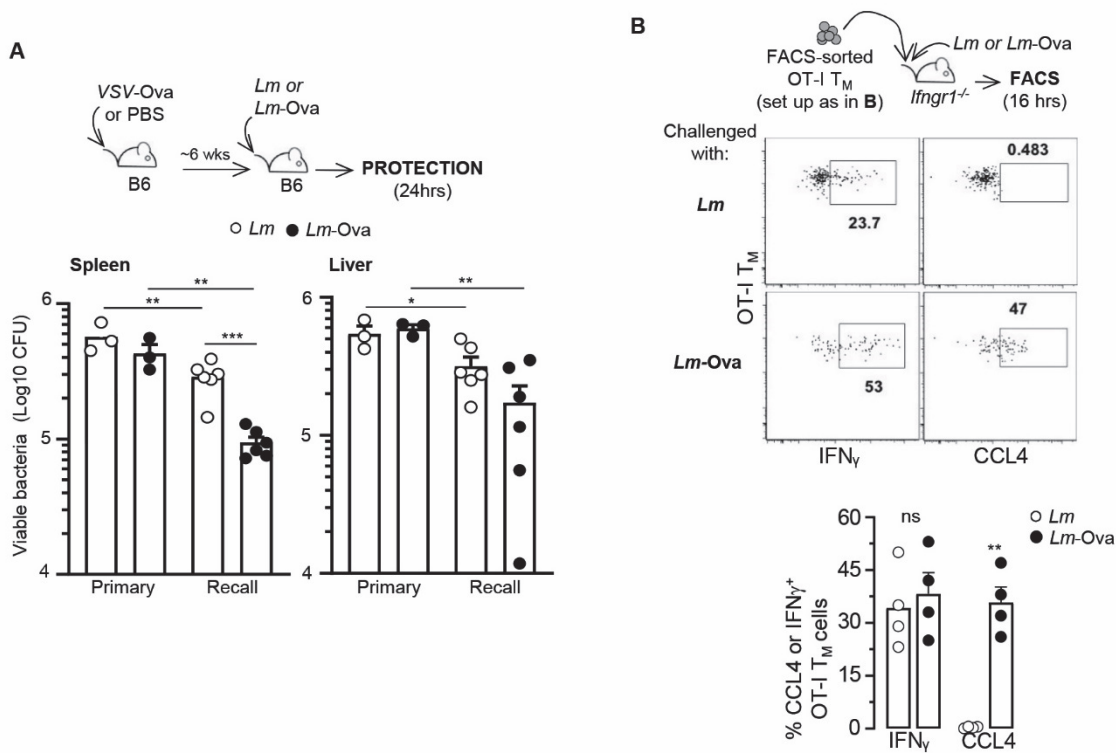
**Figure S5, related to Figure 6.** (A-B) *Lm-Ova*-immunized mice were co-transferred with naive OT-I Td<sup>+</sup> cells and challenged or not ~6 wks later with *Lm-Ova* for 16 hrs. 1 hr before sacrifice, mice were injected i.v. with Ly6C-PE mAb. (A) Representative tiles of reconstructed mouse spleens in 1 of 2 replicate experiments are shown with Ccr2<sup>CFP</sup> monocytes in green and Ly6C-PE<sup>+</sup> monocytes in red. Green and red signals are merged in yellow. Bar graphs represent the intensity of Ly6C-PE staining (MFI) on non-clustered and clustered CCR2<sup>+</sup>Ly6C<sup>+</sup> monocytes at 1 or 16 hrs

post Ly6C-PE mAb injection across 2 replicate experiments (n=2-4 mice). *r* corresponds to the ratio of MFI between clustered and non-clustered CCR2<sup>+</sup>Ly6C<sup>+</sup> monocytes. (B) Gating strategy to identify by flow cytometry Ly6C-PE<sup>hi</sup> and Ly6C-PE<sup>low</sup> monocytes, after gating on Ly6C-PerCpCy5.5<sup>+</sup> monocytes following the experimental design as described above. (C) Representative dot plots and FACS histograms of cell-surface ICAM-1 and CD86 expression on Ly6C-PE<sup>hi</sup> and Ly6C-PE<sup>low</sup> monocytes are shown. Bar graphs pool 2 independent replicate experiments with each symbol corresponding to one mouse and indicated p-value (n=5 mice).

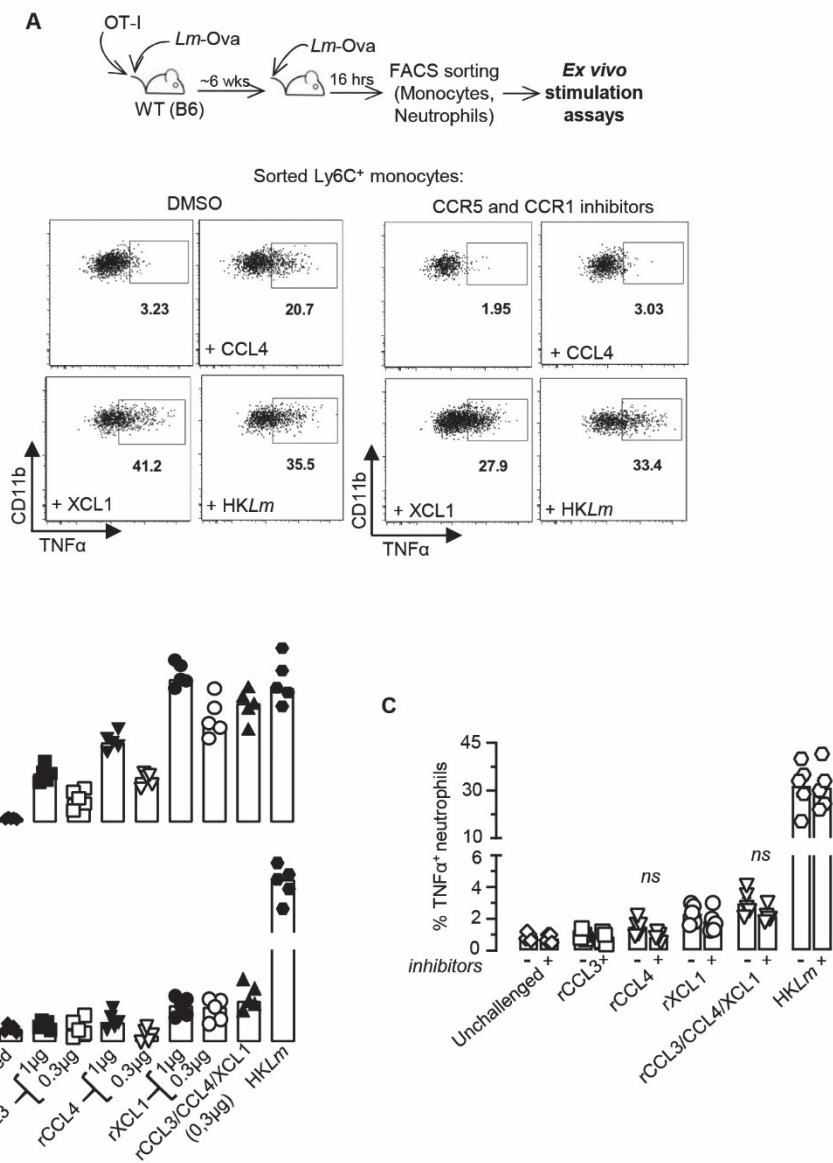


**Figure S6, related to Figure 7.** *CX3CR1*<sup>CreERT2</sup>*Rosa26*<sup>LSL-PTX</sup> mice transferred with OT-I cells were immunized with 10<sup>4</sup> *Lm-Ova*. 6 wks later, mice received Tx or vehicle i.p. daily for 5 days prior to 10<sup>6</sup> *Lm-Ova* recall infection. 16 hrs post challenge infection, BM cells (femur) were isolated and stained for cell surface CD11b, Ly6C-PerCpCy5.5 and the proportion of CCR2<sup>+</sup>Ly6C<sup>+</sup> monocytes were quantified. Representative FACS dot plots are shown and bar graphs pool 2 independent replicate experiments with n=6. A naive group of aged-matched mice (“unchallenged”) were included as control of BM monocyte proportions. Bar graphs show 1 of 2 independent experiments with n=3-4 mice. P-values are indicated.





**Figure S7, related to Figure 8.** (A) Mice were immunized with  $2 \times 10^5$  PFU *VSV*-Ova or injected with PBS, and ~6 wks later challenged with  $10^6$  *Lm* or *Lm*-Ova. Spleens and livers from challenged mice were harvested 24 hrs later and *Lm* CFUs determined after plating. Bar graphs show 1 of 2 representative experiments with each symbol corresponding to 1 individual mouse. (B)  $2 \times 10^5$  OT-I  $T_M$  cells induced using the depicted experimental set up, were transferred in *Ifngr1*<sup>-/-</sup> mice, and mice were next challenged with  $10^6$  *Lm* or *Lm*-Ova for 16 hrs. OT-I Td<sup>+</sup>  $T_M$  cells were stained for cell surface CD8, CD3 and intracellular CCL4 and IFN $\gamma$ . Representative FACS dot plots are shown and bar graphs pool 2 independent replicate experiments (n=4 mice) with indicated p-values.



**Figure S8, related to Figure 9.** (A, B) Mice grafted with OT-I cells were immunized with  $10^4$  *Lm-Ova* and 6 wks later challenged with  $10^6$  *Lm-Ova*. 16 hrs post-challenge, CCR2<sup>+</sup>Ly6C<sup>+</sup> monocytes and neutrophils were sorted from spleen and stimulated for 4 hrs with or without recombinant chemokines at indicated concentrations, or with HKLm and with or without CCR1/5 inhibitors (as depicted in Figure 9B). Cells were next stained for cell surface expression of CD11b, Ly6C, Ly6G and intracellular TNF $\alpha$ . Representative FACS dot plots are shown. (B) Graphs show TNF $\alpha$ <sup>+</sup> monocytes and neutrophils frequency after 4 hrs incubation with recombinant chemokines (1 and 0.3 $\mu$ g) or HKLm. Bar graphs (each symbol is 1 mouse) represent the pool of 2 independent replicate experiments with p-values indicated. (C) TNF $\alpha$  expression in FACS-sorted neutrophils as depicted in Figure 6C, incubated with CCR5 and CCR1 inhibitors or DMSO vehicle. Bar graphs (each symbol is 1 mouse) represent the pool of 2 independent replicate experiments with p-values indicated.

**Movie S1. Dynamic behavior of cognate antigen- versus inflammation-stimulated memory CD8<sup>+</sup> T cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters during recall infection.** Representative time-lapse movie showing cognate antigen (OT-I, red) and inflammation-stimulated gBT-I (green) CD8<sup>+</sup> T<sub>M</sub> cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters (CCR2<sup>CFP</sup>, blue) at ~16 hrs post challenge with *Lm*-Ova.

**Movie S2. Dynamic behavior of inflammation-stimulated memory CD8<sup>+</sup> T cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters during recall infection.** Representative time-lapse movie showing inflammation-stimulated (OT-I, red and gBT-I, green) CD8<sup>+</sup> T<sub>M</sub> cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters (CCR2<sup>CFP</sup>, blue) at ~16 hrs post challenge with *Lm*.

**Movie S3. Dynamic behavior of cognate antigen-stimulated memory CD8<sup>+</sup> T cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters during recall infection.** Representative time-lapse movie showing cognate antigen (OT-I, red and gBT-I, green) CD8<sup>+</sup> T<sub>M</sub> cells in CCR2<sup>+</sup>Ly6C<sup>+</sup> monocyte clusters (CCR2<sup>CFP</sup>, blue) at ~16 hrs post challenge with *Lm*-Ova-gB.

**Table S1. List of genes up/down regulated for Ag/Infl., Infl. and Ag/Inf./Infl CD8<sup>+</sup> T<sub>M</sub> cells as defined in Figure 1.**

**Table S2. GO pathways for Figure 1E**

**Table S3. Table for antibodies and other reagents**