Supplementary Materials for

Most Microbe-specific Naïve CD4$^+$ T Cells Produce Memory Cells During Infection

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Materials and Methods

Mice

Six-8 week-old C57BL/6 (CD45.2/2 CD90.2/2), B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (CD45.1/1 CD90.2/2), and B6.PL-*Thy1a*/CyJ (CD45.2/2 CD90.1/1) mice were purchased from the Jackson Laboratory or the National Cancer Institute Mouse Repository (Frederick, MD, USA). CD45.1/1 CD90.2/2 mice and CD45.2/2 CD90.1/1 mice were bred to obtain CD45.1/2 CD90.1/2 F_1 progeny, whick were bred to obtain F_2 mice, each of which inherited 1 of 9 possible allelic combinations of the congenic *Cd45* and *Cd90* genes. All mice were housed in specific-pathogen-free conditions in accordance with the guidelines of the University of Minnesota and the National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Minnesota approved all animal experiments.

Tetramers

Biotin-labeled I-A^{b} molecules containing the LLOp (NEKYAQAYPNVS) or Lymphocytic choriomeningitis virus (LCMV) GP66-77 peptide (DIYKGVYQFKSV) covalently attached to the I-A^{b} beta chain were produced with the I-A^{b} alpha chain in *Drosophila melanogaster* S2 cells, then purified and made into tetramers with streptavidin (SA)-phycoerythrin (PE) or SA-allophycocyanin (APC) (Prozyme, San Leandro, CA, USA) as described previously (9, 10).

Infections

The ActA-deficient *L. monocytogenes* strain was described previously (22). Mice were injected intravenously with 10^7 bacteria or intraperitoneally with $2x10^5$ plaque-forming units of the LCMV Armstrong strain.

Partial Splenectomy

Mice were anesthetized with an isoflurane vaporizer (VetEquip) and placed on a sterile surface in a vaporizer nosecone to maintain anesthesia. A 1 cm incision was made on the left side of the dorsal torso, under the last rib. A 0.5 cm incision was then made in the peritoneal wall. The third of the spleen proximal to the pancreas was removed for analysis with care taken to leave the splenic artery intact. The cut surface of the spleen was closed by cauterization. The peritoneal wall was closed and stitched using perma-hand silk 5-0 sutures (Ethicon). The skin was closed using 9 mm wound clips (Clay Adams brand, Becton Dickinson). Mice were injected subcutaneously with Ketoprofen (Fort Dodge) (5 mg/kg) at the end of the procedure before removal of anesthesia. The mice were transferred to a new clean cage with a heating pad. The animals had access to ibuprofen treated drinking water for 48 hours after the operation. Wound clips were removed 10-14 days after surgery.

Cell enrichment and flow cytometry

Single cell suspensions of spleens, spleen pieces, or lymph nodes were stained for 1 hour at room temperature with PE and/or APC-conjugated I-A^b tetramers

and 2 µg of anti-CXCR5-PE or anti-CXCR5-BV421 (2G8; Becton-Dickinson). Cells were then incubated with antibodies specific for the fluorochrome in the tetramer (PE or APC) conjugated to magnetic beads (Miltenyi Biotec). Samples were then enriched for bead-bound cells and enumerated as described previously (9). Enriched samples were stained with Fixable viability dye eFluor780 (eBioscience) to exclude dead cells, and antibodies specific for B220 (RA3–6B2), CD11b (MI-70), CD11c (N418), CD8α (5H10, Invitrogen, Carlsbad, CA, USA), PD-1 (J43), CD4 (RM4–5), CD3ε (145-2C11), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD90.1 (HIS51), and/or CD90.2 (53-2.1) each conjugated to a different fluorochrome. All antibodies were from eBioscience unless noted. Cells were then analyzed on an LSR II or Fortessa (Becton Dickinson) flow cytometer. Data were analyzed with FlowJo software (TreeStar).

Limiting dilution cell transfer

$CD4^+$ T cells were isolated from the spleens and lymph nodes of donor mice with the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). For limiting dilution experiments, $7x10^5$ $CD4^+$ T cells from each donor were injected intravenously into each recipient, such that on average less than 1 LLOp:I-A^b-specific cell would engraft per recipient as previously described (12). Recipient mice were then injected intravenously with 10^7 *L. monocytogenes* bacteria. Eight days later intact spleens or spleen pieces were analyzed by tetramer-based cell enrichment as described above to identify LLOp:I-A^b tetramer-binding $CD4^+$ T cells of donor or recipient origin. If more than 75% of recipient mice contained an LLOp:I-A^b tetramer-

binding population derived from a congenic donor (determined at the end of the experiment), cells from that donor were excluded to ensure that clonal populations were analyzed. Because one third of the spleen was analyzed on day 8, the total number of LLOp:I-A^b tetramer-binding cells present at that time was calculated by multiplying the number of cells detected in a spleen third by 3. The mice were euthanized 54 or 56 days later and examined for LLOp:I-A^b tetramer-binding memory cells in the remaining two-thirds of the spleens. The total number of LLOp:I-A^b tetramer-binding cells that would have been present in the full spleen was calculated by multiplying the number of cells detected in two thirds of each spleen by 1.5. To be considered genuine, clonal populations had to have greater than 5 detectable events at day 8 post infection based on the observation that B6 mice that did not receive transferred cells had 0.6 ± 1.8 events per gate (data not shown).

Statistical analysis

Statistical tests were performed using Prism (Graphpad) software.

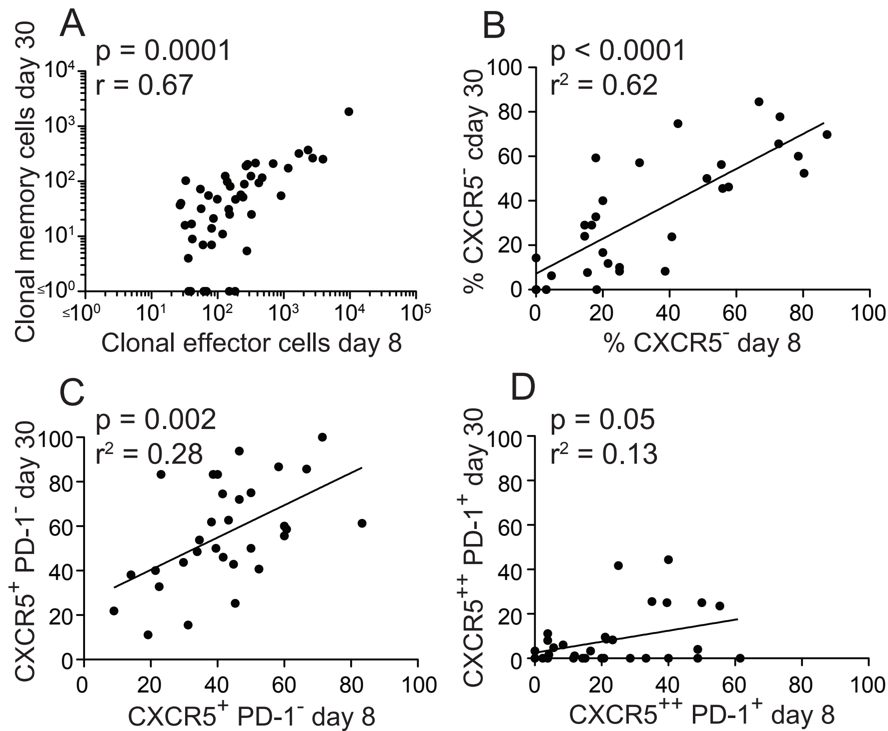

Fig. S1. **Most clonal effector populations give rise to similar memory populations 30 days after infection**. (A) Number of cells produced by different single LLOp:I-A^b tetramer-binding naïve cells at days 8 and 30 after infection. Statistical values were calculated with the Spearman correlation test. (B-D) Percentages of cells in LLOp:I-A^b tetramer-binding populations that were $CXCR5^-$ (B), $CXCR5^+$ (C), or $CXCR5^{++}$ $PD\text{-}1^+$ (D) on day 8 plotted versus the percentages of those subpopulations 30 days after infection. Each dot represents a clonal population. Statistical values and trend lines from linear regression analyses are shown. Pooled data from 2 experiments are shown.

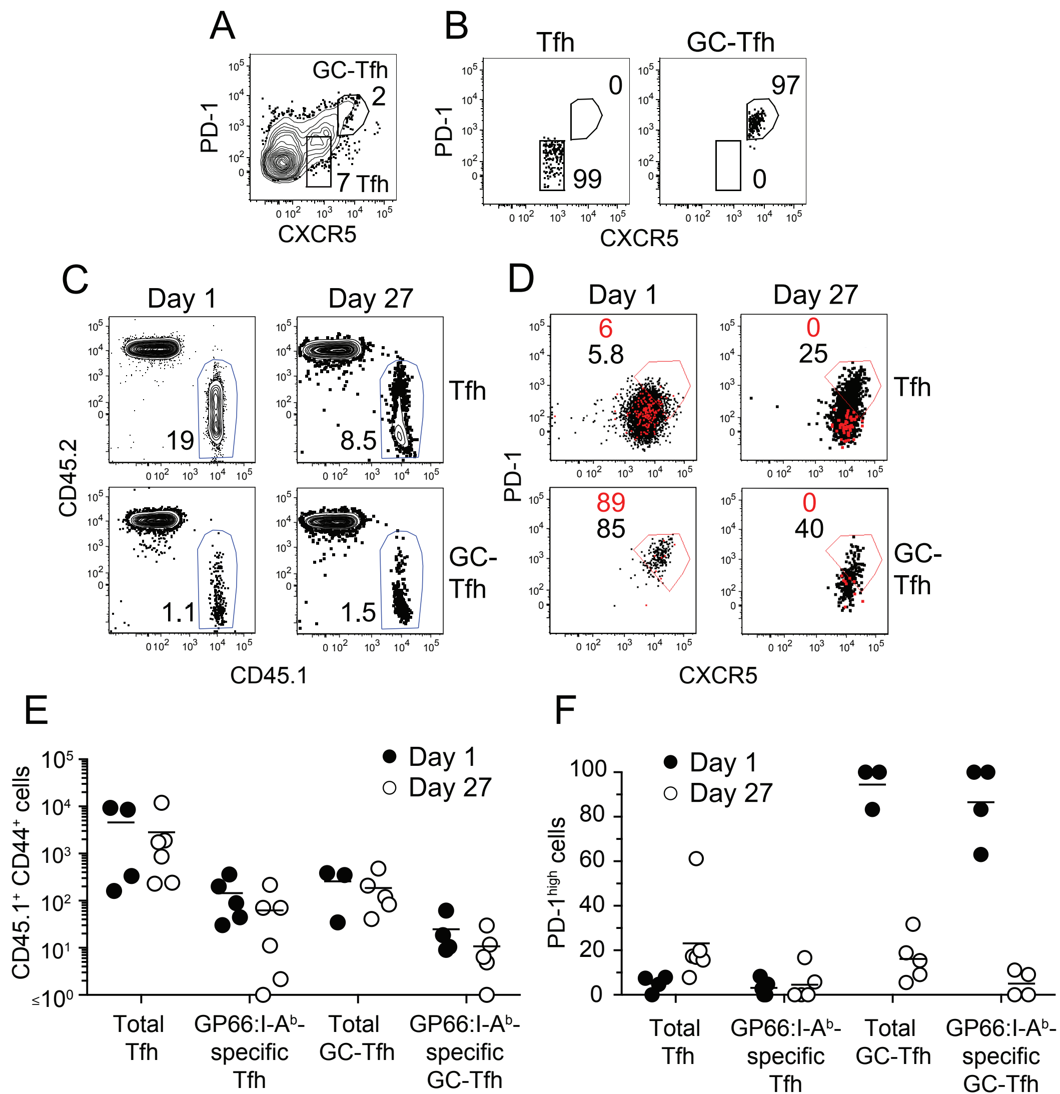

Fig. S2. **GC-Tfh effector cells convert to Tfh-like memory cells.** An adoptive transfer approach was used to determine whether GC-Tfh effector cells produce memory cells. LCMV infection was used for this purpose because it induces a very large number of LCMV glycoprotein peptide 66-77:I-A^b (GP66:I-A^b) tetramer-binding GC-Tfh cells (23). CXCR5$^+$ PD-1$^-$ Tfh cells and CXCR5^{++} PD-1$^+$ GC-Tfh cells were sorted from day 13 LCMV-infected CD45.1/1 mice using the gates shown in (A). (B) Post sort analysis of the indicated populations. Sorted cells

were transferred into uninfected CD45.2/2 recipients. (C) Detection of donor-derived CD45.1/1 $CD4^+$ T cells from both sorted populations 1 or 27 days after transfer. (D) CXCR5 and PD-1 expression by all (black dots) or GP66:I-A^b tetramer-binding (red dots) T cells in the donor-derived populations identified in (C). Numbers indicate the percentages for $CXCR5^{++}$ PD-1^+ GC-Tfh-phenotype cells in each population. (E) Number of total or GP66:I-A^b tetramer-binding T cells of the indicated types 1 or 27 days after transfer. (F) Percentages of PD-1^+ total or GP66:I-A^b tetramer-binding T cells of the indicated types 1 or 27 days after transfer. Each symbol represents an individual mouse. Pooled data from 3 experiments are shown. The results show that Tfh and GC-Tfh effector cell populations both contracted 27 days after transfer and produced memory cells (E), but many GC-Tfh effector cell-derived memory cells, especially the GP66:I-A^{b-}specific ones, did not express PD-1 (F).